THIRD EDITION

FISH NUTRITION

Edited by

JOHN E. HALVER · RONALD W. HARDY

Third Edition

Fish Nutrition

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Preface

This third edition of *Fish Nutrition* was reviewed and updated with selections from the myriad of publications which have appeared in the literature on fish nutrition since the previous 1989 edition. During this decade aquaculture continued to advance more rapidly than any other field of animal production in the world, and it is expected to continue to expand to provide fish for a growing world population. As aquaculture production increases, it must contend with rapidly approaching limits on key feed ingredients and on increasing sensitivity to the effects of aquaculture on the aquatic environment. Many of these effects are associated with diet, so fish nutrition research must focus on increasing the efficiency of production and on lowering environmental effects through increased nutrient retention. This will provide safe and nutritious fishery products in a sustainable and environmentally compatible fashion.

Over 200 fish species have been examined as potential targets for fish production to utilize the special advantages of an animal capable of growing efficiently in a wide variety of temperatures and ionic-strength waters. Universities, research centers, and various government agencies have adopted fish as an important agricultural animal, with a resultant plethora of publications from scientists in many countries focused on an increasing number of fishes and their nutritional requirements. Since it would have been impossible to include all these reports in this book, the authors have focused on selected demonstrations of nutrient requirements and metabolism which summarize the basic and applied principles of fish nutrition.

The chapter "Bioenergetics" has been entirely rewritten to include the rapid advancements made since the last edition. "The Vitamins" chapter has been updated and reflects the conclusion that many of the principles discussed previously still apply, even as new species of fish are examined. The previous focus on teleost fish has been extended to include other types with unique or different metabolic capabilities. The "Amino Acids and Proteins" chapter has been expanded to include the many new species studied. "The Lipids" chapter has been extensively revised as national and international focus is aimed at understanding these compounds and their effects on animal metabolism and health. More information is included in "The Minerals" chapter to reflect the importance of minerals as activators

for many anabolic and catabolic reactions and to provide basic information concerning the importance of proper mineral balance, especially of phosphorus, for lowering the environmental impacts of fish culture. The chapter "Intermediary Metabolism" has been condensed to the principles involved, with more extensive discussions to be found in other nutrient chapters. "Nutritional Physiology" has been rewritten, extending the discussions to the larval stages of the life history of many species of fish, as well as to juvenile and grow-out stages. The chapters "Nutritional Pathology" and "Nutrition and Fish Health" have been rewritten. "Adventitious Toxins" are reviewed, and the roles of new toxins encountered discussed. "Diet Formulation and Manufacture" has been expanded to include some of the latest techniques in fish husbandry production and in feed manufacturing processes, and the "Special Feeds" chapter outlines new possibilities in fish feeds for new species and environments. Finally, the practical applications of fish nutrition to "Diet and Husbandry" have been extended to include new areas of fish production.

The Appendix reflects the many changes encountered in fish species and diet database assembly during the past decade.

We hope this treatise continues to review *"what we know and what we know we do not know"* to stimulate research and better understanding of nutrient requirements and their role in growth, reproduction, and fish health as more and more effort is concentrated on using fish as the best animal for protein and food production. Dividends from understanding nutrient metabolism in fish at the cellular level can be extended to similar functions in terrestrial animals, including humans.

This book would not have been possible without the dedicated and demanding efforts of the chapter authors to condense fragmented and often contradictory information in the literature and from their own laboratories into succinct discussions and presentations of the principles of fish nutrient requirements and metabolism. Their efforts are sincerely appreciated. The reader is invited to compare the developments in fish nutrition which have occurred since the first edition appeared in 1972.

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1

Bioenergetics

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1.1 Introduction

The catabolism of food is organized within the animal to harness chemical (free) energy and substrates for use in anabolic and other life-sustaining processes. The physiological mechanisms which achieve this are very complex, allowing the catabolism of a large variety of food molecules using the finite number of enzyme systems which are found in animal tissues (Krebbs and Kornberg, 1957). To look quantitatively at the utilization of all dietary components is extremely complex. However, since feeding, growth, and production can be described in terms of partition of dietary energy yielding components between catabolism as fuels and anabolism as storage in tissues, the study of the balance among dietary energy intake, expenditure, and gain offers a relatively simple way of looking at dietary component utilization by animals. This approach is called bioenergetics or nutritional energetics.

This chapter is a nonexhaustive review of current knowledge, methods, applications, and limitations of fish bioenergetics or nutritional energetics. It focuses mostly on fish bioenergetics in an aquaculture setting. Energy flow in the animal is presented based on the energy partition scheme and nomenclature proposed by the U.S. National Research Council (NRC, 1981) (Fig. 1.1).

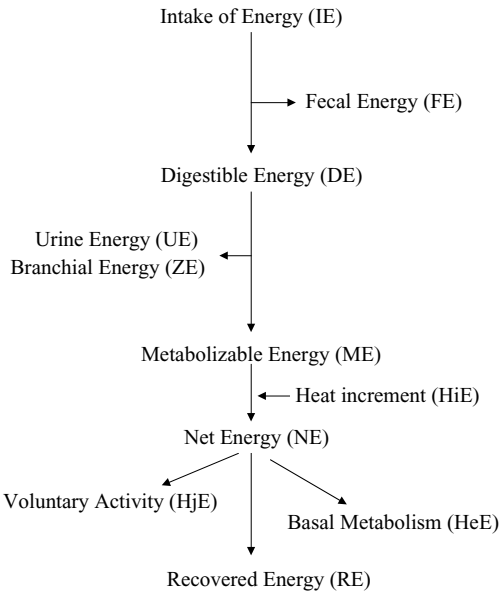

FIG. 1.1

NRC (1981) energy partitioning scheme and nomenclature.

1.2 History of Nutritional Energetics

Nutritional energetics has been studied for more than 200 years. In 1779, Adair Crawford observed that the amount of air a man "phlogisticated" in a minute was the same as that altered by a burning candle. Despite the fact that Crawford formulated ideas about the origin of animal heat in terms of the *phlogiston* theory that was popular at the time, his observations were some of the first showing a relationship among gas exchanges, heat production, and chemical reactions in animals. In 1783, Antoine Lavoisier and Pierre Laplace performed a series of exceptional experiments, considered as the foundation of bioenergetics and modern nutrition. They observed that heat produced by a guinea pig could be measured by the amount of ice melted and that the heat produced could be related to the respiratory exchange in a quantitative way. Based on this series of studies Lavoisier formulated his classical conclusion that life is a process of combustion. Lavoisier was, thus, the first to recognize the true role of oxygen in the generation of heat by animals. Lavoisier's contribution to the study of animal energetics was not limited to his elucidation of the relationship between respiration and the

production of heat but also included several aspects of energy metabolism of animals. His studies with Séguin on the metabolism of man, which involved the measurement of oxygen consumption and carbon dioxide production, showed that oxygen consumption is increased by the ingestion of food, by the performance of muscular work, and by exposure to cold. Lavoisier also measured the minimal metabolism in the resting, postabsorptive state and showed proportionality between pulse frequency and metabolism. He also showed that within a species, oxygen consumption is proportional to body size (Blaxter, 1989).

Lavoisier believed that the site of heat production was located in the lungs and that heat was carried throughout the body by the blood. It was only in 1847 when Magnus showed that arterial blood carried more oxygen and less carbon dioxide than did venous blood, and in 1848, when von Helmholtz demonstrated that isolated muscle produced heat, that the belief of Lavoisier was shown to be erroneous (Blaxter, 1989).

Nutritionists working at the Weende Agricultural Experimental Station in Germany, in the nineteenth century, recognized that the components of foods which make a significant contribution to the energy supply of the animal could be characterized as three classes of compounds: proteins, fats, and carbohydrates. The stoichiometry of the oxidation of these classes of compounds allowed the calculation of the energy released as heat from measurements of respiratory exchange, oxygen consumption, and carbon dioxide production, along with measurements of urinary nitrogen excretion. This method of measuring heat production is referred to as indirect calorimetry (or respirometry). In 1894, Rubner validated this approach to calorimetry by showing that the heat produced by a dog is equal to the heat of combustion of the fat and protein catabolized minus the heat of combustion of the urine. Rubner, thus, was the first to demonstrate the fundamental laws of thermodynamics applied to intact living animal systems (Blaxter, 1989).

Rubner is also credited with making the first systematic experimental analysis of the effect of size on metabolism. He showed in 1883 that the fasting metabolism of dogs of different body weights was approximately constant when expressed per unit area of body surface. In 1901, Voit, Rubner's student, showed that the fasting metabolisms of a number of species were also proportional to their surface areas. Kleiber, and Brody and Proctor, almost simultaneously in 1932, showed that metabolism was related directly to body weight and metabolism was proportional to a power of weight higher than 2/3, that is, about 0.75. Kleiber came to the conclusion that the 3/4 power of body weight was the most reliable basis for predicting the basal metabolic rate of animals and for comparing nutrient requirements among animals of different sizes. He also provided the basis for the conclusion that the total efficiency of energy utilization is independent of body size. In 1945, Brody published *Bioenergetics and Growth*, and in 1961, Kleiber published

The Fire of Life, two books, discussing several aspects of energy metabolism of animals, that remain very influential to this day.

Ege and Krogh (1914) were the first to apply the principles of bioenergetics to fish. Ivlev (1939) worked with carp. Since then, there have been several hundred reports on studies of energy utilization and expenditure for several species of fish. Many reviews have also been made on fish bioenergetics, including those by Phillips (1972), Brett and Groves (1979), Cho *et al.* (1982), Elliott (1982), Cho and Kaushik (1985), Tytler and Calow (1985), Smith (1989), Cho and Kaushik (1990), Kaushik and Médale (1994), Cho and Bureau (1995), and Médale and Guillaume (1999), which are most relevant to aquaculture.

1.3 Energy Exchange in Biological Systems

The first law of thermodynamics, also known as the law of conservation of energy, states that the total energy (E) of a system, including its surroundings, remains constant unless there is input of energy (heat or work). It implies that within the total system, energy is neither lost nor gained during any changes. However, within that total system, energy may be transferred from one part to another or may be transformed into another form of energy (heat, electrical energy, radiant energy, or mechanical energy). Thermodynamic principles as they apply to biological systems are reviewed in several textbooks (e.g., Patton, 1965; Blaxter, 1989; Mayes, 2000). Readers are invited to refer to these for a more comprehensive presentation of these principles.

All biological organisms must obtain supplies of free energy from their environment to sustain living processes. Nonbiological systems may utilize heat energy to perform work, but biological systems are essentially isothermic and use chemical energy to sustain life processes. Autotrophic organisms couple their metabolism to some simple processes in their surroundings, such as sunlight and inorganic chemical reactions, such as the transformation of Fe^{2+} to Fe^{3+}. Heterotrophic organisms obtain free energy from the breakdown of organic molecules in their environment. Bioenergetics, or biochemical thermodynamics, is the study of the energy changes accompanying such biochemical reactions (Mayes, 2000).

Life processes (e.g., anabolic reactions, muscular contraction, active transport) obtain energy by chemical linkage. This chemical coupling results in some energy being transferred to synthetic reaction and some energy lost as heat. As some of the energy liberated in the degradative reaction is transferred to the synthetic reaction in a form other than heat, the normal chemical terms “exothermic” and “endothermic” cannot be applied.

The terms exergonic and endergonic are used to indicate that a process is accompanied by the loss or gain, respectively, of free energy (Mayes, 2000). In practice, an endergonic process cannot exist independently but must be a component of a coupled exergonic–endergonic system where the overall net change is exergonic. The exergonic reactions are termed catabolism, whereas the synthetic reactions are termed anabolism. The combined catabolic and anabolic processes constitute metabolism. A method of coupling an exergonic to endergonic process is to synthesize a compound of high-energy potential in the exergonic reaction and to incorporate this new compound into the endergonic reaction, thus transferring free energy from the exergonic to the endergonic pathway. Adenosine triphosphate (ATP) is one of the compounds serving as a transducer of energy from a wide range of exergonic reactions to an equally wide range of endergonic reactions or processes (Mayes, 2000).

ATP is a phosphorylated nucleotide containing adenine, ribose, and three phosphate groups. ATP has an intermediate standard free energy of hydrolysis among high-energy phosphate molecules, whose characteristics allow it to play an important role in energy transfer. As a result of its position midway down the list of standard free energies of hydrolysis, ATP is able to act as a donor of high-energy phosphate to form compounds with lower free energies of hydrolysis (Mayes, 2000). Likewise, provided the necessary enzymatic machinery is available, ADP can accept high-energy phosphate to form ATP from compounds with high energies of hydrolysis. In effect, an ATP/ADP cycle connects those processes that liberate free energy to those processes that utilize it. Thus, ATP is continuously consumed and regenerated. However, it is worth recalling that the total ATP/ADP pool is sufficient to maintain an active tissue for only a few seconds (Mayes, 2000).

The system that couples respiration to the generation of the high-energy intermediate, ATP, is termed oxidative phosphorylation. Oxidative phosphorylation enables aerobic organisms to capture a far greater proportion of the available free energy of respiratory substrates compared with anaerobic organisms. The mitochondrion is the organelle in which most of the capture of energy derived from respiratory oxidation takes place. The mitochondria contain the series of catalysts known as the respiratory chain that collect and transport reducing equivalents and direct them to their final reaction with oxygen to form water. Also present is the machinery for trapping the liberated free energy as high-energy phosphate. Mitochondria also contain the enzyme systems responsible for generating the reducing equivalents (such as NADPH) in the first place, i.e., the enzymes of β-oxidation and of the citric acid cycle. The latter is the final common pathway for the oxidation of all the major foodstuffs.

As mentioned earlier, the coupling of exergonic and endergonic reactions does not harness all the energy, and a significant portion of the energy

is dissipated as heat. One mole of glucose, for example, contains about 2803 kJ of free energy. When it is combusted in a calorimeter to CO_2 and water, 2803 kJ is liberated as heat.* When oxidation occurs in the tissues, some of the energy is not lost immediately as heat but is captured in high-energy phosphate bonds. Under aerobic conditions, glucose is completely oxidized to CO_2 and water, and the equivalent of 36 high-energy phosphate bonds is generated per molecule. The total energy captured in ATP per mole of glucose oxidized is 1398 kJ, or the equivalent of roughly 50% of the enthalpy of combustion. The rest is dissipated as heat. In turn, when ATP generated by the catabolism of glucose is hydrolyzed during coupling with an endergonic reaction, only a fraction of the free energy may be retained in the synthesized compounds and the rest is liberated as heat. Therefore, ultimately the free energy liberated by exergonic reactions that is not captured in the products of anabolism (protein, lipids, carbohydrates, nucleic acids, etc.) is liberated as heat by biological organisms.

A very important aspect from a bioenergetics point of view is that heat produced by a chemical reaction is always the same, regardless of whether the process went directly or proceeded through a number of intermediate steps (Blaxter, 1989). This means that the amount of heat produced by an animal depends on the chemical nature (energy content) of the compounds catabolized or the overall reaction and not the chemical reaction pathways over which this catabolism occurred.

1.4 Energy Utilization and Requirements

The study of the balance among dietary energy supply, expenditure, and gain offers a relatively simple way of looking at dietary component utilization by animals. Study of the energy transactions in animals requires that components be expressed in compatible terms. Classically, all measurements of energy transactions made by animal nutritionists were expressed in terms of calories. The calorie used in nutrition is the 15°C calorie (the energy required to raise the temperature of 1 g water from 14.5 to 15.5°C). However, the joule (J) was adopted in the Système International des Unités (International System of Units) as the preferred unit for expression of electrical, mechanical, and chemical energy and by most nutrition journals as the basic unit for expressing dietary energy. One joule is defined as 1 kg-m^2/sec^2 or 10^7 erg. One 15°C calorie is equivalent to 4.184 J.

* *Editors note.* The authors prefer to use the joule to measure energy content and reactions, whereas many other authors use the calorie for energy measurements. These are convertible: 1 cal = 4.184 J, or 1 kcal = 4.184 kJ. See below.

Many terms have been invented and applied to describe energy transactions occurring in animals. Historical terms, such as "specific dynamic action of food," are still used, even though they imply nothing about the underlying relationships; others such as "work of digestion" have specific but incorrect implications regarding underlying relationships (Baldwin and Bywater, 1984). Different groups have tended to adopt and defend alternative systems of nomenclature to describe the partition of energy in animals. This is especially apparent in fish biology, where nomenclatures and mode of expression of energy transaction are extremely diverse. In 1981, a subcommittee of the Committee on Animal Nutrition of the U.S. National Research Council was appointed to develop a systematic terminology for description of energy utilization by animals, including fish (NRC, 1981). This system is presented schematically in Fig. 1.1 and has been widely adopted by animal nutritionists. This rational nomenclature has also been adopted by a number of fish nutrition researchers and is used in this chapter. Its various components are discussed below.

1.4.1. Gross Energy: Dietary Fuels

Gross energy (GE) is the commonly used term for the enthalpy (ΔH) of combustion in nutrition. However, as opposed to enthalpy, GE is generally represented by a plus (+) sign. The GE content of a substance is usually measured by its combustion in a heavily walled metal container (bomb) under an atmosphere of compressed oxygen. This method is referred to as bomb calorimetry. Under these conditions, the carbon and hydrogen are fully oxidized to carbon dioxide and water, as they are *in vivo*. However, the nitrogen is converted to oxides, which is not the case *in vivo*. The oxides of nitrogen interact with water to produce strong acids, an endergonic reaction. These acids can be estimated by titration, allowing a correction to be applied for the difference between combustion in an atmosphere of oxygen and catabolism *in vivo* (Blaxter, 1989).

The GE content of an ingredient or a compounded diet depends on its chemical composition. The mean GE values of carbohydrates, proteins, and lipids are 17.2, 23.6, and 39.5 kJ/g, respectively (Blaxter, 1989). Minerals (ash) have no GE because these components are not combustible. IE is the notation adopted by the NRC (1981) for an animal's intake GE of (Fig. 1.1). IE is simply the product of feed consumption and GE.

1.4.2. Fecal Energy and Digestible Energy

Before the feed components can serve as fuels for animals, they must be digested and absorbed (sometimes called "assimilated," a term whose use

should be discouraged) from the digestive tract. Some feed components resist digestion, and these pass through the digestive tract to be voided as fecal material. Egestion (excretion through feces) of components containing GE is referred to as fecal energy (FE) losses. The difference between the GE and the FE of a unit quantity of this diet is termed the digestible energy (DE). DEI was adopted by the NRC (1981) to represent the intake of DE, the product of feed intake and DE of the feed, or IE minus FE (Fig. 1.1).

Variation in the digestibility of foods is generally a major factor affecting the variation in their usefulness as energy sources to the animal, since FE is a major loss of ingested GE. Therefore, values for DE and values for the digestibility of individual nutrients should be used to estimate levels of available energy and nutrients (as opposed to GE or crude nutrients) in feed ingredients for diet formulation (Cho and Kaushik, 1990). Formulation on a GE or crude nutrients (e.g., crude protein) basis, rather than formulation on a DE or digestible nutrients basis, is still very common in fish nutrition, but sufficient information on DE values of common fish feed ingredients is now available to allow feeds to be formulated on a DE or a digestible nutrient basis. It is, however, important to emphasize that DE is only an indication of the potential contribution of the energy from nutrients in the ingredient. These values do not serve as measures of the utilizable energy or of the productivity of the diet.

1.4.3. Measurement

The first task in the measurement of digestibility of feeds and feedstuffs is the collection of fecal samples. In aquatic animals, separating fecal material from water and avoiding contamination of the feces by uneaten feed necessitate the use of approaches that differ significantly from those commonly used to measure digestibility interrestial animals and birds.

Quantitative collection of fish feces is very difficult, and therefore, digestibility measurements using direct methods, involving total collection of fecal material, are rarely used with fish. Digestibility measurements in fish must, therefore, rely on the collection of a representative fecal sample (free of uneaten feed particles) and the use of a digestion indicator to obviate the need to quantify dietary intake and fecal output (indirect method). The inclusion of a digestion indicator in the diet allows the digestibility coefficients of the nutrients in a diet to be calculated from measurements of the nutrient-to-indicator ratios in the diet and feces (Edin, 1918).

Several techniques have been used to collect fecal material from fish. The suitability of these various techniques has been a subject of discussion and disagreement among fish nutritionists for many years (Smith *et al.,* 1980; Cho *et al.,* 1982; Cho and Kaushik, 1990; Hajen *et al.,* 1993a; Smith *et al.,*

1995; Guillaume and Choubert, 1999). Some early, yet still widely used, techniques are the collection of feces from the lower part of the intestine by stripping (Nose, 1960), by suctioning fecal material, or by dissecting the fish (Windell *et al.,* 1978). It is generally agreed that forced evacuation of fecal material from the rectum results in the contamination of the samples with physiological fluids and intestinal epithelium that would otherwise have been reabsorbed by the fish before natural defecation. This affects the reliability of this type of approach and, in general, leads to underestimation of digestibility (Cho *et al.,* 1982; Hajen *et al.,* 1993; Guillaume and Choubert, 1999).

Techniques involving the collection of feces voided naturally by the fish are, therefore, preferable. Smith (1971) developed a metabolic chamber to collect feces samples voided naturally into the water by fish. With this method, the fish need to be force-fed, and they frequently regurgitate and may not be in a positive nitrogen balance status. This technique clearly imposes an unacceptable level of stress on the fish and produces estimates of digestibility of questionable reliability (Cho *et al.,* 1982). Other techniques, such as the periodical collection of feces by siphoning from the bottom of a tank, are also likely to yield inaccurate estimates of digestibility since the breakup of feces by fish movement may lead to leaching of nutrients and, therefore, overestimation of digestibility of nutrients.

To prevent these problems, specific devices were developed by Ogino *et al.* (1973), Cho *et al.* (1975), and Choubert *et al.* (1979) to collect fecal material passively. Ogino *et al.* (1973) collected feces by passing the effluent water from fish tanks through a filtration column (TUF column). Cho and Slinger (1979) developed a settling column to separate the feces from the effluent water (Guelph system) and Choubert *et al.* (1979) developed a mechanically rotating screen to filter out fecal material (St. Pée system). These systems are convenient and have been adopted in many laboratories around the world. They are widely recognized as producing meaningful estimates of digestibility of nutrients if used correctly, despite the fact that differences of opinion about the accuracy of these systems remain. In a study comparing the TUF column and the Guelph system, very similar apparent digestibility coefficients (ADC) of dry matter, protein, lipid, and energy were obtained with both methods for two reference diets (Satoh *et al.,* 1992).

It is clear that differences exist in the estimates of digestibility with the various techniques currently used (Cho *et al.*, 1982). It is difficult to reach objective conclusions about the accuracy and reliability of the various techniques, as there are relatively few solid experimental studies allowing serious comparisons. Direct measurements of energy and nutrient deposition and various losses (nonfecal losses, heat production, etc.) are virtually the

only way of objectively comparing the accuracy of the various approaches. However, measurements of the various components of the energy or nutrient budgets (e.g., nonfecal losses, heat production) of fish also require specific expertise and are subject to errors.

The differences in estimates of apparent digestibility measured with the most common techniques (stripping, St. Pée system, TUF column, Guelph system) tend to be fairly stable when these techniques are used in a standardized fashion. This suggestion comes from examination of the results of studies examining energy or nutrient depositions of groups of fish at different measured intakes of various practical diets (Kaushik *et al.,* 1981; Médale *et al.*, 1995; Azevedo *et al.,* 1998; Ohta and Watanabe, 1998; Médale and Guillaume, 1999; Rodehutscord and Pfeffer, 1999). Regressions of energy and N depositions as a function of DE or digestible nitrogen (DN), measured with different techniques (stripping, St-Pée system, TUF column, Guelph system), show very significant linear relationships within studies ($R^2 > 0.96$). This suggests that digestibility measurements appear to be consistent within techniques and that, if investigators adopt one technique and apply it in a standard fashion, very meaningful (informative) energy or nutrient budgets can be constructed.

1.4.4. Apparent versus True Digestibility

Feces are composed of the undigested food components and the unreabsorbed residues of body origin. These residues are the remains of mucosal cells, digestive enzymes, mucoproteins, and other secretions released into the digestive tract by the animal, together with the residues of the microflora which inhabit the digestive tract (Nyachoti *et al.,* 1997). The enthalpy of combustion of these materials represents a loss of energy which is not derived from the food. This energy loss is designated fecal energy of metabolic origin (FmE) and is influenced by the characteristics of the food and the level of feed intake. Estimates of FmE allow the description of "true" digestible energy values, which are greater than "apparent" digestible energy values. The term "true" digestibility may be misleading since, to the animal, FmE losses are real and inevitable. The term "standardized digestibility" is slowly replacing "true digestibility" in the vocabulary of animal nutritionists.

$$\text{Apparent digestible energy (ADE)} = \text{IE} - \text{FE}$$

$$\text{True (or standardized) digestible energy} = \text{IE} - (\text{FE} - \text{FmE})$$

Measurement of FmE of fish has received little attention. The FmE that has been mostly studied in fish and other animals (swine and poultry) has

been associated with endogenous protein/nitrogen losses. The most common approach for measuring metabolic fecal nitrogen (MFN) representing endogenous nitrogenous losses is by determining the fecal nitrogen output of fish fed a protein-free (nitrogen-free) diet. The MFN of fish fed a protein-free diet has been estimated as about 2.7–3.3 mg/100 g live body weight per day or 123–144 mg/100 g dry diet consumed in common carp at 20°C (Ogino *et al.,* 1973). FmE as protein (probably contributing the most to FmE) can, therefore, be estimated to be about 0.4 kJ/100 g live body weight per day or 20 kJ/100 g dry matter intake. This is relatively small, being equivalent to about 1% of the IE or about 10–20% of the FE of animals fed good-quality practical diets.

Fish will generally eat very little of a protein-free diet, making it very difficult to calculate meaningful estimates of MFN. Moreover, there is evidence that the amount of MFN produced by animals receiving a semipurified protein-free diet can differ significantly from that of animals fed practical diets containing protein (Nyachoti *et al.,* 1997). Several other dietary constituents (fiber, antinutritional factors) can enhance MFN (Nyachoti *et al.,* 1997). For these reasons, it is reasonable to doubt the accuracy of "true" protein digestibility coefficients calculated using estimates of MFN obtained from fish fed protein-free diets. Accurate estimation of MFN may require the use of sophisticated techniques (for review see Nyachoti *et al.,* 1997). This type of work remains to be carried out with fish.

In digestibility studies with swine and poultry, fecal samples must be collected from the ileum or from cecectomized animals because of the significant activity of the intestinal microflora in the large intestine or cecum of these animals (Levis and Bayley, 1995). Reabsorption of endogenous material (e.g., enzymes) in the hindgut is thus prevented. Correction for endogenous losses is, therefore, essential to obtain the additive estimates of the apparent digestibility of nutrients for these animals. Endogenous losses from naturally voided fecal material in fish are probably small and consequently of little concern since the intestinal flora activity is generally considered negligible in most fish species (Clements, 1996) and a large proportion of endogenous material is reabsorbed prior to egestion of feces. This view is supported by the higher values for the ADC of protein of most feed ingredients measured in salmonids (e.g., Cho and Bureau, 1997) compared to the ileal ADC of protein of the same ingredients in swine and poultry (Levis and Bayley, 1995).

In fish maintaining a high feed intake, the contribution of MFN to the total fecal nitrogen is probably small. Under these conditions, the difference between the "true" and the apparent digestibility of protein is probably negligible. If poor feed intake or poor growth is observed in a digestibility trial, it is preferable to discard the fecal samples collected since these samples may

contain a high proportion of MFN and could produce unreliable estimates of apparent digestibility (Cho *et al.,* 1982).

1.4.5. Digestibility of Whole Diets versus Digestibility of Ingredients

As discussed above, knowledge of the digestibility of energy and nutrients of diets is a very important aspect of any study on nutritional energetics. Because digestibility measurements require specialized equipment and are time-consuming, it is impossible to measure the digestibility of all diets. Because a diet is a combination of various ingredients, knowing the digestibility of a variety of potential fish feed ingredients may allow estimation of the digestibility of an infinite variety of diets formulated using these ingredients (Cho *et al.,* 1985). This, however, requires, that estimates of apparent digestibility of nutrients of different ingredients are additive, an assumption that generally holds true (Cho and Kaushik, 1990; Watanabe, 1996a,b).

Very few feed ingredients can be fed voluntarily as the sole component of a diet to fish. First, certain fish feed ingredients may not be very acceptable (palatable) for fish as a sole component of the diet. Second, it is not possible to produce feed particles with proper physical characteristics (water stability) with many individual ingredients. Third, most fish feed ingredients do not contain all the essential nutrients required by fish and feeding diets containing many of these ingredients as the sole component for more than a few days may dramatically affect the feed intake and the overall physiological status of the fish.

The use of the protocol proposed by Cho and Slinger (1979) generally solves these problems. This protocol involves comparison of the digestibility of a reference diet with that of a test diet, this test diet being a mixture of the reference diet and a test ingredient, generally at a 70 : 30 ratio. Using this protocol, palatable, water-stable, and nutritionally adequate test diets can be produced with most potential fish feed ingredients. This allows the fish to maintain a high feed intake and good growth rate, which in turn allow the measurement of apparent digestibility values that are reliable and repeatable. Also, adoption of this procedure allows the measurement of feed intake and growth rate, allowing confirmation of the nutritional adequacy of the experimental diets.

Inclusion of a digestion indicator in the reference diet allows the ADC of the energy and nutrients in the diets to be calculated from measurements of the ratios of nutrient to indicator in the diet and feces. The corresponding ADC can be calculated for the energy and nutrients in the tested ingredient by simple calculation from the ADC of the reference and test diets. The use of a reference diet, however, assumes that there are no interactions between

the components of the diet during digestion. Hence, much care is warranted in formulating such a test diet.

The apparent digestibility coefficients (ADC) for the nutrients and energy of the test and reference diets can be calculated as follows:

$$\text{ADC} = 1 - [(F/D) \times (\text{Di}/\text{Fi})] \quad (1)$$

where D is the percentage nutrient (or kJ/g gross energy) of the diet; F, the percentage nutrient (or kJ/g gross energy) of the feces; Di, the percentage digestion indicator of the diet; and Fi, the percentage digestion indicator of the feces.

The ADC of the test ingredients (ADCI) is then calculated based on the digestibility of the reference diet and the test diets as follows:

$$\text{ADCI} = \text{ADCT} + [((1-s)\,\text{DR})/s\,\text{DI}]\ (\text{ADCT} - \text{ADCR}) \quad (2)$$

where ADCI is the apparent digestibility coefficient of the test ingredient; ADCT, the apparent digestibility coefficient of the test diet; ADCR, the apparent digestibility coefficient of the reference diet; DR, the percentage nutrient (or kJ/g gross energy) of the reference diet; DI, the percentage nutrient (or kJ/g gross energy) of the test ingredient; s, the proportion of test ingredient in the test diet (i.e., 0.3); and $1-s$, the proportion of reference diet in the test diet (i.e., 0.7).

1.5 Digestible Energy of Feedstuffs

Data on the DE of commonly used feedstuffs in fish diets are essential for optimization of feed formulation. The additive nature of the ADC of energy and nitrogen (Cho and Kaushik, 1990; Kim, 1989; Watanabe *et al.,* 1996a,b) makes DE values very useful in optimization of dietary formulations. Table 1.1 lists estimates of the ADC of dry matter, crude protein, lipid, and energy for a number of feed ingredients fed rainbow trout (fecal samples collected with the Guelph system). Useful estimates of ADC and DE for a large number of fish feed ingredients are also available from a number of sources (Hajen *et al.*, 1993b; Guillaume *et al.,* 1999).

The DE values approach the GE values for the high protein materials such as spray-dried blood meal, herring meal, cooked full-fat soybeans, and soybean protein concentrate, indicating a high degree of digestion and absorption. However, for feed ingredients that contain a substantial level of carbohydrate and fiber, such as yellow corn, corn gluten feed, and rapeseed meal, the DE values are less than half of the gross energy values, confirming

Table 1.1

Apparent Digestibility Coefficients and Digestible Energy Values of Feed Ingredients[a]

Ingredient	Apparent digestibility coefficient (%)			
	Dry matter	Crude protein	Lipid	Energy
Alfalfa meal	39	87	71	43
Blood meal				
Ring-dried	87	85	—	86
Spray-dried	91	96	—	92
Flame-dried	55	16	—	50
Brewer's dried yeast	76	91	—	77
Corn yellow	23	95	—	39
Corn gluten feed	23	92	—	29
Corn gluten meal	80	96	—	83
Corn distiller, dried, soluble	46	85	71	51
Feather meal	77	77	—	77
Fish meal, herring	85	92	97	91
Meat and bone meal	70	85	—	80
Poultry by-products meal	76	89	—	82
Rapeseed meal	35	77	—	45
Soybean, full-fat, cooked	78	96	94	85
Soybean meal, dehulled	74	96	—	75
Wheat middlings	35	92	—	46
Whey, dehydrated	97	96	—	94
Fish protein concentrate	90	95	—	94
Soy protein concentrate	77	97	—	84

[a]Fed to rainbow trout based on fecal samples collected with the Guelph system. Adapted from Cho and Bureau (1997).

that starch and fiber are poorly digested by rainbow trout. The DE values of the feed ingredients are also closely correlated with the dry matter digestibility. There are some feed ingredients in which as much as 50–80% of IE is lost as FE. Some grain by-products, such as ground corn, wheat, and wheat middlings, are included mainly as binders and fillers to enhance the physical characteristics of the pelleted feed. Clearly the appropriate level at which to include these poorly digested materials represents a compromise between the nutritional value of the diet and practical considerations such as the mechanical durability of the feed aggregate.

Most fish species digest protein and lipid very well. Available data suggest that for most practical ingredients, the digestibility values for protein are

comparable between species, differences being more apparent with regard to carbohydrate sources (Kaushik, 1998).

Simple sugars are also well absorbed by fish (Buddington and Hilton, 1987). However, starch in its native form, found in cereals and tubers, is poorly digested by rainbow trout (Cho and Slinger, 1979; Bergot and Breque, 1983). Thermal treatment under moist conditions (hydrothermal treatment) can lead to swelling and partial breakdown of the organization of the starch granule in a process referred to as gelatinization (Swinkels, 1985). It has been shown in numerous studies that gelatinization of starch by cooking or extrusion markedly increases the digestibility of starch for fish (Kim, 1989; Pfeffer *et al.*, 1990, Bergot, 1993), probably as a result of easier access for amylolytic enzymes to individual glucose molecules in the starch granules. It was also observed that the apparent digestibility of gelatinized starch varies inversely with the level in the diet in salmonids (Bergot and Breque, 1983; Kim, 1989; Pfeffer, 1995). Consequently, the apparent digestibility coefficient (ADC) of complex carbohydrates may not be as additive as the ADC of protein and lipid sources (Kim and Kaushik, 1992). Work by Médale *et al.* (1991) indicates that starch digestibility, and hence the provision of DE from starch to rainbow trout, can also be affected by water temperature. The ADC of carbohydrates was higher at 18°C than at 8°C. These results suggest that DE should be different at different water temperatures, especially for diets containing high starch levels.

1.6 Effect of Biological and Environmental Factors

The effect of various factors on apparent digestibility of nutrients and energy has been the topic of several investigations, yet results are often contradictory. Water temperature, salinity, fish size, and feeding level are all factors that have been the subject of studies or speculations.

1.6.1. Feeding Level and Frequency

Any change in feeding levels due to changes in water temperature or to other husbandry practices may alter the amount of total digested and absorbed energy and nutrients from feed consumed. At lower feeding levels, the apparent digestibility of protein may decrease as a result of greater relative levels of MFN. Cho and Kaushik (1990), however, presented evidence that neither the frequency of feeding nor the level of feed intake affects the ADC of dry matter, crude protein, lipid, and gross energy. The lack of effect of feeding frequency on digestibility is not surprising since the rate of passage

of feed is determined not by the frequency of feeding but rather by the fish itself, based on its needs and the chemical characteristics of the feed.

1.6.2. Water Temperature

The primary response of fish to an increase in temperature is to increase their feed intake. They also increase their metabolic rate, and one constant feature is an increase in the rate of transit of ingesta through the digestive tract with increasing water temperature (Fange and Grove, 1979), which may affect the digestibility of the diet. On the other hand, the longer gastric evacuation time may compensate for a less efficient digestive process at lower water temperatures (Fauconneau *et al.*, 1983).

Numerous studies were undertaken with rainbow trout to determine the effect of different temperatures on digestibility. Azevedo *et al.* (1998) observed a significant decrease in the digestibility of dry matter, protein, and energy of a practical diet fed to rainbow trout as the temperature decreased from 15 to 6°C. This significant decrease in the apparent digestibility coefficient of dry matter, nitrogen, and energy with decreasing temperature is in agreement with the results of Choubert *et al.* (1982), who observed a significant increase in the apparent digestibility of dry matter, nitrogen, and energy for trout when the water temperature increased from 10 to 18°C. Results from other studies, using a different feces collection system (Watanabe *et al.*, 1996a,b), also suggest that the apparent digestibility of nitrogen and energy by rainbow trout increases with increasing water temperature (from 5 to 15°C). These observations are in disagreement with the results of Cho and Kaushik (1990) and Médale *et al.* (1991), who did not observe any effect of temperature on the apparent digestibility of protein, lipid, and energy for rainbow trout between 9 and 18°C. Comparisons between results on the pattern of ADCs of nutrients and energy with changes in water temperature must be interpreted carefully. Different results from the literature on this subject could have been caused by different dietary compositions of the experimental diets, mainly different amounts of undigestible or low-digestible components, such as raw starch. Also, the amount of antinutritional factors, the technological processes used in the processing of ingredients, or the feces collection method used could also have caused these differences. Differences between studies could be due to experimental (methodological) errors. As discussed above, digestibility measurements are of relatively uncertain accuracy and reliability. Experimental errors may also be associated with differences in feed intake and, consequently, feces production between the fish at different water temperatures.

A reduction in the activity of digestive enzymes at low water temperatures or progressively greater endogenous gut losses per gram of diet could also be

responsible for the decrease in apparent digestibility with a decrease in water temperature seen in some studies. It has been shown that the effect of water temperature on apparent digestibility is the result of an effect of feed intake rather than an effect of water temperature itself (Kaushik, 1980). Feed intake of fish varies significantly with changes in ambient water temperature. At a low feed intake, endogenous gut losses can represent a greater proportion of the fecal waste. This effect would result in a decrease in the apparent, but not the "true," digestibility of the diet (Azevedo *et al.,* 1998). This hypothesis requires further investigation.

Studies have suggested differences in digestibility and nutritive value at different water temperatures of lipid sources with different fatty acid profiles. Cho and Kaushik (1990) presented the results of an experiment indicating that fish oil and plant oils (rapeseed, soybean, and linseed) were well digested (ADC, 80–95%) over a wide range of water temperatures (5 to 15°C). However, the ADCs of lard and tallow (lipid sources high in saturated fatty acids) were affected by the water temperature and lower than that of oils, suggesting a strong interaction between the melting point of the lipid employed and the water temperature on the apparent digestibility of lipids. The ADCs of lard and tallow were clearly lower at lower water temperatures, in contrast with the lack of effect of water temperatures on the lower-melting point oils. Recent observations suggest, however, that beef tallow is highly digestible and of a high nutritive value for rainbow trout provided the diet contains a certain amount of fish oil (Table 1.2). This is likely due to the synergetic effect of polyunsaturated fatty acids on the digestibility of saturated fatty acids, as found in poultry (Sibbald, 1978).

1.7 Urinary and Branchial Energy and Metabolizable Energy

Digestion of a diet leads to the absorption of amino acids, fatty acids, and sugars, which are the principal metabolic fuels for the body. Catabolism of fats and carbohydrates results in the formation of carbon dioxide and water. The catabolism of amino acids yields ammonia in addition to carbon dioxide and water. Excretion of nitrogenous waste compounds, of which ammonia amounts to about 85% in most fish species (Kaushik and Cowey, 1991), results in nonfecal energy losses since these compounds contain energy. Although ammoniotelic, fish excrete small amounts of urea. Urea cycle enzymes have been detected in several species of fish. Purine catabolism is, however, the main source of urea production in fish. Urinary excretion of

Table 1.2

Performance of Rainbow Trout (Initial Weight = 7 g/Fish) Fed Practical Diets[a]

Parameter	Water temperature			
	7.5°C		15°C	
	Diet 1	Diet 2	Diet 1	Diet 2
Lipid sources				
Fish oil, herring	16	8	16	8
Beef tallow, fancy, bleachable	—	8	—	8
Composition				
Digestible protein (DP) (%)	44.0	43.5	44.9	44.4
Digestible energy (DE) (MJ/kg)	19.5	19.9	20.9	20.8
DP/DE (g/MJ)	22.6	21.9	21.5	21.3
Apparent digestibility coefficients (%)				
Crude protein	93	93	95	95
Lipid	93	94	98	95[b]
Energy	83	85	89	89
Performance				
Weight gain (g/fish)	13.7	13.1	38.1	39.2
Feed efficiency (gain : feed; as is)	1.32	1.27	1.22	1.15
Retained energy (% digestible intake)	47	47	50	48

[a]Diets contained fish oil or a fish oil and tallow combination; reared at 7.5 or 15°C for 12 weeks. From D. P. Bureau, A. M. Harris, and C. Y. Cho. (unpublished data, 1996).

[b]Significantly different from diet 1.

other types of combustible materials, such as trimethylamine (TMA) and trimethylamine oxide (TMAO), in certain marine teleosts is also known to occur but has not been quantified under intensive culture conditions (Kaushik and Cowey, 1991). All these nonfecal energy losses, mainly through the gills (branchial energy loss; ZE) and some through the kidneys (urinary energy loss; UE), are unaccounted for by the DE value of a diet, meaning that the DE value of a diet overestimates its actual energy value to the fish. The physiologically available fuel value of the diet to the fish is the metabolizable energy (ME) value, defined as follows:

$$ME = IE - (FE + UE + ZE)$$

In the rainbow trout, endogenous (branchial and urinary) nitrogen excretion (UNe + ZNe) rates measured in fish after 3 to 4 days of fasting have been found to vary between 80 and 130 mg N/kg body weight/day (endogenous UE + ZE = 2.0–3.2 kJ/kg/day), affected most by water temperature

and body weight (Watanabe and Ohta, 1995; Kaushik, 1998). Some recent studies with Atlantic salmon suggest that the values might be much lower (Forsberg, 1997). With regard to marine fish, data of Ballestrazzi *et al.* (1994) and of Dosdat *et al.* (1996) also show that the UNe rates in European seabass, gilthead seabream, or turbot would be in the range of 100 to 160 mg N/kg/day (endogenous UE + ZE = 2.5–4.0 kJ/kg/day), comparable to the values found for rainbow trout (Kaushik, 1998).

Although nonfecal nitrogen losses contribute significantly to the environmental load in terms of ammonia nitrogen, from the point of view of energy balance, their contribution is small, generally no more than 3% of the ME (Kaushik, 1998).

1.7.1. Measurement

Direct determination of the ME values of fish diets is technically difficult because of the need to measure both branchial and urinary losses released into the aquatic environment in which the fish live. Smith (1971) attempted to overcome these difficulties and developed a procedure which allowed the estimation of the ME values of a number of feedstuffs using rainbow trout 165–530 g in body weight. Before the assays, the fish were anesthetized to allow the insertion of a cannula for urine collection. The fish were then confined in a tank with a diaphragm separating the front from the rear portion of the body; they were force-fed the feed as a single daily meal under anesthetic. The ME values determined by this procedure as a fraction of the DE values ranged from 0.72 to 0.93 (mean = 0.87). The procedures employed to separate and collect nitrogen excreted via the gills and kidneys (including force-feeding) involved considerable handling and were stressful to the fish, which increased the loss of nitrogen (Hunn, 1982) and combustible matter. The increase in nitrogen output, together with the low food intake attained by force-feeding of a single daily meal, might be expected to result in a negative nitrogen balance and a low ratio of ME-to-DE values for many of the feed ingredients studied. This strongly suggests that energy losses via the gill and kidney were greater than would be the case for unrestrained fish feeding normally (Cho and Kaushik, 1990).

Monitoring waste in water in the rearing environment is a commonly used approach. Brett and Zala (1975) determined the diurnal pattern of nitrogen excretion of young sockeye salmon (*Oncorhynchus nerka*) by allowing ammonia to build up during alternate periodical closed–open circuit cycles. Kaushik (1980a,b) was the first to estimate the postprandial excretion rates in a flow-through system in a continuous manner using an autoanalyzer. This method allows continuous monitoring of ammonia and urea nitrogen excretion under normal physiological conditions even in larval fish (Kaushik

et al., 1982). Under these conditions, however, attention should be paid to the maintenance of a constant flow rate and the precise measurement of low concentrations of ammonia in the outlet water. Application of such a technique has revealed postprandial patterns of ammonia nitrogen excretion to be very similar among phylogenetically different species (Dosdat *et al.*, 1996; Chakraborty and Chakraborty, 1998).

Urinary cannula or noninvasive measurement of the urine flow rate in conjunction with spot sampling of urine (Curtis and Wood, 1991) is another approach that has been used to estimate the urinary excretion of glucose and UE of fish (Bureau *et al.*, 1998; Deng *et al.*, 2000).

Because direct measurement of UE + ZE requires sophisticated and time-consuming techniques, the use of an indirect method to estimate UE + ZE based on nitrogen losses by the fish is considered simpler (Cho and Kaushik, 1985). Since UE + ZE occurs mainly as nitrogenous product losses, the total nonfecal nitrogen loss, branchial and urinary, is estimated by the difference between digested nitrogen and recovered nitrogen as shown in the following expression:

$$\mathrm{ZN} + \mathrm{UN} = \mathrm{DN} - \mathrm{RN}$$

$$\mathrm{ZE} + \mathrm{UE} = (\mathrm{ZN} + \mathrm{UN})\ 24.9\ \mathrm{kJ\ g^{-1}\ N}$$

$$\mathrm{ME} = \mathrm{DE} - (\mathrm{ZE} + \mathrm{UE})$$

where ZN is branchial N loss; UN, urinary N loss; DN, digestible N intake; RN, recovered tissue N; ZE, branchial energy loss; UE, urinary energy loss; ME, metabolizable energy; and DE, digestible energy.

It has been determined that, in general, ammonia represents at least 85% of the nitrogenous wastes, whereas urea represents less than 15% (Kaushik and Cowey, 1991). The energy of combustion value of ammonia (82.3% N, by weight) and urea (46.7% N, by weight) is 20.5 kJ/g (24.9 kJ/g N) and 10.5 kJ/g (22.5 kJ/g N), respectively (Bradfield and Llewellyn, 1982). Because most nitrogen losses are as ammonia, and the difference in the amount of energy loss per gram of nitrogen between ammonia and urea is small, it has been proposed that the loss of 1 g of nitrogen by fish under normal conditions can be equated to an energy loss of 24.9 kJ.

1.8 Factors Affecting Metabolic Waste Output

The egestion of combustible matter (i.e., FE) depends on the susceptibility of the feed components to digestion and absorption by the fish, and there are few significant interactions between the feed ingredients of diets that might influence their digestibility. Thus, the DE value of an ingredient

is relatively independent of the composition of the diet in which it is fed. In contrast, the loss of combustible matter through the gills, or in the urine, depends upon a variety of factors, such as the composition of the diet (overall balance of the amino acids and digestible energy content) and other factors (physiological state of the animal, stress, etc.). As a consequence, the ME content of a given ingredient is not independent of the diet composition and conditions of the fish to which it is fed. As mentioned by Cho and Kaushik (1990), ME has significance only as long as it has been measured with respect to an animal's response to a complete diet under a given set of biological and environmental conditions.

1.8.1. Dietary Factors

The main factors affecting nonfecal energy losses are those that influence the retention of protein by the body and hence govern the loss of nitrogenous end products through the gills or in the urine. One such factor is the balance between digestible protein (available amino acid) energy and nonprotein energy of the diet. This balance is represented by the ratio of digestible protein (DP) to DE of the diet (DP/DE). Numerous studies have shown that an increase in dietary DE by an increase in dietary nonprotein energy led to a decrease in ammonia nitrogen excretion, UE + ZE, and hence to an increase in ME (Kaushik and Oliva-Teles, 1985). Studies with rainbow trout have shown that the regression slopes between nitrogen intake and nitrogen excretion as well as the basal nitrogen excretion levels are affected by the DP/DE of the diet. At a dietary DP/DE ratio of 18 mg/kJ, the relation between nitrogen excretion (Ne) and nitrogen intake (NI) was $75.1 + 0.307 \times$ NI, and at the higher DP/DE ratio of 23 mg/kJ, the relation was 84.9 + 0.343 x NI (Kaushik, 1998). With regard to marine species, there is a relative lack of quantitative data on N excretion rates as affected by dietary DP/DE levels. Available data, however, indicate that as with the salmonids, N excretion is reduced with decreasing DP/DE ratios in species such as seabass (*Dicentrachus labrax)* and seabream (*Sparus aurata)* (Kaushik, 1998; Lupatsch *et al.,* 2000). It can therefore be concluded that, in general, UE + ZE decreases as DP/DE decreases, at least within a certain range of DP/DE.

This decrease in nonfecal N excretion and UE + ZE is due to the utilization of nonprotein energy sources for meeting energy requirements, resulting in a reduction in catabolism of a certain proportion of amino acid for energy purposes. This phenomenon is referred to as "protein-(amino acid) sparing." Protein-sparing by lipids has been shown to occur in a majority of fish species. Protein-sparing by digestible carbohydrates such as glucose and gelatinized starch is more limited and the object of continuing studies.

The amino acid composition of the diet is another factor that has a determinant effect on the efficiency of nitrogen utilization and UE + ZE. Feeding amino acids in excess of the requirement will result in catabolism of the amino acid, with associated excretion of ammonia and loss of energy. The total digestible nitrogen retention efficiency rarely exceeds 50% in rainbow trout (60% in Atlantic salmon) fed diets with very low DP : DE ratios (16 g DP/MJ DE) with a good amino acid balance. It is not clear to what extent this significant catabolism of amino acids, despite an ample supply of nonprotein energy, is related to maintenance requirements, imbalances, or inevitable catabolism of amino acids.

It has been observed that fish exhibit persistent postprandial hyperglycemia, either after being fed an excessive amount of digestible carbohydrates or after experimental administration of glucose (Bergot, 1979; Furuichi, 1988). Excretion of glucose in the urine (Yokote, 1970; Kakuta and Namba, 1989; Furuichi, 1988; Bureau *et al.,* 1998; Deng *et al.,* 2000) as well as through the gills (Hemre and Kahrs, 1997) has been detected in hyperglycemic fish. Bureau *et al.* (1998) showed that rainbow trout that had levels of blood glucose exceeding a certain threshold for renal excretion (ca. 5–10 m*M*) excreted very significant amounts of glucose in their urine and consequently had significantly increased UE+ZE values. The excretion of glucose in the urine means that diets containing high levels of digestible carbohydrate may have a ME content lower than that calculated only on the basis of nitrogenous waste energy excretion (Bureau *et al.,* 1998).

1.8.2. Other Factors

Feeding level and water temperature do not appear to have any effect on the ME/DE ratio of diets (Kaushik, 1980a; Azevedo *et al.,* 1998; Rodehutscord and Pfeffer, 1999). Interspecific differences in nitrogen excretion and consequently ME are little studied. Dias *et al.* (1999) observed significant differences in efficiency of N retention in seabass and rainbow trout fed similar diets. Marine fish species appear to retain a much lower proportion of the digestible protein fed to them than do salmonid fish species and therefore have significantly higher UE + ZE values (Kaushik, 1998). Differences in N retention efficiency are also evident between salmonid fish species. Atlantic salmon appear to retain a greater proportion of the digestible protein than do rainbow trout when these two species are fed similar diets (Azevedo, 1998). Available data do not appear to indicate any significant influence of genetic origin (strain, family, ploidy) on nitrogen excretion per unit N intake (Kaushik *et al.*, 1984; Oliva-Teles and Kaushik, 1988).

1.9 Heat Production

As discussed earlier, heat is liberated by animals as a consequence of the transfer of the chemical energy of nutrients to energy-rich molecules, during the metabolic transformation of dietary substrates into biologically important substances, or during the hydrolysis of ATP to do physical or chemical work. The rate at which heat is liberated is an indication of the intensity of ongoing reactions. This is designated the metabolic rate (Kleiber 1975). Heat is liberated by animals as a consequence of the metabolic transformation of dietary substrates into tissue components, as a result of tissue turnover, and as a result of physical activity. The metabolic rate varies with the qualitative and quantitative intakes of energy and nutrients and the activity of the animal. An important concept is that of basal metabolism, which is the minimum rate of metabolic activity needed to sustain the structure and function of the body tissues. This has also been termed the minimal metabolism (Blaxter, 1989). The ingestion of feed increases the metabolic rate as a consequence of the extra work required for the ingestion, digestion, and metabolic utilization of the components of the diet. This increase is termed the "heat increment of feeding" (HiE). Physical activity also increases the metabolic rate due to work done. These three components of animal metabolism lead to the release of energy as heat from the ME derived from the diet, and clearly, energy released as heat is not available for growth (increase in body energy).

1.9.1. Methodological Approaches

The nutrients absorbed from the digestive tract are either catabolized or stored as new tissue components. The energy released by catabolism of the nutrients is ultimately released as heat, so that the energy balance can be determined either by measuring the heat production (HE) or by estimating the change in total body energy content from weight and whole-body chemical composition. The latter system is referred to as body balance and requires observations over an appreciable portion of the animal's growth phase. Animal calorimetry is the method preferred by nutritionists for measuring the energy balance of animals, notably over short time periods. Classically, calorimetry is the measurement of heat flow between two objects, in this case from the animal to the environment.

HE can be measured by two main methods: direct calorimetry and indirect calorimetry. Direct calorimetry measures the heat dissipated by an animal (conduction, convection, or radiant), whereas indirect calorimetry, as its name suggests, estimates HE indirectly, generally through measurement

of respiration (oxygen consumption, carbon dioxide production), but can also, by definition, include other approaches (e.g., comparative carcass analysis).

Comparisons of direct and indirect calorimetry using respiration calorimeters, i.e., systems that permit simultaneous determination by direct and indirect calorimetric techniques, have been carried out for both animals and humans. In general, comparisons demonstrate a good agreement between the two measurements, confirming that the body has no source of energy other than that released by oxidation. A number of reviews have evaluated the relative merits of both direct and inirect calorimetry to measure the HE of fish (Cho and Kaushik, 1985, 1990; Tytler and Calow 1985). For fish bioenergetic studies, the indirect calorimetric studies are reliable, more practical, and less expensive than direct calorimetry.

1.9.2. Direct Calorimetry

A direct calorimeter was designed by Smith *et al.* (1978a,b) to measure the heat production of fish. However, the estimates of HE measured with this calorimeter appear to be unrealistically high, which suggests that this type of calorimeter is less sensitive than methods based on measurements of oxygen consumption (Brett and Groves, 1979; Cho *et al.,* 1982). Direct determination of HE requires the measurement of small temperature changes in water resulting from changes in metabolic rates after feed ingestion. This technique, although elegant, is very difficult to apply to fish, as fish produce only a limited amount of heat and the heat absorption capacity of water is very high. This requires very precise instruments to record temperature changes. The needs to exchange water to eliminate metabolic wastes and to provide oxygen through aeration of the water are also factors that make it extremely difficult to ensure that changes in temperature are really due to the metabolism of the fish and not to other factors.

1.9.3. Indirect Calorimetry

An indirect method of estimating heat production is to monitor respiratory exchanges (oxygen consumption, carbon dioxide excretion) and nitrogenous waste excretion of animals. The total heat production is the sum of the enthalpies of oxidation of the nutrients utilized by the animal. Heat production can therefore be estimated from the stoichiometry of nutrients catabolized. Heat production of a mixture of compounds representative of the three classes of energy-yielding nutrients can, therefore, be calculated from equations where the molar enthalpies of the reactions are determined

by the molar amount of oxygen consumed, carbon dioxide produced, and nitrogen excreted. A comprehensive demonstration of this is presented by Blaxter (1989).

In the majority of studies with fish, carbon dioxide production is ignored, and only oxygen consumption is measured. The amount of heat produced for each liter of oxygen (oxycalorific coefficient) used in metabolism changes little, whether lipid, carbohydrate, or protein is oxidized. The highest figure (21.2 kJ per liter of oxygen for carbohydrate metabolism) and the lowest (19.2 kJ per liter of oxygen for protein) differ by less than 10%. An intermediate value of 20.1 kJ per liter of oxygen has been used to estimate the heat production of warm-blooded animals (Blaxter, 1989). Because a larger proportion of the heat produced by fish is derived from the catabolism of amino acids and lipids than in warm blooded animals, the value of 19.4 kJ per liter of oxygen (13.6 kJ/g oxygen) was adopted to estimate the heat production of fish (Cho and Kaushik, 1990). Since the determination of HE is never very precise, application of a single oxycalorific coefficient for all conditions (fish species, diet composition, fed or fasting measurement) is considered acceptable (Cho and Kaushik, 1990) and measurements of carbon dioxide and N excretion, in most cases, are unnecessary (Dersant-Li *et al.,* 2000).

Measurement of both oxygen consumption and carbon dioxide production, nevertheless, allows the calculation of respiratory quotients (volume or mole of carbon dioxide produced per volume or mole of oxygen consumed). Knowledge of the respiratory quotient (RQ) can allow estimation of the relative proportions of fats and carbohydrates used for energy purposes. Precise knowledge about the substrate used for energy purposes depends on measurement of the excretion of nitrogenous waste output to estimate the amount of protein metabolism and subsequent calculation of the nonprotein respiratory exchange to estimate the proportion of lipids and carbohydrates metabolized. On the assumption that all the nonfecal (gill and urinary) nitrogen comes from protein and that protein contains 16% nitrogen, the mass of metabolized protein can be calculated by multiplying the mass of nonfecal nitrogen by 6.25. The liters of oxygen consumed in protein metabolism can be calculated by multiplying the mass of branchial and urinary nitrogen in grams by 5.91; the liters of carbon dioxide produced, by multiplying the mass of branchial and urinary nitrogen in grams by 4.76. Subtraction of these volumes from the total leaves the nonprotein respiratory exchange. A nonprotein RQ of 0.7 would indicate that 100% of the heat produced was from lipid catabolism, whereas a nonprotein RQ of 1.0 would indicate 100% carbohydrate metabolism. A nonprotein RQ >1 would indicate lipid synthesis from carbohydrate. There are, nevertheless, numerous limitations to the estimation of substrate utilization through RQ analysis. These estimates should be used with caution.

Several indirect calorimeters for fish have been described (O'Hara, 1971; Brett, 1972; Solomon and Brafield, 1972; Pierce and Wissing, 1974; Cho *et al.*, 1975; Hogendoorn *et al.* 1981). Most of these calorimeters allow measurements of oxygen consumption of fish under varying degrees of physical constraint. Some earlier investigations have been concerned with the energy cost of swimming by the fish and have largely ignored the level of feeding and the type of diet. Characteristically, these studies measured oxygen consumption by a fish swimming involuntarily either without food or after receiving a single meal, often from a poorly characterized diet. Calculation of heat production from oxygen consumption measured in these circumstances is of highly questionable value (Cho *et al.*, 1982). An undefined fraction of the heat is derived from the catabolism of feed components, with the balance being provided by breakdown of body tissue. This would preclude definitive statements about the energy cost of swimming for fish in their natural surroundings or under the conditions employed for intensive fish culture. Data derived from restrained fish or fish forced to swim may also not always be representative of fish under culture conditions. A relatively secure environment for the fish is, therefore, desirable to produce meaningful results (Cho *et al.*, 1982). A complete description of the energy balance of the fish requires the measurement of food intake together with fecal and other excretal losses to allow energy to be partitioned on the basis of DE or ME intake.

1.9.4. Comparative Carcass Analysis

For practical purposes, it is not always feasible to measure heat losses because of the complexity and cost of fish respirometry. Cho and Kaushik (1985) presented a simple scheme based on the use of comparative carcass analyses (Table 1.3). Using this technique, HE of fish is estimated

Table 1.3

Comparative Carcass Analysis Scheme[a]

1.	Estimate digestible N (DN) and digestible energy (DE) intakes
2.	Measure recovered nitrogen (RN) and recovered energy (RE) in carcass
3.	Measure carcass N (Nf) and energy (HEf) losses during fasting (ca. 7–10 days)
4.	Calculate nonfecal N losses (UN+ZN) = DN − RN
5.	Estimate nonfecal energy losses (UE+ZE) = (UN+ZN) 24.9 kJ g^{-1}
6.	Calculate metabolizable energy (ME) intake = DE − (UE+ZE)
7.	Calculate heat production (HE) = ME−RE
8.	Calculate heat increment of feeding (HiE) = HE − HEf
9.	Calculate net energy (NE) = ME−HiE

[a]Proposed by Cho and Kaushik (1985).

by the difference between IE and FE, UE + ZE, and energy deposition in the carcass (RE). Partition of energy between basal metabolism can be estimated by measuring the carcass energy loss during fasting. Generally this rather simple slaughter technique is very suitable and reliable for fish compared to terrestrial animals, because large numbers of fish can be sampled and easily homogenized for the analyses. The results are in good agreement with those of indirect calorimetry. Examples of studies that have used such approaches are those by Cho and Kaushik (1985), Azevedo *et al.* (1998), Lupatsch *et al.* (1998), and Rodehutscord and Pfeffer (1999).

1.9.5. Other Approaches

A number of other methods can be used to study the energy metabolism of fish. Some of the most interesting involve stable isotopes. The occurrence in nature of stable isotopes of certain elements which are chemically indistinguisable, but which can be separated and measured on the basis of their different mass, has been exploited in biological research for over 50 years (Prentice, 1999). The existence of stable isotopes of the chief elements of organic molecules (C, H, O, and N) allows many applications for stable isotopes, notably in the quantification of nutrient flux and turnover and energy expenditure. Doublylabeled water (DLW) has been widely used in the study of energy metabolism of terrestrial animals (Prentice, 1999). Food or ingredients naturally or artificially enriched in certain stable isotopes have been used to study the nutrition of fish and shrimp in the wild and under aquaculture conditions (Preston *et al.,* 1996, Primavera, 1996). These methodological approaches are bound to gain a lot of popularity in fish nutrition and bioenergetics research.

Nuclear magnetic resonance (NMR) of certain elements, such as phosphorus and hydrogen (^{31}P NMR and ^{1}H NMR), has been used for short-term studies of tissue energetics of animals (Gadian, 1992; Ginneken, *et al.,* 1995). Elements, such as phosphorus and hydrogen, have intrisic magnetic properties and will resonate differently depending on the local magnetic field experienced by their nuclei. NMR spectroscopy makes use of the observation that nuclei in different chemical environments give rise to signals of slightly different frequencies (Gadian, 1992). The concentration of different compounds, such as inorganic P, ATP, and phosphocreatine, can be measured in intact organisms or biopsies and used to estimate the energy expenditure of tissue over short periods of time.

Other methods, such as the use of physiograph and transponders to measure muscle activity or heart rate, have also been suggested for estimating HE of fish and crustaceans (Villarreal, 1990; Höjesjö *et al.,* 1999).

1.10 Minimal Metabolism

Animals require a continuous supply of energy for those functions of the body immediately necessary for maintaining life, regardless of whether or not feed is consumed. A major portion of this energy is spent for basal metabolism (HeE); a smaller portion of energy is spent for voluntary or resting activity (HjE), such as minor bodily movements and muscular activity, and regulation of body temperature (HcE) in the case of homeotherms.

HeE represents the use of energy for things such as circulation of the blood, pulmonary ventilation, repair and replacement of cells, membrane transport of ions (especially of sodium and potassium), and muscle tone. Under basal conditions all the energy released by these processes appears as heat. In fish, HeE is clearly related to both body weight and temperature. To make comparisons between animals or between species, the conditions under which the measurements are made must be standardized. This is achieved by attempting to measure a minimum rate of heat production free of the effects of any controlling factors known to increase it. Such factors include exercise (voluntary movement), the consumption of feed and its subsequent metabolism, and the physical environment. The object of standardization is to ensure comparability of estimates rather than to establish some absolute minimum value of metabolism that is compatible with life. A number of terms have thus arisen to describe these standardized measurements of "minimal metabolism." With domesticated animals, and hence fish under aquaculture conditions, what is usually measured is the fasting heat production (HEf) (Blaxter, 1989). HEf is also know as standard metabolism in the fish biology literature (Elliott, 1982).

It is difficult to ensure that fish are in a state of muscular repose because they need to maintain their orientation in the water and this entails some muscular activity. It has been suggested that basal metabolism be measured by extrapolation to zero activity from fish swimming at different rates (Smith, 1989). However, fish such as rainbow trout will spend considerable periods resting on the bottom of their tanks, maintaining their position in quiet water with minimal activity. Similar situations arise more commonly with flatfish, such as turbot and halibut. HEf has been regarded as a close approximation of minimal or basal metabolism (Cho and Kaushik, 1990). Oxygen consumption of free-swimming fish fasted for 3 to 7 days to eliminate the effect of the feed consumed and its subsequent metabolism is the most common approach for measuring HEf (Kaushik and Médale, 1994; Cho and Bureau, 1995). Measuring carcass energy losses during fasting is another common method of estimating HEf and, consequently, HeE

Table 1.4

Minimal Metabolism of Rainbow Trout (15°C) and Homeotherms (37°C)[a]

Live weight (g/fish)	Minimal Metabolism (kJ fish^{-1} day^{-1})[b]					
	1	2	3	4	5	6
1	0.1			0.2	1.3	1.6
5	0.5	8.6	1.1	0.7	2.6	5.5
10	0.8	13.3	3.8	1.0	3.5	9.3
50	3.1	36.5	6.5	2.8	7.0	31.0
100	5.5		21.6	4.3	9.5	52.1
500	20.7			11.9	19.0	174.0
1000	36.6			18.4	25.5	293.0

[a]According to different sources. Adapted from Cho and Bureau (1998).

[b]Key to References: (1) Cho (1991): HEf = $(-1.04 + 3.26T - 0.05T^2)$(kg BW$^{0.824}$) kJ day^{-1}; fasting heat production (HEf) estimated by indirect calorimetry. (2) NRC (1981): 241 (kg BW$^{0.63}$) kJ day^{-1}; estimate of HeE. (3) Smith *et al.* (1978a): 201 (kg BW$^{0.75}$) kJ day^{-1}; HEf estimated by direct calorimetry. (4) Smith (1989): 18.4 (kg BW$^{0.63}$) kJ day^{-1}; estimate of HeE. (5) Rodehutscord and Pfeffer (1999): 1.31 (g BW$^{0.43}$) kJ day^{-1}; estimate of HEm obtained through comparative carcass analysis and factorial model, DE = HEm + (RE/K_{pf}). (6) Kleiber (1975): 293 (kg BW$^{0.75}$) kJ day^{-1}; estimate of HEf for homeotherms.

(Cho and Kaushik, 1985). Both approaches have been shown to result in similar estimates of HeE for rainbow trout (Bureau, 1997).

There are literally hundreds of reports on the HEf of different species of fish under various conditions. Unfortunately, there are serious disagreements in the estimates of HEf or HeE of fish in the literature (Table 1.4). This variation is often due to differences in the methodologies involved and the weight scaling exponent (metabolic body weight). Available data on the HEf values of fish show that, for a given weight, they are 5- to 20-fold lower than that of terrestrial vertebrates. Data from Kaushik and Gomes (1988), Cho (1991), and Bureau (1997) suggest a HEf of approximately 30–40 kJ (BW$^{0.824}$)$^{-1}$ day^{-1} for rainbow trout at between 15 and 18°C. In comparison, a HEf between 170 and 590 kJ (BW$^{0.75}$)$^{-1}$ day^{-1} has been reported for domestic animals (Blaxter, 1989). The low HEf of fish can be attributed to the lack of expenditure for thermoregulation; the lower sodium

pump activity; their aquatic mode of life, experiencing neutral buoyancy, which eliminates the need to oppose gravity; and the mode of nitrogen excretion (ammoniotelism).

Analysis of energy loss and the corresponding changes in energy-yielding substrates under fasting or protein-free feeding conditions shows that in all fish, amino acid catabolism contributes greatly to the provision of energy. From a comparative point of view, while the HeE of fish is low, the participation of body protein degradation in meeting such needs is higher in fish than in all terrestrial vertebrates studied so far. The proportionate loss in terms of N per unit energy loss is about 0.2 mg N kJ^{-1} in higher animals, whereas a number of studies undertaken with different fish species including salmonids show that the values are more than 10-fold higher (2 to 5 mg N $kJ^{-1)}$. The variability observed results from the differences in the duration of fast, water temperature, and size of fish (Kaushik and Luquet, 1977; Kaushik and Médale, 1994).

The values reported in the literature for marine fish differ from one species to another more than those for freshwater species; flatfish are considered to have reduced metabolic rates. The influence of water salinity on the basal metabolism of fish is the subject of controversy. Most experimental data suggest that the energy cost of osmoregulation is very low, equivalent to less than 5% of the basal metabolism (Morgan and Iwama, 1999).

1.10.1. Effect of Body Weight

In poikilotherms as well as in homeotherms, the HeE in absolute term (kJ $animal^{-1}$ $day^{-1)}$ increases with the mass of the animal. The logarithm of the HeE increases in a linear way with the logarithm of the body mass (Blaxter, 1989). However, the slope of this relation is <1. This means that in all the species, animals of smaller size spend more energy per unit of mass than larger animals.

The relationship of body weight to metabolic rate in animals can be described by the general equation $Y = aW^b$, where Y is the metabolic rate, W is the body weight, and a is a constant which is dependent on species and temperature. The value of the exponent for fish has been reported to range from 0.50 to 1.00. Hepher (1988), who reviewed experimental data from the literature, concluded that the exponent 0.8 describes, with reasonable accuracy, the change in metabolic rate with size of several fish species. Detailed observations by Brett and Groves (1979) and Hogendoorn (1983) for African catfish (*Clarias gariepinus)*, Cui and Liu (1990) for six teleost species *(Cyprinus carpio, Oreochromis mossambicus, Pseudobagrus fulvidraco, Carassium*

auratus, Macropodus chinensis, Pseudorasbora parva), Cho (1991) for with rainbow trout, Sanchez *et al.* (1993) for turbot *(Scophthalmus maximus)*, Lupatsch *et al.* (1998) for gilthead sea bream (*Sparus aurata* L.), and Liu *et al.* (2000) for mandarin fish *(Siniperca chuatsi)* and Chinese snakehead *(Channa argus)* suggest that across species the exponent is >0.7 and <0.9. It thus appears reasonable to assume that metabolic body weight (MBW) can, in practice, be calculated as $kg^{0.8}$ for most species.

1.10.2. Effect of Temperature

Fish are poikilothermic animals and water temperature, therefore, is the major factor determining their metabolic rate and energy expenditure. Variations in water (environmental) temperature have a great effect on their basal metabolism. Based on a mathematical analysis of oxygen consumption data from several trials, Cho and Kaushik (1990) observed that the HEf of rainbow trout as a function of water temperature could be described as:

$$\text{HEf} = (-1.04 + 3.26T - 0.05T^2)\ (\text{BW}^{0.824})^{-1}\ \text{day}^{-1} \qquad (3)$$

where HEf is the fasting heat production (kJ), T is the water temperature (°C), and BW is the body weight (kg).

Clarke and Johnson (1999) observed a curvilinear relationship between metabolic rate and temperature based on analysis of data from 69 teleost fish species. This suggests that the HeE of warm-water species (e.g., tropical species) is considerably higher than that of cold-water fish (e.g., Arctic fish species). This, however, does not appear to be the case when one examines experimental evidence for salmonids (Cho and Kaushik, 1990) and other cold-water species (Kaushik, 1998) and warm-water species such as mandarin fish [*Siniperca chuatsi,* (Liu *et al.,* 2000)], Chinese snakehead [*Channa argus* (Liu *et al.,* 2000)], and gilthead sea bream (Lupatsch *et al.,* 1998). At their optimal growth temperature the basal metabolisms of these cold and warm-water species appear to be fairly similar (Kaushik, 1998; Médale and Guillaume, 1999). These different conclusions may be related to the fact that the analysis by Clarke and Johnson (1999) was based on a survey of published data from 69 species with only one temperature per species, defined as the "experimental temperature most representative of that experienced in the wild." Using this approach, a statistically significant curvilinear relationship is seen but is mostly the result of low metabolic rates for polar species (water temperature, <5°C) and higher metabolic rates for certain fish species between 35 and 40°C. More detailed studies with polar, temperate, and tropical species show no clear relationship between preferred environmental temperature and HEf or HeE across species (Médale and Guillaume, 1999).

Within a species, increasing water temperature results in a curvilinear (almost-linear) increase in HeE up to a certain level (generally around the optimum temperature for growth), where an increase in temperature results in no increase in or, even, a depression of HeE. When the temperature is elevated further, fish stop feeding, and with further elevation metabolic disorders lead to death (upper lethal temperature). The upper lethal temperature varies with species and strains within a species. For some species it may be as high as 40°C.

Conversely, the metabolic rate of fish is reduced when the water temperature and consequently the body temperature of the fish are reduced. This reduction continues until the lower lethal limit is reached and the fish dies. This lower limit differs with species. For some species it may be as low as about 0.5°C and for some Polar fish it is below the freezing point of water.

1.10.3. Maintenance Requirement

The maintenance energy requirement (HEm) is generally defined as the amount of energy required for an animal to maintain zero energy balance (zero energy gain, RE = 0). This amount of energy can be defined as the basal metabolism plus the heat increment of feeding (HiE) associated with utilizing nutrients from feed to cover energy losses associated with basal metabolism.

The most commonly used method for estimating HEm consists of feeding fish at different levels and using regression of RE as a function of DE or ME intake that results in zero carcass energy gain (RE = 0). In a study with rainbow trout (18°C; IBW, 150g), the estimation of HEf based on oxygen consumption was found to be between 46 and 60 kJ kg^{-1} day^{-1} (Kaushik and Gomes, 1988). The calculated HiE in these fish fed a maintenance ration was 16 to 24 kJ kg^{-1} day^{-1}. Maintenance energy requirements calculated using comparative carcass analysis were between 85 and 110 kJ ME kg^{-1} day^{-1} (Kaushik and Medale, 1994). Data from Storebakken *et al.* (1991) suggest a HEm of 42 kJ ME kg^{-1} day^{-1} for rainbow trout (300-g live weight) at 15°C. The HEf of these fish should be about 29 kJ ME kg^{-1} day^{-1} according to the equation of Cho and Kaushik (1990), above [Eq. (3)]. Lupatsch *et al.* (1998) observed a HEf of 42.5 kJ $(BW^{0.83})^{-1}$ day^{-1} and a HEm of 55.8 kJ DE $(BW^{0.83})^{-1}$ day^{-1} for gilthead sea bream *(Sparus aurata)* at 24°C. McGoogan *et al.* (1998) observed, a HEm of 92–97 kJ DE BW^{-1} day^{-1}, for red drum *(Sciaenops ocellatus)* weighing about around 50 g and kept at 25°C. This corresponds to about 50 kJ DE $(BW^{0.8})^{-1}$ day^{-1}. Watanabe *et al.* (2000) observed a HEm of 82 kJ DE BW^{-1} day^{-1} for 700 g yellowtail *(Seriola quinqueradiata)* at 15°C, corresponding to 77 kJ DE $(BW^{0.8})^{-1}$ day^{-1}.

Table 1.5

Estimates of Maintenance (HEm), Cost of Protein (K_p), and Lipid Deposition (K_f)[a]

Species	HEm	K_p	K_f	Ref.
Common carp	42 kJ $kg^{0.75}$ day^{-1}	0.56	0.72	Schwartz and Kirschgessner (1995)
European sea bass	42 kJ $kg^{0.79}$ day^{-1}	0.54	0.91	Lupatsch (2000)
Gilthead sea bream	59 kJ $kg^{0.83}$ day^{-1}	0.47	0.66	Lupatsch (2000)
Grouper	35 kJ $kg^{0.83}$ day^{-1}	0.44	0.91	Lupatch (2000)
Rainbow trout	1.37 kJ $g^{0.39}$ day^{-1}	0.54	0.90[b]	Rodehutscord and Pfeffer (1999)

[a]Determined using the factorial approach of Kielanowski (1965) for different fish species.
[b]Fixed.

The balance of evidence suggest that the HEm of fish reared at their optimum growth temperature is about 40–60 kJ ME $(BW^{0.8})^{-1}$ day^{-1} (see Table 1.5). Estimates of HEm obtained across studies (i.e., within species) may have relatively large variances. However, this variance, in absolute terms (i.e., kilojoules), is, in practice, negligible due to the low HeE and HEm of fish. The variance of HEm can be of about the same order of magnitude as the experimental errors associated with its measurement. Several factors, such as diet composition and methodological approach (approach, scaling factor used to calculate metabolic weight, regression model used, use of arithmetic or geometric means, etc.), may have a significant impact on the estimate of both HeE and HEm. It must also be noted that at zero energy gain (RE = 0), fish still deposit body protein (positive proteinenergy gain) and mobilize body lipids (negative nonprotein energy gain) and, thus, still gain live weight. This phenomenon is observable in all young animals fed a maintenance ration that is adequate in protein (Blaxter, 1989). This illustrates well that it is rather meaningless to feed a "maintenance ration" as is done in many studies. Many have argued that the concept of maintenance is an illogical concept for growing animals and that it should be discarded or replaced. Others have argued that the concept of maintenance, while far from perfect, is still very useful in practice (Baldwin and Bywater, 1984).

1.10.4. Heat Losses Associated with Activity

Fish have an efficient mode of transportation. Their bodies are supported by water and they do not need to expend energy to oppose gravity like land animals. A streamlined body moving through the water is the most efficient form of transportation. Many bioenergetics studies have focused on the cost of swimming for fish (see Brett and Groves, 1979). Generally these studies have been conducted in swim tunnel respirometers. A study by

Krohn and Boisclair (1984) suggests that the costs associated with the activity of free-swimming fish are considerably higher than those predicted using metabolic cost and speed from forced-swim respirometers. This work also provides indications that the metabolic costs of turning and accelerating may be substantial.

Estimation of the energy costs of activity may be very significant for fish in the wild, as these animals often need to expend a considerable amount of energy to acquire food. Under aquaculture conditions, the amount of energy spent on activity is probably fairly small. Energy losses associated with activity are difficult to estimate separately from basal metabolism, as there is always a certain amount of voluntary activity in any group of fish (Cho *et al.*, 1982). It may therefore be concluded that when constructing the energy budget of free-swimming fish under normal aquaculture conditions, the cost of activity is rather negligible and is already included in the estimate of HeE.

1.11 Heat Increment of Feeding

Ingestion of feed by an animal that has been fasting results in an increase in the animal's HE. This expenditure of energy due to feeding is referred to as the heat increment of feeding (HiE). This component of the energy budget is also known as extra heat, specific dynamic action (SDA), calorigenic effect, and dietary thermogenesis.

The length of time for which consumption of diet exerts an influence upon HE depends upon many factors; chief among these are the quantity and quality of the diet, the water temperature, and growth (nutrient deposition) by the animal. Saunders (1963) found that the oxygen consumption of Atlantic cod (*Gadus morhua*) remained above the fasting level for 7 days at 10°C following the consumption of a large meal of herring, whereas in water at 15°C the effect lasted for only 4–5 days. Kaushik and Dabrowski (1983) similarly found that in developing larvae and juveniles, the increase in oxygen uptake corresponded more or less to the rate of transit of feedstuffs in the digestive tract. The HiE depends to a large extent upon the balance of dietary nutrients and the plane of nutrition (Brody, 1945). Therefore, attempts to measure the HiE of individual feed ingredients which give a nutritionally unbalanced diet (Smith et al., 1978b; Tandler and Beamish, 1979) or measurements of the effect of fish size (Beamish, 1974) and fish density (Medland and Beamish, 1985) made under forced-activity conditions have very doubtful meaning. Similarly, to estimate the HiE of animals without reference to the growth and nutrient deposition (energy or protein and lipid deposition) (e.g., Ross *et al.*, 1992) is also inappropriate.

HiE has also been related to digestible nitrogen intake, equaling about 27 to 30 kJ per gram of digestible nitrogen intake (Cho and Kaushik, 1990). Data on common carp, or tilapia, suggest figures much higher than those found for rainbow trout (Kaushik, 1998). In the European sea bass, much lower values, in the range of 13 to 15 kJ per gram of digestible nitrogen intake, are found (Kaushik, 1998). Data obtained for rainbow trout having different growth or protein deposition rates (Médale and Guillaume, 1999) seem to indicate that there is a good correlation between protein retention and HiE.

Many studies have shown highly significant linear (or largely linear) relationships between ME intake and RE as well as between digestible N (DN) intake and carcass N gain (RN). In most studies, the slope of RE as a function of ME intake is between 0.40 and 0.70 (Meyer-Burgorff *et al.,* 1989; Cui and Liu, 1990; Azevedo *et al.,* 1998; Lupatsch *et al.,* 1998; Médale *et al.,* 1998; Ohta and Watanabe, 1998; Rodehutscord and Pfeffer, 1999; Lupatsch *et al.,* 2000; Peres and Oliva-Teles, 2000). The slope is often identified as the "efficiency of metabolizable utilization for production," K_g or K_{pf}. HiE appears, therefore, to be equivalent to 30–60% of ME intake or 40–150% of RE in fish fed balanced diets. This is a significant range, and much of the variability seen is likely associated with species, diet composition, digestibility estimation method (e.g., stripping vs Guelph system), estimate of HEm, and composition of weight gain (protein vs lipid).

Interestingly, for a given diet, HiE expressed as a proportion of ME intake or RE does not appear to vary significantly with water temperature or feeding levels (Azevedo *et al.,* 1998; Lupatsch *et al.,* 1998, 2000; Rodehutscord and Pfeffer, 1999), as show in Fig. 1.2. There is some evidence that at very high feeding levels, protein deposition tends to level off (plateau), while lipid deposition continues to increase linearly. At that point, HiE as a proportion of ME or RE appears to increase significantly.

It is noteworthy that the highly linear relationship between ME intake and RE (and consequently HiE) is purely empirical in nature and appears to be applicable only to a certain set of conditions (same species, same diet composition). Studies with farm animals suggest that HiE may be more appropriately quantified as a factorial function of protein and lipid deposition rates (Emmans, 1994). This is discussed later in this chapter. Protein and lipid oxidation rates also appear to contribute to HiE (Cho and Kaushik, 1990). There is a need to understand and quantify better the various factors determining HiE in fish. The factors contributing to HiE have classically been separated into three categories: (1) digestion and absorption processes (HdE), (2) formation and excretion of metabolic wastes (HwE), and (3) transformation and interconversion of substrates and their retention in tissues (HrE).

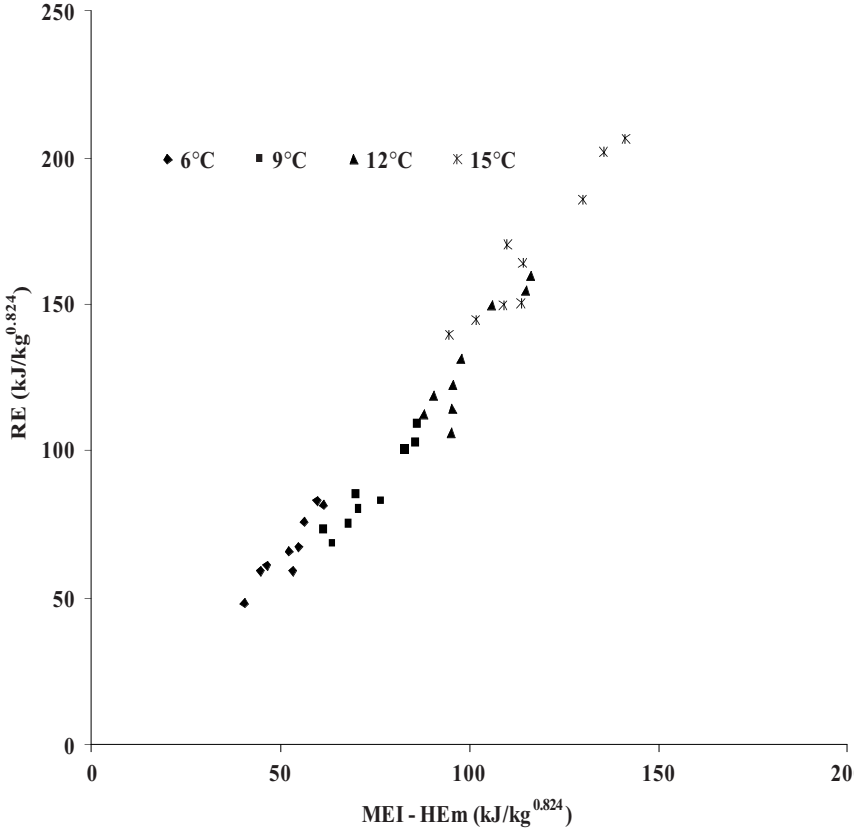

FIG. 1.2

Recovered energy (RE) as a function of the metabolizable energy intake above maintenance (MEI − HEm) of rainbow trout at various water temperatures and feeding levels. From Azevedo *et al.* (1998).

1.12 Digestion and Absorption Processes (HdE)

There have been some studies on HiE designed to separate the biochemical aspects of postabsorptive processes from the physical or mechanical aspects of feeding and digestion in fish. These studies involved either "sham feeding" or feeding nondigestible materials such as kaolin or cellulose. A practical diet was diluted to 1:5 with cellulose, and the effects of increasing meal volumes of the practical and diluted diets on the HiE of largemouth

bass (*Micropterus salmoides*) were compared. The difference between heat production resulting from the two types of diet was referred to as "mechanical SDA" (Tandler and Beamish, 1979). The mechanical SDA approached 10–30% of the total heat increment. However, Jobling and Davies (1980), in plaice (*Pleuronectes plattesa*), found that neither sham feeding nor kaolin feeding increased the resting metabolic rate of their fish. Clearly, one effect of providing feed to fasting fish is an increase in physical activity, but the magnitude of this effect in relation to the physiological effects of "normal" feed ingestion depends on the experimental procedures employed. Sham feeding and nondigestible materials elicit minimal response compared to the use of the "diluted" low-protein diet used by Tandler and Beamish (1979).

As early as 1902, Rubner showed that the "chemical work of the glands" was the source of heat, in contrast with the original belief that the increased heat production was due to "mechanical work." Rubner (1902) demonstrated that feeding bone meal to dogs did not increase heat production. This conclusion was reinforced by the observation that intravenous infusion of amino acids increased heat production to the same extent as oral administration of the amino acids (Benedict and Emmes, 1912; Borsook, 1936).

Heat losses associated with the enzymatic hydrolyses of lipid, polysaccharides, and proteins in the lumen of the gut have been estimated to be about 0.1 to 0.2% of the GE of the substrate hydrolyzed (Blaxter, 1989). Heat loss arising from anaerobic fermentation in the gut is another factor contributing to HdE. However, fermentation in most fish species is very limited, perhaps with the exception of certain marine herbivorous fish species (Clement, 1996).

The absorption of certain products of digestion, such as amino acids, peptides, and glucose, by the intestinal mucosa often occurs through an energy-dependent transport system known as active transport. Carrier proteins simultaneously transport the target molecule and a sodium ion. The maintenance of a sodium gradient across the membrane is achieved by an ATP-dependent sodium transporter working in the opposite direction. This transporter hydrolyzes one ATP molecule per every three sodium ions extruded. The cost of transport of glucose through active transport is one-third of an ATP, which is equivalent to less than 1% of the GE of glucose or about 1% of the potential amount of ATP generated by the aerobic metabolism of glucose, i.e., 36 ATPs. Absorption of lipid digestion products differs significantly: triacylglycerides (TAG) are hydrolyzed to free fatty acids (FFA) and monoacylglycerol (MAG) in the lumen. FFA and MAG are absorbed passively and TAG are resynthesized in the mucosa and exported as chylomicrons to the circulations. Synthesis of TAG and chylomicrons requires a certain amount of energy, but again, this amount represents only a small proportion of the energy content of these molecules (Blaxter, 1989).

Overall, the heat losses associated with diet ingestion and digestion (HdE) are very small compared to that associated with metabolic work (HrE + HwE) (Brody, 1945). The physiological bases of this increased heat production are the postabsorptive processes related to an ingested diet. These processes are primarily the metabolic work required for the synthesis of proteins and lipids in the tissues from the newly absorbed nutrients, metabolism of amino acids, fatty acids, and glucose, and detoxification of nitrogenous wastes.

1.12.1. Formation and Excretion of Metabolic Waste

Deamination and catabolism of amino acids lead to ammonia production. Because ammonia is toxic and cannot be rapidly eliminated by mammals and birds, these animals synthesize urea and uric acid, which are less toxic. The energy cost of synthesis of these products is 13 and 10 kJ/g N, respectively, for urea and uric acid (Martin and Blaxter, 1965). Urea and uric acid are concentrated for further excretion by the kidneys in terrestrial animals, requiring the expenditure of further energy. In contrast, ammonia is the primary waste product of protein catabolism in fish (Kaushik and Cowey, 1991). As this waste is efficiently excreted by the gills, fish generally do not require energy to detoxify or concentrate it. As a result, HwE should represent only a very small fraction of the HiE of fish. Nevertheless, a few fish species have been shown to transform significant amounts of ammonia into urea under certain conditions (Wood *et al.,* 1989; Saha *et al.,* 1999). HwE may be a more significant contributor to HiE for these fish under these very specific conditions.

1.12.2. Transformation of Substrates and Retention in Tissues

Most theoretical and experimental evidence suggests that the heat losses associated with transformation of the substrates and their retention in tissues (HrE) represent a very large proportion of the HiE in animals. There is concurring evidence that the efficiency of utilization of metabolizable energy varies with the chemical nature of the energy-yielding nutrients absorbed (Blaxter, 1989). When a fasting animal is refed, nutrients absorbed by the animal replace body constituents as the source of energy. The efficiency of utilization of ME is proportional to the ATP yield of the nutrients absorbed (Blaxter, 1989).

Growing animals accrete new tissues and some of the energy supplied by the diet is stored as protein, lipid, and glycogen. The theoretical efficiency of transformation or retention of substrates in tissue has been calculated for higher vertebrates (Blaxter, 1989; Flatt, 1992). As the metabolism of fish is very similar to that of higher vertebrates, it may be assumed that these

theoretical costs are also valid for fish. Converting glucose into glycogen costs 5% of the energy of glucose as HiE, whereas converting glucose into lipids entails an increase in HiE equal to about 30% of its GE (Blaxter 1989). Conversion of dietary lipids into body lipids is, in theory, about 96%, therefore 4% of the GE of lipids is dissipated as HiE. Conversion of dietary amino acids into body proteins is, in theory, 86% efficient, entailing a HiE of 14% (Blaxter, 1989). Conversion of amino acids into body lipids is, in theory, only 66% efficient, or 34% of energy is lost as HiE.

Determining the cost of nutrient deposition and interconversion in practice is not an easy task. Many studies have approached the problem in an empirical manner by trying to relate ME to RE (or HiE) and then trying to delineate the various determinants of HiE. The most popular approach is a factorial one that was first proposed by Kielanowski (1965): multiple regression of ME intake as the independent variable, with protein and lipid energy deposition rates as the dependent variables to determine the partial energy costs for protein and lipid deposition (Reeds, 1991). In the classical factorial approach, the energy cost of lipid and protein deposition is simply defined as the ME required to promote a defined increment in body protein or lipid. The partial efficiency of ME utilization for whole-body growth (K_{pf}), protein deposition (K_p), and lipid deposition (K_f) is the ratio of the net energy retained to the corresponding ME intake components.

$$ME = HEm + (REp/K_p) + (REf/K_f)$$

Using this approach, Emmans (1994) concluded that the net energy cost of protein retention is 2.54 kJ/kJ of protein retained (that is, 1.54 kJ of heat expended for each 1 kJ of protein deposited), equivalent to a K_p of 39.5%. According to Emmans (1994), when all related factors are accounted for in the analysis, the energy cost of protein deposition does not appear to differ between species. The calculated energy costs of lipid retention were 1.4 and 1.1 kJ/kJ of lipid deposited (i.e., heat losses of 0.4 and 0.1 kJ for each 1 kJ of lipid deposited) when deposited from nonlipid and lipid, respectively. These are equivalent to a $K_f = 90\%$ when deposited from lipid and a $K_f = 70\%$ when deposited from nonfat substrates.

A few studies have used the factorial approach to estimate the HEm, K_p, and K_f of fish. Results of these studies are summarized in Table 1.5. The estimates of K_p varied between 0.44 and 0.56, and those of K_f between 0.72 and 0.91. These values appear to be similar to what has been observed with mammals and birds.

The factorial approach has been criticized because it is much easier to control ME intake than it is to control protein and lipid depositions (Emmans, 1995). Moreover, when different levels of ME intake are used in an experimental design, within a genotype and (or) body weight there may

be a strong correlation between protein and lipid deposition. Therefore, it may be difficult to estimate parameters accurately using a factorial analysis. A multivariate model has recently been proposed by Van Milgen and Noblet (1999) to circumvent these problems. This model appears to give more accurate and reliable estimates of K_p, K_f, and HEm but has never been applied to fish.

It is worth emphasizing that both the factorial and the multivariate approaches are purely empirical and, to some extent, an oversimplification of reality. Protein and lipid deposition is not merely the deposition of energy but a highly complicated process whose rates and efficiencies are governed by dietary factors (nutrient balance and utilization) and biological factors (genetics, types of tissue made, etc.). Factorial and multivariate approaches are, nevertheless, interesting tools for insight into the cause of possible differences between theoretical and observed protein and lipid deposition costs.

The cost of lipid deposition determined by the factorial approach appears to be close to the theoretical chemical cost. Results from Emmans (1994) showed that the source of lipid deposition (lipid or nonlipid) affected the efficiency value, and this might contribute to differences in K_f estimates between studies. Since K_f differs depending on the origin of the lipid deposited, the composition of the diet might affect the efficiency of lipid deposition. Dietary intake of pre-formed lipids will lead to a very good efficiency of utilization for lipid deposition, whereas *de novo* synthesis of lipid from dietary carbohydrates will lead to a slightly lower efficiency. The pathways of lipogenesis in fish are qualitatively similar to those in other vertebrates. In salmonids, variation in the dietary lipid level appears to be a more effective modulator of fatty acid synthesis than the level of dietary carbohydrate (Sargent *et al.,* 1989). Some recent data have confirmed that an increase in dietary lipid levels decreases hepatic lipogenic enzyme activities (Dias *et al.,* 1998) as well as *de novo* fatty acid synthesis (Brauge, 1994). In fish fed high-fat diets, where almost all the lipid deposited is of dietary origin, the cost of lipid deposition is very low.

There are large differences in the energy cost of protein deposition based on theoretical assumptions (86% efficient) and that calculated using the factorial approach (40–60% efficient). The reasons for this difference have not been clearly identified. In growing animals, even at nitrogen equilibrium (zero body protein gain), protein is synthesized and energy is consumed for maintaining protein turnover (Milligan and Summers, 1986). According to Reeds *et al.* (1985), the amounts of protein and energy needed at zero nitrogen retention might account for as much as 20% of the maintenance ATP utilization. The ATP cost of Na^+/K^+ pumping accounts for a similar proportion of ATP utilization at energy equilibrium (Gill *et al.,* 1989). The

rate of protein synthesis greatly exceeds that of protein deposition (Reeds *et al.,* 1981), the efficiency of protein deposition in fish ranging between 40 and 60%. Therefore, changes in protein turnover are a possible explanation for the variable energy cost of protein deposition. On the other hand, when problems associated with structural and kinetic heterogeneity of amino acid pools are involved (Watt *et al.,* 1991) in addition to the difficulty of measuring the synthesis of rapidly turning-over proteins (Wheatley *et al.,* 1988), one is led to conclude that current estimates of protein synthesis *in vivo* may be underestimates, and consequently the energy cost of protein deposition may also be underestimated. No single cause is responsible for the apparent higher cost of protein deposition that is predicted in theory. Energy is expended in the biochemical pathways that lead to protein synthesis, and protein degradation and in regulating and integrating the various cellular metabolic activities involved in protein deposition. All these components need to be quantified so reliable K_p values can be obtained from *in vivo* studies.

It is also evident that the amino acid balance of the diet will contribute to K_p. Catabolism of amino acids due to amino acid excess or imbalances in the diet or a low non protein energy content will result in waste of energy (higher HiE associated with catabolism of amino acids than with that of lipids) and a decrease in K_p. Diets with an imbalanced amino acid composition will result in less energy retained as protein and, consequently, result in K_p values lower than those that would occur if the diet was perfectly balanced.

Cho *et al.* (1976) showed that an increase in dietary fat levels led to a decrease in HiE. LeGrow and Beamish (1986) confirmed that the increase in oxygen uptake with increasing dietary protein levels was consistent, irrespective of the dietary fat levels, thus highlighting the importance of dietary DP/DE ratios. In addition, the efficiency of the energy (i.e., net energy) derived from the catabolism of amino acids by the fish is unclear.

It has been suggested that digestible carbohydrates are possibly also significant contributors to HiE (Beamish *et al.,* 1986; Hilton *et al.,* 1987). Beamish *et al.* (1986) observed that fish fed a diet with a high glucose content consumed more oxygen than fish fed a diet with the same protein level but rich in lipids, suggesting that an increase in digestible carbohydrate intake results in an increase in heat production. The fish fed the diet high in glucose had a lower N retention efficiency (N gain/N intake) than the fish fed the diet high in lipids (Beamish *et al.,* 1986). These data suggest that the effect on HiE observed was in fact related more to the variation in dietary lipids and the sparing of protein than to the digestible carbohydrate itself. However, Helland and Grisdale-Helland (1998) observed that at low intake levels, an increase in digestible starch at the expense of digestible protein resulted in an increase in the oxygen consumption of Atlantic salmon but

no change in the N retention efficiency. Bureau *et al.* (1998) observed a very poor retention of ME of digestible carbohydrate fed to rainbow trout. These data suggest that utilization of carbohydrate can be associated with a significant HiE, especially when the DP/DE ratio of the diet is less than 20–21 g /MJ.

1.13 Recovered Energy and Growth

Dietary ME taken in as diet which is not dissipated as heat is retained in the body as new tissue constituents. The difference between the enthalpy of combustion (i.e., GE) of the body at the beginning and that at the end of a period of time is referred to as "recovered energy" (RE) according to the NRC (1981) nomenclature.

The most direct way of estimating RE is to determine the GE of a number of individuals (representative samples) from a group of experimental animals at the beginning and at the end of a study. This method of determining RE is termed comparative carcass analysis or slaughter technique and was discussed earlier in the chapter. Alternatively, RE can be estimated by the difference between IE and FE, UE + ZE, and HE (Blaxter, 1989), which is also known as the energy balance technique.

RE can be either positive or negative and represents the enthalpy of combustion of organic compounds stored or lost by the animal. Protein and lipids are the main energy-yielding components of the body, whereas glycogen generally represents only a small proportion of the body of the animal (<1%) and consequently of RE. RE is not always a quantitative measure of weight gain because deposition of lipid reduces the water content of the body, thus changing the energy value per unit weight of the living animal. The great difference in the energy value of lipid and protein also exaggerates the difference in the energy content of body weight gain. However, RE remains a useful and simple measure of growth and nutrient deposition provided that one recognizes its limitations. RE is only an estimate of the deposition of energy-yielding nutrients (mostly protein and lipids) achieved through different systems and regulated by different factors. Consequently, RE measurements should always be accompanied by measurements of nitrogen gain (RN) and interpreted with care.

There are 3–6 grams of water associated with each gram of protein tissue deposited (Cho and Kaushik, 1990). On a wet weight basis protein tissue is only about 25% dry matter and the dry matter has an energy value of 23.6 kJ/g, giving a value for protein of 6 kJ/g wet tissue. Lipid is usually deposited in adipose tissue in association with relatively little water. Fat tissue is about

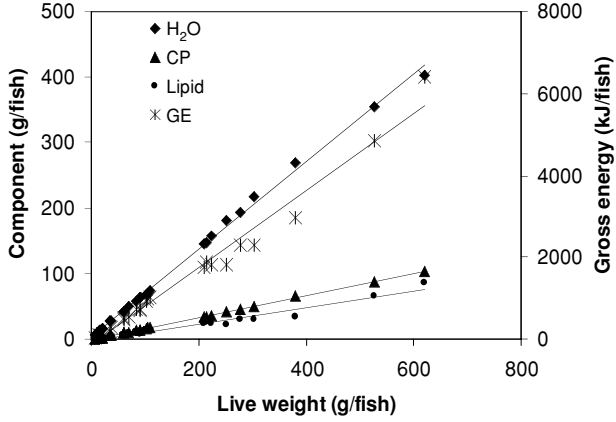

FIG. 1.3

Chemical composition (absolute; g/fish or kJ/fish) of rainbow trout of various sizes fed practical diets with 20–22 g DP/MJ DE. Data from Bureau *et al.* (unpublished). Regressions: H_2O (g/fish) = 0.670 BW (g/fish) – 3.13 ($R^2 = 1.00$); Crude protein (CP) (g/fish) = 0.169 BW (g/fish) – 0.07 ($R^2 = 1.00$); Lipid (g/fish) = 0.125 BW (g/fish) – 2.52 ($R^2 = 0.96$); Gross energy (GE) (kJ/fish) = 8.6 BW (g/fish) −40.1 ($R^2 = 0.98$).

85% dry matter, with an energy value of approximately 39 kJ/g. Fat tissue therefore contains about 33 kJ/g of tissue deposited (Smith, 1989). In addition, lipid is stored in tissues generally substituting for water. RE varies greatly with the type of tissue being produced.

Figures 1.3 and 1.4 show the absolute (gram or kilojoule per fish) and relative (percentage or kilojoule per gram) compositions of rainbow trout of various sizes fed a practical diet with 20–22 g digestible protein (DP)/MJ digestible energy (DE). Figure 1.3 shows that the absolute contents of water, protein, lipid, and gross energy of fish increase linearly with the weight of the animal. Figure 1.4 shows that, expressed as the relative composition (e.g., percentage of body weight), the protein content of the fish increases slightly, then remains approximately constant. Moisture tends to decrease rapidly with fish size up to about 100 g live weight, then decreases more slowly. Lipid and GE increase rapidly with size also up to 100 g live weight, then increase linearly very slowly. Similar observations and figures are reported by Shearer (1994) and Lupatsch *et al.* (1998).

Shearer (1994) concluded that the protein content of growing salmonids is determined solely by fish size, that the lipid level is affected by both endogenous (fish size, growth rate) and exogenous (dietary, environmental) factors, and that the ash content is homeostatically controlled. The protein

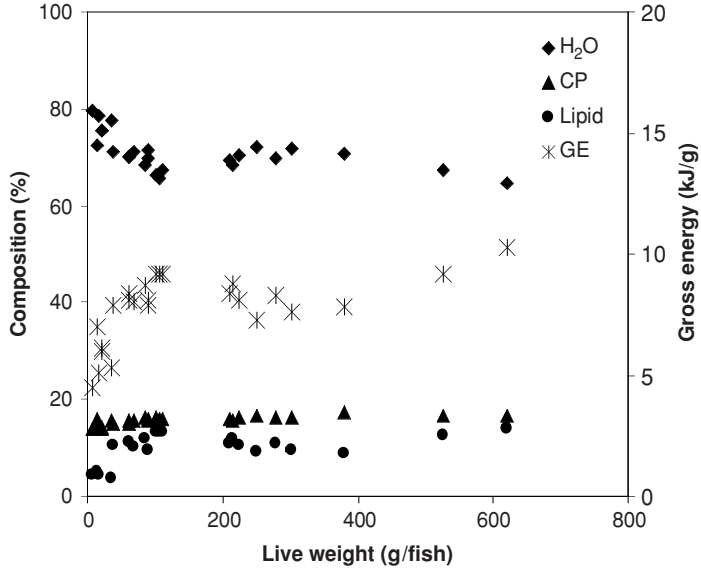

FIG. 1.4

Chemical composition (relative; % or kJ/g) of rainbow trout of various sizes fed practical diets with 20–22 g DP/MJ DE. d^{-1}, day^{-1}. Data from Bureau *et al.* (unpublished, 2000).

or ash contents of the whole body appear to vary little with the growth of a given species of fish, whereas the whole-body energy content varies considerably over time. Whole-body GE content increase is due mainly to increasing lipid content with increasing body weight.

The results from a number of studies on protein and lipid deposition clearly show that this increase in lipid content of fish of increasing body weight, fed to near-satiety, is generally not due to enhanced deposition of lipid compared to that of protein as the animal increases in size. Azevedo *et al.* (1999) observed that rainbow trout fed the same diet at different ration levels (from 70 to 100% of near-satiety) deposited protein and lipid according to the same ratio regardless of fish size and water temperature. Their data showed a very good proportionality between protein and fat gain; for each unit of protein energy gain, the fat energy gain was 1.4 times higher. A similar proportionality of protein and lipid deposition with increasing feed intake has been reported in domestic animals (Boekholt *et al.,* 1994, 1997).

Severe feed restriction results in a significant alteration of the protein-to-lipid deposition ratio in fish. Protein deposition has, in general, priority over

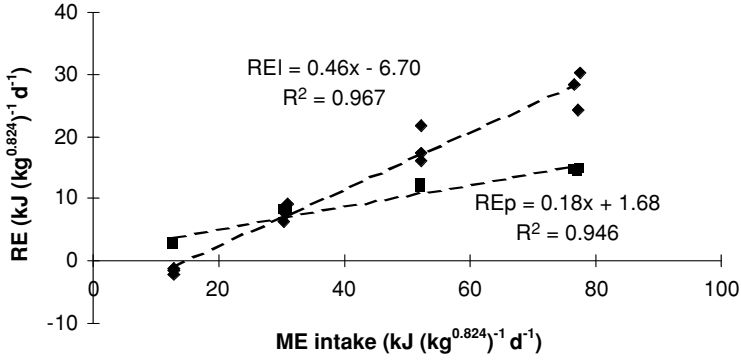

FIG. 1.5

Recovered energy (RE) as protein (REp; squares) and lipid (REl; diamonds) as a function of the metabolizable energy (ME) intake of rainbow trout reared at 8.5°C. d^{-1}, day^{-1}. Data from Bureau *et al.* (unpublished observations).

lipid deposition. As mentioned earlier, fish fed a ration allowing a RE = 0 can still deposit body protein (positive protein energy gain) while mobilizing body lipids. Clearly, live weight gain is driven by protein deposition. Studies on the effect of feeding levels on fish have shown that protein and lipid depositions increase linearly with feed allocation but that protein and lipid energy depositions have different slopes and intercepts (Fig. 1.5). Under severe feed restriction, protein deposition greatly exceeds lipid deposition. Energy deposition as lipids often exceeds that as protein at moderate to high feeding levels. A number of studies have also shown that protein deposition tends to plateau at high feeding levels, whereas lipid deposition does not appear to level off (increases linearly). A decreasing protein-to-lipid deposition ratio can be observed at feeding levels approaching maximum protein deposition.

The relative importance of protein and lipid deposition depends on a great number of nutritional factors. The balance of available amino acids, particularly essential amino acids, in the dietary protein and the digestible protein-to-digestible energy ratio in the diet are the major factors. Proteins of high biological value may promote greater protein deposition than those of lower value. Large excesses of energy intake and improper balance of protein to energy result in the deposition of a larger proportion of RE as lipid. Seasonal changes in body composition, in relation to specific physiological stages or endocrine status, are also known to occur. That there are considerable interspecific differences in lipid deposition and tissue distribution should also be recognized. Nutrient deposition and temporal changes

in body composition of fish, and effects of all the factors mentioned above, should be examined more closely.

1.14 Reproduction

The most taxing period of life for many organisms is maturation and reproduction, when resources are diverted from procurement of food and into tasks necessary for successful breeding (Thorpe, 1992; Hendry and Berg, 1999). This is the case at least for many fish species, such as Atlantic and Pacific salmon, which often migrate long distances from feeding areas in the open ocean to their natal spawning sites. Females develop large gonads and engage in vigorous competition for access to spawning sites. Males undergo extensive morphological changes and fight incessantly for access to spawning females. Feeding ceases at entry into fresh water, often months before spawning commences, and in many species fish die after a single spawning season (Thorpe, 1992; Hendry and Berg, 1999).

Under aquaculture conditions, expenses associated with reproduction may not be as dramatic. However, reproduction is a critical part of the production cycle for farm fish and involves fairly dramatic changes in energy acquisition and partitioning by the fish. Reproduction involves the synthesis and temporary storage of new tissues that are formed almost regardless of the level of dietary energy intake, the necessary energy being withdrawn from other body tissues if the dietary supply is insufficient (Jonsson *et al.,* 1991, 1997). Consequently the redistribution of tissue energy which takes place in the breeding season can complicate measurements of energy balance.

The energy content of the eggs [termed OE by the NRC (1981)] of rainbow trout is about 27 kJ/g dry matter, irrespective of the size of the eggs. The average energy content of eggs, measured in about 50 teleosts, is 23.5 kJ/g dry matter whatever the size of the eggs. The total amount of energy stored in the eggs would represent 8 to 15% of the gross energy of the whole body, very much correlated with the gonadosomatic index. (Kaushik and Médale, 1994). The values reported by Wooton (cited by Tytler and Calow, 1985) relate to ornamental species and vary from 10 to 18% for the medaka to 16 to 30% for the three-spine stickleback. For the majority of species, the male gonads represent only a small proportion of the body mass. On the other hand, mature ovaries can represent up to 30% of the body mass in certain species. In some multiple spawners such as the gilthead sea bream, the total egg production over a single season can even reach 100% of the body mass (Tandler *et al.,* 1995).

In pike, just before emission of the gametes, the total energy content of the male gonads is of 75 kJ, whereas the content of the ovaries exceeds 1 MJ.

While the composition and energy content of gonads is fairly well characterized for a number of fish species, there are no studies on the actual cost of gonad formation (e.g., HiE associated with nutrient deposition in gonads). This cost can be evaluated only at the end of a long-term study, by the effort of reproduction, which corresponds to the quantity of energy stored in the gametes (OE) compared to the energy intake.

The total cost of the reproduction exceeds that of the production of the gametes. The development of secondary sexual characters, production of mucus, nuptial behaviors and activities, construction of nest for certain species, and migration all involve expenditure of energy. Energy expended in swimming represents a significant amount of energy. The energy expended in swimming can exceed the dietary energy intake, the balance being supplied by the mobilization of body tissue reserves. In Atlantic salmon, the total energy loss due to migration and spawning has been estimated to be about 60–70% of body reserves prior to upstream migration, being similar in males and females (Jonsson *et al.,* 1997). In Pacific salmon, only 7–8% of the total energy expended during migration and maturation is acconted for by gonadal growth, in both wild and farmed salmon (Idler and Bitners, 1959; Hardy *et al.,* l984).

Both Atlantic and Pacific salmon cease feeding more than a month before spawning but then migrate upstream, develop large ovaries (female) or secondary sexual characters (males), and compete for spawning sites (females) or mates (males). Sockeye salmon female lose about 13 MJ of carcass energy from freshwater entry (live weight, about 2.5 kg) to death after spawning (live weight, approximately 1.5 kg), representing about 75% of the energy reserve of the animal. In male sockeye salmon, about 65% of the total energy content of the fish is used during reproduction, from freshwater to death, this energy being channeled mostly into the development of secondary sexual characters.

1.15 Integrating and Using Information from Bioenergetics

The essential thrust of studies on bioenergetics of animals is to provide a basis for diet formulation and evaluation, and develop convenient and accurate systems to predict the energy balance of animals based on body weight, sex, activity, physiological state, environment, and amount and nutritive value of feed consumed (Baldwin and Bywater, 1984).

Several bioenergetics models have been developed to predict energy requirements and growth of fish under a variety of conditions (reviewed by Cui and Xie, 1999). In several bioenergetics models developed mostly by fish ecologists, FE, UE + ZE, HiE, HeE, and the GE content of the carcass are considered a fixed fraction of IE regardless of the composition of the feed and performance of the fish. A basic understanding of nutrition should indicate that these are unrealistic assumptions.

It is common to observe energy requirements expressed as the absolute amount of DE required per kilogram body weight per day for maximal production or energy expenditure, and deposition expressed as a proportion of the maximum feed consumption ($C_{max)}$ in numerous fish bioenergetics studies (e.g., Gatlin *et al.,* 1986; McGoogan *et al.,* 1998; Ohta and Watanabe, 1998; Cui and Xie, 1999; Elliott and Hurley, 1999; Watanabe *et al.,* 2000). It is important to recognize that the maximal production and C_{max} of an animal are factors of genetics, diet, environmental conditions (e.g., temperature), husbandry practices, health status, and other factors. Maximum production and C_{max} are, therefore, highly variable parameters. Consequently, the energy requirement for maximum production calculated in some studies (i.e., energy requirement expressed as an absolute term such as kJ $fish^{-1}$ day^{-1}) can be valid only for the specific conditions (diet composition, strain, temperature, culture conditions, etc.) encountered in the study. Fish growing at different rates will deposit nutrients at different rates and, consequently, have different energy and feed requirements. Energy requirements should therefore be calculated for explicitly expressed levels of performance (e.g., expected or achievable level of performance), feed composition, and life stage (Cho, 1991, 1992; Cho and Bureau, 1998; Kaushik, 1998). In addition, this should be done using factorial approaches (Cho and Bureau, 1998, Lupatsch *et al.,* 1998), i.e., approaches that divide energy requirements into different components or fractions, as opposed to lumping them into one estimate as is commonly done.

Cho (1991) proposed factorial models to determine energy requirements of fish based on expected level of performance, diet composition, and expected body composition. These models were updated by Cho (1992) and Cho and Bureau (1998). Using this approach, calculation of the total energy requirement and, consequently, the feed requirements (or allocation) can be accomplished as follows.

1. Characterization of diet (including DE content)
2. Calculation of expected live weight gain and RE
3. Allocation of HeE based on fish size and water temperature
4. Allocation of HiE for maintenance and energy deposition
5. Allocation of UE + ZE
6. Calculation of minimum DE requirement
7. Calculation of feed requirement

Determination or estimation of DE, HeE, HiE, and UE + ZE can be done using the approaches described above or by carefully analyzing the literature. It is imperative to take into account the composition of the diet and type of fish used (species, life stage, etc.) rather than blindly applying values reported in the literature.

Accurate prediction of the growth potential of a fish stock under given husbandry conditions is an inevitable prerequisite to estimation of the energy or feed requirement (e.g., weekly ration). The formula most commonly used for fish growth rate expression is the instantaneous growth rate, known as the "specific growth rate" (SGR), which is based on the natural logarithm of body weight:

$$\mathrm{SGR} = (\ln\mathrm{FBW} - \ln\mathrm{IBW})/\mathrm{D}. \tag{4}$$

where FBW is the final body weight (g); IBW, the initial body weight (g); and D, the number of days.

The SGR has been widely used by most biologists to describe the growth rate of fish. However, the exponent of the natural logarithm underestimates the weight gain between the IBW and the FBW used in the calculation and it grossly overestimates the predicted body weight at weights higher than the FBW used. Furthermore, the SGR is dependent on the IBW, making comparisons of growth rates among groups meaningless unless the IBW are similar.

A more accurate and useful coefficient for fish growth prediction in relation to water temperature is based on the exponent 1/3 power of body weight (Iwama and Tautz, 1981).

$$\text{Thermal-unit growth coefficient (TGC)} = [\mathrm{FBW}^{1/3} - \mathrm{IBW}^{1/3}]/\Sigma[T\,D]\ 100 \tag{5}$$

$$\text{Predicted final body weight} = [\mathrm{IBW}^{1/3} + \Sigma\ (\mathrm{TGC}/100\,T\,D)]^3 \tag{6}$$

where T is the water temperature (°C).

This model equation has been shown to represent very faithfully the actual growth curves of rainbow trout, lake trout, brown trout, chinook salmon, and Atlantic salmon over a wide range of temperatures. Figure 1.6 shows the growth curve of rainbow trout fed to near-satiation and reared at 8.5°C. Live weight increases curvilinearly, whereas the cubic root of live weight increases in a highly linear fashion, supporting the observations of Iwama and Tautz (1981) and the validity of the TGC model.

Since these TGC values and growth rate are dependent on species, stock (genetics), nutrition, environment, husbandry, and others factors, it is

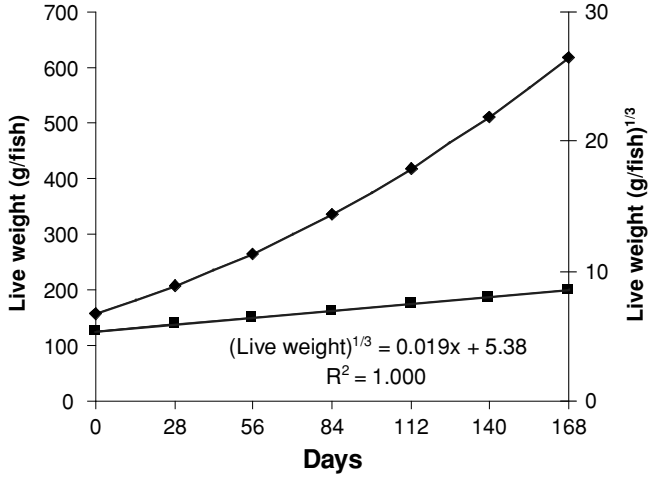

Fig. 1.6

Live weight and cubic root of live weight of rainbow trout fed to satiation and reared at 8.5°C for 168 days. Data from Bureau *et al.* (unpublished observations, 2000).

essential to calculate the TGC for a given aquaculture condition using past growth records or records obtained from similar stocks and husbandry conditions. Once the weight gain is known, RE can quite easily be predicted using simple models (as shown by Figs. 1.3 and 1.4, for example). Development of such models can be done relatively easily, as it may quite simply involve sampling animals at different sizes and determining their chemical composition.

Using an approach similar to that of Cho (1991), the energy, oxygen, and feed requirements and expected feed efficiency of fish of different sizes reared under different conditions or rearing periods can be calculated (Cho and Bureau, 1998). Table 1.6 lists the energy and oxygen requirements of rainbow trout reared at 12°C and fed a diet with 44% DP and 20 MJ DE at different sizes or growing from 1 to 1000 g with a TGC = 0.220. The DE to produce a 1-kg biomass (e.g., 1000 10-g fish gaining 1 g each) varies from about 9.5 MJ for 1-g fish to 23.6 MJ for 1-kg fish. It is of utmost importance to understand that these estimates are valid only for the given set of conditions (species, water temperature, TGC, diet composition, etc.) and should not be applied blindly.

Data from Azevedo *et al.* (1998) show that the DE requirement is largely independent of water temperature, since as the temperature increases, DE and RE increase but the efficiency of ME and DE utilization does not change.

Table 1.6

Energy and Oxygen Requirements[a] and Expected Feed Efficiency of Rainbow Trout[b]

Live weight (g fish$^{-1)}$	GE (kJ g^{-1}) live weight)[c]	RE[d] (MJ kg^{-1} weight gain)	HeE[e] (MJ kg^{-1} weight gain)	HiE[f] (MJ kg^{-1} weight gain)	UE + ZE[g] (MJ kg^{-1} weight gain)	DE[h] (MJ kg^{-1} weight gain)	Oxygen[i] (g kg^{-1} weight gain)	Feed efficiency[j]
1	4.4	4.4	1.1	3.7	0.3	9.5	357	2.10
5	4.8	4.8	1.6	4.3	0.3	11.1	433	1.81
10	5.2	5.2	1.8	4.6	0.3	11.9	472	1.68
50	6.8	6.8	2.4	6.2	0.5	15.8	623	1.28
100	6.9	6.9	2.7	6.5	0.5	16.6	675	1.21
500	8.1	8.2	3.5	7.9	0.6	20.2	840	1.00
1000	9.8	9.8	4.0	9.2	0.7	23.6	968	0.85
1–1000	—	8.7	3.6	8.2	0.6	21.1	869	0.95

[a]MJ or g kg^{-1} weight gain.

[b]At various sizes or growing from 1 to 1000 g, based on the assumption that the fish are reared at 12°C, growing with a TGC= 0.220, and fed a diet with 20–22 g DP/MJ DE and 20 MJ/kg DE.

[c]GE, gross energy content of carcass. Calculated from experimental data (Bureau *et al.*, unpublished) as follows: for fish 30 g or less: GE (kJ g$^{-1)}$ = −0.0006 (live weight)^2 + 0.0948 (live weight) + 4.31; for fish more than 30 g, GE (kJ g$^{-1)}$ = 0.0031 (live weight) + 6.61.

[d]RE = (live weight gain; g fish$^{-1)}$ (GE content).

[e]HeE = $[-1.04 + 3.26(T) - 0.05(T)^2](0.0200.824)^{-1}$ day^{-1} (Cho, 1991).

[f]HiE = 0.67 (HeE + RE) (Azevedo, 1998).

[g]UE + ZE = 0.03(HeE + RE + HiE) (Kaushik, 1998).

[h]DE requirement = (RE + HeE + HiE + UE + ZE).

[i]Oxygen requirement = (HeE + HiE)/13.6 kJ g^{-1} O_2.

[j]Expected feed efficiency = weight gain/feed.

The total energy requirement should ideally be expressed as DE, since FE and, consequently, IE are highly dependent on the composition of the diet fed. FE is mainly from undigestible starch, fiber, and some protein in the diet and is influenced by the quality of ingredients. Less expensive commercial diets tend to contain higher levels of undigestible plant products, diluting digestible nutrients and increasing the amount of FE.

1.16 Limitations and Perspectives of Bioenergetics

The student of bioenergetics should always remember that organisms ingest and metabolize organic compounds, not "energy" per se. It should be apparent from a careful reading of this chapter that nutrients have different fates and that energy losses are governed by how these nutrients are utilized. Energy is not a nutrient per se, but the sum of a number of processes resulting in the assimilation of specific energy-yielding nutrients.

There is a necessity to move to approaches based on a clearer understanding of the role of specific absorbed nutrients and their metabolism for determining the productive responses of an animal (Reynolds, 1999). More scientifically correct feed evaluation and requirement systems should be based on the characterization of nutrient fractions relevant to their actual digestion, metabolism, and utilization in the animal under varying practical conditions (Boisen and Verstegen, 1998).

Some attempts have been made to develop nutrient-based growth models for fish (Machiels and Henken, 1986; Conceicao, 1997). The models may provide a promising alternative to current bioenergetics modeling. However, these research models are highly theoretical (causal) and incorporate considerable physiologic and metabolic details. As a result, they are too complex and expensive to use as predictive models or as components of feeding or management systems. However, they can be used to identify and characterize elements for incorporation into more empirical, predictive models for use under applied conditions (Baldwin and Bywater, 1984; DeLange, 1997). The second approach has been the development of predictive models of energy transactions in animals that also consider the digestion of feed components and metabolism of absorbed nutrients explicitly rather than in the aggregate as energy and crude protein. In addition, they should also consider the turnover of macromolecules in the animal and partitioning of nutrients among tissues and functions within the animal (Baldwin and Bywater, 1984; DeLange, 1997).

Although there may be argument for describing a diet in terms of chemical composition, this should not obscure the fact that some of the animal

control mechanisms may, in effect, perceive the substrates as contributors to the energy supply rather than identifying them as specific chemicals (McLeod, 1999). Bioenergetics has made a great contribution to the greater understanding of nutrient utilization by fish over several decades and is likely to remain a useful discipline for decades to come.

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2

The Vitamins

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2.1 Historical Introduction

2.1.1. Pioneers and Concepts

Diet deficiency diseases have been recognized in humans and animals for several hundred years. Vedder (1912), who worked on beriberi, traced the recognition of this disease by the Chinese to as early as 2697 B.C., but it was Eijkman and Grijns at the end of the nineteenth century who showed how polyneuritis could be reduced by the addition of rice husks or pericarp to the diet and Vorderman (Williams, 1961) who correlated fowl polyneuritis with human beriberi. Casimir Funk (1912) isolated the water-soluble antiberiberi compound in a partially purified state and, in a paper on the etiology of deficiency diseases published in 1912, used the term "vitamine" for the first time. He reviewed the literature on beriberi, pellagra, and scurvy with the view that all were deficiency diseases (Funk, 1922).

Scurvy was also an ancient disease, and the first treatise on its description and experimental cure was published in 1753 by Lind, who showed that oranges, lemons, and apple cider contained a scurvy preventive factor. One hundred fifty years later, Holst and Frölich (1907) produced a deficiency disease similar to scurvy in guinea pigs; 25 years thereafter, King and Waugh (1932; Waugh and King, 1932) identified vitamin C as ascorbic acid.

A similar scientific history applies to the description of pellagra in peasants in Spain by Casal in 1735 (Harris, 1919). A long delay followed before Spencer, in 1916, and Chittenden and Underhill, in 1917, produced black tongue in dogs as a deficiency disease similar to pellagra. Twenty more years elapsed before Elvehjem and co-workers (1937, 1938) cured black tongue with nicotinic acid. The new vitamin was immediately applied to the treatment of pellagra by Fouts *et al.* (1973) and Smith *et al.* (1937) and, finally, shown to be effective by Nakao and Greenberg (1958).

Fish have been confined and fed for several thousand years. Mosaic walls in ruins of ancient Greece and Rome depict fish being fed in impoundments. Whenever intensive fish culture was employed with more purified, artificial diets, many of the fish became anemic and died. McCay and Dilley (1927), at Cornell, worked industriously to identify the antianemic factor H which was present in fresh meat and which was necessary in the diet when trout were held for more than 10–12 weeks on the best purified diets then available for animal nutrition studies. Twenty years elapsed, however, before this water-soluble factor was identified as a combination of vitamin B_{12} and folic acid (Halver, 1953).

One of the first reports of a specific vitamin deficiency in fish was in 1941, when Schneberger, at the Thunder River Hatchery in Wisconsin, reported that paralysis in rainbow trout (*Oncorhynchus mykiss*) which were fed carp (*Cyprinus carpio*) could be cured only by injecting crystalline thiamin into individual fish or by feeding them dried brewer's yeast. Fish diet disease was reported by Louis Wolf (1942) to be due to thiaminase present in fresh fish tissue which would hydrolyze thiamin in the commercial meat–meal mixtures fed to fish in the hatchery. That same year Tunison *et al.* (1942) measured levels of thiamin, riboflavin, and nicotinic acid in the liver, pyloric caeca, kidney, and muscle to establish baselines for experiments to measure requirements of trout for these vitamins. Dietary gill diseases were also reported during this decade and could be reduced by incorporating fresh liver or dried yeast in the diet (Wolf, 1945). McCay and Tunison (1934) observed scoliosis and lordosis in brook trout (*Salvelinus fontinalus*) fed formalin-preserved meat, but the symptoms took nearly 1 year to develop and were not correlated with the recently identified vitamin C. Many of the fish disease reports of the 1940s inferred that dietary deficiencies may have caused or augmented the symptoms observed when a specific pathogen and Koch's postulates could not be followed for a specific disease episode.

2.1.2. Avitaminosis

Typical avitaminosis symptoms of Chastek-type paralysis, cataracts, convulsions, scoliosis, anemia, slime patch disease, clubbed gills, poor growth, anorexia, and increasing mortality were reported wherever fish were concentrated and intensive fish cultural practices were used. As diets became manufactured more from agricultural products, anemia and diet disease symptoms become more common, but specific cause-and-effect relationships were often difficult to define.

2.1.3. Hypervitaminosis

Hypervitaminosis D and A were reported when seal and whale liver were used as one of the fresh meat components in salmon diets (Burrows *et al.,* 1952). An analogy was drawn between symptoms observed in fish and those reported for other experimental animals, but no good experimental diets were available with positive experimental control over the particular vitamin that investigators wished to study.

2.1.4. Test Diets and Conditions

Barbara McLaren, working with Conrad Elvehjem and others in Wisconsin, developed a vitamin test diet containing crystalline vitamins, casein, dextrin,

and oils, with crab meal or dried liver as the source of the antianemic factor (McLaren *et al.,* 1947b). These pioneer fish nutritionists were able to report tentative qualitative, then quantitative requirements of rainbow trout for thiamin, riboflavin, pyridoxine, pantothenic acid, inositol, biotin, folic acid, choline, and niacin (McLaren *et al.,* 1947a). These values were based on fish growth response and food conversion. The test diet did furnish some control over many vitamins but assays revealed low levels of several of the water-soluble vitamins present in the dietary components, and when the diet excluded dried liver or crab meal, fish became anemic and died (McLaren *et al.,* 1946). Xanthopterin was once thought to be the fish antianemic factor because young chinook salmon (*Oncorhynchus tshawytscha*) showed an improvement in hematology when this material was injected (Simmons and Norris, 1941). Tunison *et al.* (1943) reported that riboflavin, pyridoxine, and pantothenic acid would improve anemic brook trout and could be part of McCay's factor H. Three years later, in 1946, Phillips *et al.* (1946), working in the same laboratory, could not repeat the response with these three vitamins even when folic acid was added to the vitamin mixture. These workers could, however, cure the anemia when dehydrated liver powder was added. Norris and Halver tested xanthopterin again in 1949 at the University of Washington and injected this material alone and in combination with folic acid and vitamin B_{12} into young anemic chinook salmon. A measurable response was obtained from xanthopterin and a larger one from folic acid, but the most dramatic stimulation of erythropoieses was obtained by Halver in 1949 and 1950 when folic acid and vitamin B_{12} were injected in the combination of 1 part vitamin B_{12} to 100 parts folic acid. That winter, Louis Wolf (1951) developed a test diet for rainbow trout which contained commercial casein, gelatin, potato starch, hydrogenated cottonseed oil, α-cellulose flour, minerals, cod liver oil, and crystalline vitamins. The diet was used successfully to induce several vitamin deficiency syndromes by deleting one vitamin at a time from the vitamin supplement and feeding the resultant diets to trout (Wolf, 1951). During the summer of 1951, this crude test diet was improved by changing to vitamin-free casein, purified gelatin, white dextrin for potato starch, corn oil for Crisco, and a simplified mineral mix (Halver, 1953a). Chinook salmon fingerlings grew as well on this diet as on Wolf's test diet (Halver, 1953a, 1957). Later this original, complete vitamin test diet was improved by lowering the protein content and used for short-term feeding studies with coho salmon (*O. kisutch*) and sockeye salmon (*O. nerka*) (Halver, 1966) and for long-term feeding studies for at least three reproductive cycles with rainbow trout in 8, 10, 15, and 17°C water systems (Halver, 1970; Halver and Coates, 1957). The tool had been forged to test for specific qualitative and quantitative vitamin requirements of salmonids (Table 2.1). Applications of the diet to salmon,

Table 2.1

Water-Soluble Vitamin Test Diet H-440[a]

Complete test diet	g	Vitamin mix	mg	Mineral mix	mg
Vitamin-free casein	38	Thiamin hydrochloride	5	USP XII No. 2	Plus
Gelatin	12	Riboflavin	20	$AlCl_3$	15
Corn oil	6	Pyridoxine hydrochloride	5	$ZnSO_4$	300
Cod liver oil	3	Choline chloride	500	CuCl	10
White dextrin	28	Nicotinic acid	75	$MnSO_4$	80
α-Cellulose mixture[b]	9	Calcium pantothenate	50	KI	15
				Na_2SeO_3	4
α-Cellulose 8		Inositol	200	$CoCl_2$	100
Vitamin mix 1		Biotin	0.5	per 100 g	
		Folic acid	1.5	of salt mixture	
Mineral mix	4	L-Ascorbic acid	100		
Water	200	Vitamin B_{12}^c	0.01		
Total diet as fed	300	Menadione (K)	4		
		α-Tocopherol acetate (E)[d]	40		

[a] Diet preparation: Dissolve gelatin in cold water. Heat with stirring in water bath to 80°C. Remove from heat. Add, with stirring, dextrin, casein, minerals, oils, and vitamins as the temperature decreases. Mix well to 40°C. Pour into containers; move to refrigerator to harden. Remove from trays and store in sealed containers in refrigerator until used. The consistency of the diet is adjusted by the amount of water in the final mix and the length and strength of beating.

[b] Delete 2 parts α-cellulose and add 2 parts CMC for preliminary feeding.

[c] Add vitamin B_{12} in water during final mixing.

[d] Dissolve α-tocopherol in oil mix.

trout, and other species were rapid and produced dramatic results. Specific deficiency syndromes occurred whenever one of the required vitamins was deleted from the vitamin mix and fish were fed for adequate test periods. Eleven water-soluble vitamins were soon identified as being required by salmon and trout (Halver, 1957; Coates and Halver, 1958; Kitamura *et al.,* 1967). Qualitative water-soluble vitamin requirements were also identified for catfish (*Ictalurus punctatus*) (Dupree, 1966), carp (Ogino, 1967a,b), yellowtail (*Seriola quinqueradiata*) (Sakaguchi *et al.,* 1969), and eel (*Anguilla japonica*) (Hashimoto *et al.,* 1970, NRC, 1983, 1993). Modifications to this diet have been used for many other species of fish to determine qualitative or quantitative vitamin requirements. A summary of vitamin requirements and functions in fish metabolism can be found in NAS/NRC bulletins (NRC, 1973, 1981, 1983, 1993) and in symposium reviews (Halver, 1979, 1980, 1982, 1986, 1995, 1996). Standard techniques for conducting vitamin requirement

studies were included in the EIFAC workshop on methodology for determination of nutrient requirement of fish (Halver, 1995).

2.2 The Water-Soluble Vitamins

The water-soluble vitamins include eight well-recognized members of the vitamin B complex, the water-soluble essential nutritional factors choline, inositol, and ascorbic acid, and several vitamins with less-defined activities for fish, i.e., *p*-aminobenzoic acid, lipoic acid, citrin, and undefined growth factors. The first eight are required in small amounts in the diet but play major roles in growth, physiology, and metabolism. The essential nutritional factors choline, inositol, and ascorbic acid are required at appreciable levels in the diet and sometimes are referred to not as vitamins but as dietary nutrients. The last group of growth factors is less well defined for fish and more research is needed to determine the exact role of these components in cell physiology and metabolism in fish tissues. All are included in this portion of the discussion on water-soluble vitamins. The American Institute of Nutrition names for the B complex vitamins are used, although the order of presentation suggests the more classical, historical identification of the vitamin B complex. Chemical structure, characteristics, and analogs are presented to assist the reader in identifying the particular compound discussed in the text. Since fish nutritionists are concerned with the specific role and function of the vitamins in physiology and metabolism, (1) chemical formulas are followed by (2) discussion of real or potential positive functions, (3) the deficiency syndrome observed in fish and other animals, (4) a listing of requirements, when known, for different types of fish, (5) sources of these essential nutrients, (6) antimetabolites which can interfere or inactivate the required vitamins, and, finally, (7) methods for clinical assessment of nutritional status. Since several forms of many of the particular vitamins exist, the term "vitamers" may be used, with the understanding that this terminology applies to the group of compounds with that particular vitamin activity.

2.2.1. Thiamin

Experimental beriberi was produced in fowl by Eijkman in Java in 1886 (Williams, 1961). The antiberiberi factor was crystallized by Funk in 1911 and named "vitamine" (Funk, 1912). Thiamin was isolated from rice polishings by Jansen and Donath in 1926 and was synthesized by Williams and co-workers in 1936 (Williams and Cline, 1936; Cline *et al.*, 1937). The first reported use in fish was in 1941 by Schneberger, who injected crystalline thiamin to cure "diet disease" in rainbow trout.

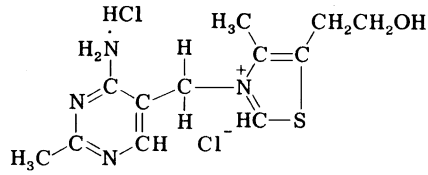


FIG. 2.1

Thiamin hydrochloride.

2.2.1.1. Chemical Structure Characteristics

Thiamin hydrochloride is a water-soluble, colorless, monoclinic, crystalline compound with the empirical formula $C_{12}H_{18}ON_4SCl_2$ and the structure shown in Fig. 2.1. It is comparatively stable to dry heat but is rapidly broken down in neutral or alkaline solutions and is split by sulfites into constituent pyrimidine and thiazole moieties. It has a characteristic yeast-like odor. The pyrimidine ring is relatively stable, but the thiazole ring is easily opened by hydrolysis. Several derivatives are stabile to heat, appear to be more completely soluble in weak alkaline solutions than thiamin itself, and still show biological activity in animals. These derivatives include thiamin propyl disulfide, benzoylthiamin disulfide, dibenzoylthiamin, and benzoylthiamin monophosphate. Both thiamin hydrochloride and thiamin mononitrate have been succesully used as the active vitamin in test diets for fish nutrition studies and for fish diet formulations.

2.2.1.2. Positive Function

Thiamin is part of the coenzyme cocarboxylase illustrated in Fig. 2.2, which participates in the oxidative decarboxylation of α-keto acids, especially pyruvic acid, eventually releasing carbon dioxide. Thiamin is an antogonist to acetylcholine. Jansen (1954) has pointed out that pyruvic acid stands

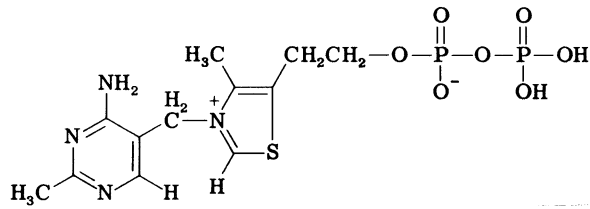


FIG. 2.2

Cocarboxylase or thiamin pyrophosphate.

at the crossroads of carbohydrate intermediaty metabolism and is the end point in the anaerobic process before decarboxylation and oxidative reactions begin in the reverse process. He assumed that the exact type of reaction depends on the protein of the apoenzyme to which thiamin pyrophosphate is combined. An interrelation between thaimin and lipoic acid has been reported (Reed, 1959), although little of this work has been confirmed in fish. Thiamin pyrophosphate, a coenzyme for the transketolase system, is part of the direct oxidative pathway of glucose metabolism occurring in the cytoplasm of cells (Handler, 1958). Brin (1963) has used this method of estimating status in experimental animals including salmon and trout. Cowey *et al.* (1975) used this technique to correlate thiamin intake with the physiological state of trout and marine flatfish. Thiamin pyrophosphate is retained longer in muscle tissue than in brain tissue (Lehninger, 1975), therefore, thiamin deficiency may progress from anorexia to neurological signs of susceptibility to shock or to the trunk-winding symptom described for the Japanese eel, *Anguilla japonica* (Hashimoto *et al.,* 1970). Similar deficiency signs have been observed in turbot, *Scophthalmus maximus* (Cowey *et al.,* 1975), yellowtail, *Seriola quinqueradiata* (Hosokawa, 1989), and red sea bream, *Chrysophrys majors* (Yone and Fuji, 1974).

Thiamin is essential for a good appetite, normal digestion, growth, and fertility. It is needed for normal function of nervous tissue, and the requirement is determined by the caloric density of the diet (Krampitz, 1969).

2.2.1.3. Deficiency Syndrome

Deficiency signs in salmonids involve impaired carbohydrate metabolism, nervous disorders, poor appetite, poor growth, and increased sensitivity to shock by a physical blow to the container or light flashes (Schneberger, 1941; Wolf, 1942; McLaren *et al.,* 1947a; Halver, 1953a,b, 1957; Coates and Halver, 1958). A trunk-winding symptom in eels has been reported together with hemorrhage at the base of the fins (Hashimoto *et al.,* 1970). Skin congestion and subcutaneous hemorrhage occur in carp fed thiamin-deficient diets (Aoe *et al.,* 1969). Typical symptoms observed in salmonids, carp, and catfish are listed in Table 2.2. Thiamin deficiency has also been reported in marine flatfish started on clam neck diets stored long enough for the thiaminase present to hydrolyze the thiamin in the ration (Shelbourne, 1970). Typical nervous paralysis occurred, with rapid mortality from physical shock. Similar thiamin deficiency signs have been reported for several marine species of fish (NRC 1983, 1993).

2.2.1.4. Requirements

Thiamin requirements for trout and salmon were determined by feeding diets containing different amounts of thiamin for periods long enough for

Table 2.2

Vitamin Deficiency Syndromes

Vitamin	Deficiency signs in salmonids, ictalurids, cyprinids, etc.
Thiamin	Poor appetite, muscle atrophy, convulsions, instability and loss of equilibrium, edema, poor growth
Riboflavin	Corneal vascularization, cloudy lens, hemorrhagic eyes, photophobia, dim vision, incoordination, abnormal pigmentation of iris, striated constrictions of abdominal wall, dark coloration, poor appetite, anemia, poor growth
Pyridoxine	Nervous disorders, epileptiform fits, hyperirritability, ataxia, anemia, loss of appetite, edema of peritoneal cavity, colorless serous fluid, rapid postmortem *rigor mortus*, rapid and gasping breathing, flexing of opercles
Pantothenic acid	Clubbed gills, prostration, loss of appetite, necrosis and scarring, cellular atrophy, gill exudate, sluggishness, poor growth
Inositol	Poor growth, distended stomach, increased gastric emptying time, skin lesions
Biotin	Loss of appetite, lesions in colon, skin coloration, muscle atrophy, spastic convulsions, fragmentation of erythrocytes, skin lesions, poor growth
Folic acid	Poor growth, lethargy, fragility of caudal fin, dark coloration, macrocytic anemia
Choline	Poor growth, poor food conversion, hemorrhagic kidney and intestine
Nicotinic acid	Loss of appetite, lesions in colon, jerky or difficult motion, weakness, edema of stomach and colon, muscle spasm while resting, poor growth
B_{12}	Poor appetite, low hemoglobin, fragmentation of erythrocytes, macrocytic anemia
Ascorbic acid	Scoliosis, lordosis, impaired collagen formation, altered cartilage, eye lesions, hemorrhagic skin, liver, kidney, intestine, and muscle
p-Aminobenzoic acid	No abnormal indication in growth, appetite, mortality
A	Impaired growth, exophthalmos, eye lens displacement, edema, ascites, depigmentation, corneal thinning and expansion, degeneration of retina
D	Poor growth, tetany of white skeletal muscle, impaired calcium homeostasis
E	Reduced survival, poor growth, anemia, ascites, immature erythrocytes, variable-sized erythrocytes, erythrocyte fragility and fragmentation, nutritional muscular dystrophy, elevated body water
K	Prolonged blood clotting, anemia, lipid peroxidation, reduced hematocrit

Table 2.3

Vitamin Requirements for Growth[a]

Vitamin	mg/kg dry diet				
	Trout	Salmon	Carp	Channel catfish	Sea bream
Thiamin	10–12	10–15	2–3	1–3	R
Riboflavin	20–30	20–25	7–10	9	R
Pyridoxine	10–15	15–20	5–10	3	5–6
Pantothenate	40–50	40–50	30–40	25–50	R
Niacin	120–150	150–200	30–50	14	R
Folacin	6–10	6–10	N	R	R
Cyanocobalamin (B_{12})	R	0.015–0.02	N	R	R
myo-Inositol	200–300	300–400	200–300	R	300–900
Choline	2000–4000	3000	1500–2000	R	R
Biotin	1–1.2	1–1.5	1–1.5	R	N
Ascorbate	100–150	100–150	30–50	60	R
A	2000–2500 IU	2000–2500 IU	1000–2000 IU	1000–2000 IU	1000–2000 IU
D	2400 IU	2400 IU	N	500–1000 IU	?
E	30	30	80–100	30	?
K	10	10	R	R	?

[a] R, required, but level not known; N, no requirement shown; ?, unknown.

the vitamin deficiency syndrome to appear. At the end of the experimental test period, storage of the vitamin in the liver was measured. Differences in growth response and food conversion were calculated (McLaren *et al.*, 1947a).

Requirements of rainbow trout for thiamin were determined by Phillips and co-workers (1946) at the fish nutrition laboratory in Cortland, New York. These requirements were determined by assaying meat–meal mixtures for thiamin content, feeding these rations to groups of young fish until positive growth differences were observed, and, finally, assaying the liver of the test fish for thiamin content. Requirements listed in Table 2.3 for chinook and silver salmon were determined by feeding duplicate lots of initial feeding fry the thiamin-deficient test diet derived from Table 2.1, to which increments of thiamin hydrochloride were added. Two year classes of fish were fed five vitamin levels for 12 weeks in 15°C water. At the end of the experimental period, 25 livers from each duplicate lot on each diet treatment were assayed microbiologically for thiamin content. The minimum dietary intake which produced the maximum liver thiamin content was selected as the diet treatment which would satisfy the vitamin requirement for that species. Similar

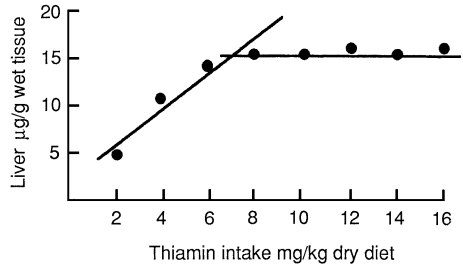


FIG. 2.3

Thiamin intake and tissue stores.

techniques were used for the other vitamin requirements listed in Table 2.3 and for ascorbate (see Section 2.2.9.4).

The requirement for carp was estimated by feeding different levels of thiamin in a modified test diet, noting growth performance and food conversion, and measuring the thiamin content in the hepatic pancreas (Aoe *et al.,* 1967b). Some considerations must be placed on the dietary ingredients in the ration. The National Research Council estimates the thiamin requirement for mammals as about 0.5 mg/1000 kcal dietary intake. The requirement for carnivorous fish does not seem much different, however, the requirement for omnivores or herbivorous fish such as the carp may explain the apparent increased requirement reported. The fat content of the diet may affect not only the caloric intake but also the thiamin requirement because cocarboxylase participates in the oxidation of fat through α-ketoglutarate. Therefore, fish on a high-fat diet and low thiamin intake might take longer to develop deficiencies, or, conversely, when test diets containing more dietary fat are used to assess the thiamin requirement, erroneously low levels of the apparent requirement might be obtained. Fish are poikilothermic and the protein requirement varies with size and water temperature (DeLong *et al.,* 1958a). Therefore thiamin requirements were calculated using a standard test diet under a standard test condition with standard temperatures of 10°C for salmon, 15°C for trout, 25°C for catfish, and 30°C for carp. Quantitative requirement studies reported for other species of fish need to be examined and equated to experimental conditions used (NRC, 1993; Halver, 1995). A typical plot of thiamin intake and tissue stores is shown in Fig. 2.3.

2.2.1.5. Sources and Protection

Common sources for thiamin are in plant seeds, dried peas, beans, soybeans, cereal bran, and dried yeast. Fresh glandular tissue is also a good

source for thiamin and other members of the B vitamin water-soluble complex but is seldom used in modern commercial fish diets. Thiamin can be easily lost by holding wet diet ingredients too long in storage or by preparing the diet under slightly alkaline conditions or in the presence of sulfite. Because thiamin is relatively stable to dry heat, dry pellet rations will retain the vitamin through the pelleting process and subsequently during dry-sealed storage. Wet or frozen diets pose a different problem because moisture content allows increased chemical reaction and subsequent increased danger for biological hydrolysis and thus destruction of thiamin. Obviously, wet or moist diet preparations containing any fresh fish or shellfish tissue must be used immediately or suffer loss of thiamin through thiaminase hydrolysis (Deutsch and Ott, 1942; Agren, 1945; New, 1987).

2.2.1.6. Antimetabolites and Inactivation

Acetylcholine is an antagonist to thiamin and pyrithiamin. Oxythiamin and normal butylthiamin are specific antimetabolites (West *et al.,* 1966). Several thiaminases occur which destroy thiamin. These rupture the thiazole ring at the sulfur bond, making the residue inactive. Freshwater fish tissues have a high thiaminase activity, as do tissues from clams, shrimp, and mussels. Thiaminases have also been found in beans and mustard seed and in several microorganisms (Goldsmith, 1964). Thiaminase activity is low in most saltwater fish tissues, however, and the enzyme is inactivated by heating or prolonged pasteurization. Thiamin present in fresh *Torula* yeast is relatively unavailable to fish, but the yeast becomes an excellent thiamin source after the cells are ruptured by steam treatment or by dehydration.

2.2.1.7. Clinical Assessment

Clinical assessment of thiamin status in fish may be made by measuring erythrocyte transketolase activity in rainbow trout and silver salmon using the method of Brin (1963). Levels of thiamin can also be assessed by microbiological assay of liver tissue from representative samples of the fish population. As an example, typical saturated levels of thiamin activity in sea salmon range from 15 to 20 μg of thiamin/g of wet liver tissue. Fingerling chinook or coho salmon reared in 10 or 15°C water on test diets containing the listed amount or more of thiamin hydrochloride assayed at 8–10 μg of thiamin/g of wet liver. These liver storage levels and normal erythrocyte transketolase activity in the absence of any deficiency sign, coupled with good growth and good food conversion, indicate an adequate thiamin intake for that fish population. Kidney or liver transketolase activity is a good indicator of thiamin status in trout (Lehnitz and Spanhof, 1977; Masumoto *et al.,* l987) and in turbot (Cowey *et al.,* l975).

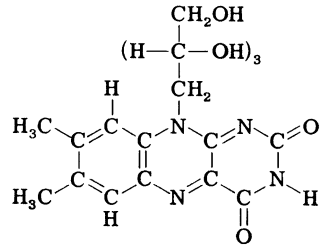


Fig. 2.4

Riboflavin.

2.2.2. Riboflavin

Growth promoting yellow–green pigments were isolated in 1879. Emmett and McKim (1917) showed two "B" vitamins present. Vitamin G deficiency was described by Goldberger and Lillie (1926). A rat assay for vitamin B_2 was designed by Bourquin and Sherman (1931), and crystalline riboflavin was isolated by Kuhn *et al.* (1933). Lactoflavin, hepatoflavin, and ovoflavin were shown to be identical to the pure riboflavin synthesized by Kuhn *et al.* (1933) and Karrer *et al.* (1935). Riboflavin was postulated as one part of factor H for fish by Tunison *et al.* in 1943. Riboflavin deficiency in trout was first described by McLaren *et al.* (1947a), in salmon by Halver (1951), in carp by Aoe *et al.* (1967), and in catfish by Dupree (1966).

2.2.2.1. Chemical Structure, Characteristics, and Analogs

Riboflavin is a yellow–brown crystalline pigment with the chemical formula $C_{17}H_{20}N_4O_6$ and the structural formula shown in Fig. 2.4. The material is slightly soluble in water with yellow–green fluorescence and is very soluble in alkali. It is insoluble in most fat solvents except alcohol. Riboflavin is stable to oxidizing agents in strong mineral acids and in neutral aqueous solutions and is heat stable in dry form. It is irreversibly decomposed on irradiation with ultraviolet rays or visible light, breaking down to lumiflavin. Riboflavin phosphate is the chemically active group in Warburg's "yellow" enzyme.

2.2.2.2. Positive Functions

Riboflavin functions in the tissues in the form of flavin adenine dinucleotide (FAD) or as flavin mononucleotide (FMN). Free riboflavin has been isolated in urine, plasma, and the retina of the eye. The flavoproteins function as enzymes of tissue respiration and are involved in hydrogen transport to catalyze the oxidation of reduced pyridine nucleotides

(NADH and NADPH). Thus, they function as coenzymes for many oxidases and reductases such as cytochrome *c* reductase, D- and L-amino acid oxidases, xanthine and aldehyde oxidase, succinate dehydrogenase, glucose oxidase, and fumaric dehydrogenase. Riboflavin is involved with pyridoxine in the conversion of tryptophan to nicotinic acid and is most important in the respiration of poorly vascularized tissues such as the cornea of the eye. Riboflavin is involved in the retinal pigment during light adaptation, and lack of it causes impaired vision and photophobia in experimental animals including fish.

2.2.2.3. Deficiency Syndrome

A summary of deficiency signs in fish is included in Table 2.2. Riboflavin tissue storage is exhausted in young salmonids after 10–12 weeks on riboflavin-deficient diets in 10–15°C water systems. Poor appetite and poor diet efficiency are the first signs, followed by photophobia, mono- or bilateral cataracts, corneal vascularization, eye hemorrhage, incoordination, and general anemia. Dark pigmentation coupled with striated constrictions of the abdominal wall in salmon has been noted. Skin atrophy has been reported for some fish species and abnormal pigmentation of both the skin and the iris has been noted (Aoe *et al.,* 1967b; Halver, 1953a,b). Short body dwarfism has been reported in catfish (Murai and Andrews, 1978b). Replacement of riboflavin in the diet reduces the symptoms except when cataracts have developed and the protein crystal structure of the lens of the eye has been lost (Halver, 1957). This irreversible condition will continue in monolateral cataracts throughout the life of the fish, whereas bilateral cataracts largely result in eventual starvation and death of the afflicted animal. The first specific signs have consistently appeared in and about the eye of the salmonids, carp (Aoe *et al.,* 1967b), catfish (Dupree, 1966), and eel (Arai *et al.,* 1972).

2.2.2.4. Requirements

The requirements of fish for riboflavin under experimental conditions in 10–15°C water supplies are listed in Table 2.3. These values have been determined for trout by assaying riboflavin content in meat–meal mixtures with microbiological assay techniques, feeding these diets to fish until growth differences were observed, and then assaying for maximum liver storage of the vitamin. Values for the trout are slightly lower than those reported for salmon which were fed the test diet containing different increments of crystalline riboflavin, determined by measuring the growth response for 10 weeks and then assaying the livers to determine the diet treatment which would induce maximum liver storage. The requirements may vary depending on the balance of other dietary ingredients, caloric density, and environmental conditions under which the fish is raised. The requirement listed under

these standard test conditions described should furnish a tentative requirement which will satisfy biological demands for normal growth, health, and physiological function. Most of these requirement studies have been made on very young fish, often initial feeding fry, with the logical assumption that the vitamin requirement of these young fish would be higher than that of larger fish having advanced metabolic enzyme systems with the ability to synthesize at least some of the requirement for these vitamins. Requirements for other species listed in Table 2.3 may have been determined using different criteria and experimental conditions.

2.2.2.5. Sources and Protection

Riboflavin is widely distributed in plants and in animal glandular tissues. Milk, liver, kidney, heart, yeast, germinated grains, peanuts, soybeans, and eggs are rich sources. Protection of finely ground raw materials and mixing processes from sunlight or intense artificial light is necessary to minimize loss of the vitamin by conversion to lumiflavin. Fortunately, the pH conditions for most diet preparations with ingredients commonly used in either dry or wet fish rations involve a relatively stable environment for retention of riboflavin activity during diet preparation. As long as the ingredients and the stored rations are protected from light in dark bags or in tight containers, most of the riboflavin activity will be carried from the raw materials into the food fed.

2.2.2.6. Antimetabolites and Inactivation

Galactoflavin is an antagonist to riboflavin and inhibits growth of rats when the diet contains this compound. Flavin monosulfate inhibits D-amino acid oxidase and appears to act as a competitor and inhibitor of riboflavin for growth of *Lactobacillus casei*. When the ribose group in the molecule is replaced by the other groups, analogs have been formed which either have some activity or become antimetabolites. The hydroxyethyl analog is an antagonist for riboflavin function in both rats and bacteria and also shows antifungal activity (West *et al.,* 1966).

2.2.2.7. Clinical Assessment

Liver tissue of actively feeding sea salmon contains between 6 and 8 μg of riboflavin/g of wet tissue. In a freshwater environment, young feeding fish fed test diets in I5°C water systems showed liver storage of 3.5–4.0 μg/g. An estimate of riboflavin content of the diet may be obtained from a microbiological assay since this vitamin is relatively stable, and one can calculate the approximate dietary intake from levels present in the food supply. The riboflavin content of blood plasma does not change significantly in riboflavin deficiency in other experimental animals. However, the erythrocyte riboflavin content has been reported to be about 10 μg/100 ml blood for

humans on a low riboflavin intake and approximately twice that level for those on a high intake (Bessey *et al.,* 1956). Urinary excretion of riboflavin has been used clinically, with 200 μg in 24 h suggesting an adequate intake and less than 100 μg indicating a low intake. Excretion of 50 μg or less daily is a strong indication of an extended dietary deficiency. Several studies indicate that the excretion of less than 200 μg of riboflavin/g of creatinine is indicative of deficiency (Goldsmith, 1964). Erythrocyte glutathione reductase in the presence of added FAD can be used to determine the riboflavin status in fish (Hughes *et al.,* 1981). Woodward (1983) reported that D-amino acid oxidase activity is a sensitive indicator of riboflavin status in rainbow trout.

2.2.3. Pyridoxine

A new factor which cured dermatitis in rats was reported by György (1935). The active material was isolated in 1938 by several groups, and pyridoxine was synthesized by Harris and Folkers (1939). Tunison *et al.* listed the first quantitative requirements for fish in 1944. Pyridoxine deficiency in trout was reported by McLaren *et al.* (1947a), in salmon by Halver (1953a,b), in catfish by Dupree (1966), in carp in by Ogino (1965), in sea bream by Kissel *et al.* (1981) in yellowtail by Sakaguchi *et al.* (1969), and in eel by Arai *et al.* (1972).

2.2.3.1. Chemical Structure, Characteristics, and Analogs

The vitamers B_6 consist of pyridoxine, pyridoxal, pyridoxamine, and several other derivatives which have biological activity or can be converted into the most biologically active form of pyridoxal. The stable pyridoxine hydrochloride form has the chemical formula $C_8H_{11}O_3N_7HCl$, and the structural formulas for the three common forms of vitamin B_6 are shown in Fig. 2.5. Pyridoxine hydrochloride is readily soluble in water and is heat

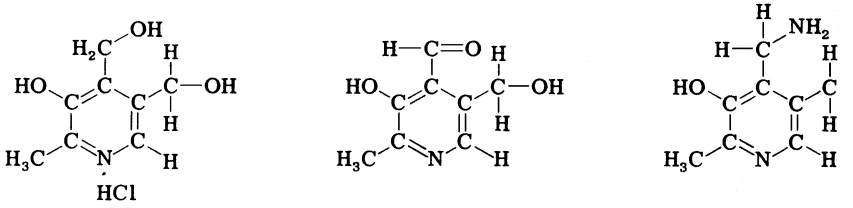


FIG. 2.5

Pyridoxine: three common forms used are (*left to right*) pyridoxine hydrochloride, pyridoxal, and pyridoxamine.

stable in either acid or alkaline solutions. Pyridoxal phosphate acts as the coenzyme in a number of systems and pyridoxic acid, deoxypyridoxine, and methoxypyridoxine are closely related compounds with varying degrees of activity. Pyridoxine is sensitive to ultraviolet light in neutral or alkaline solutions. Pyridoxamine and pyridoxal in dilute solutions are labile compounds which are rapidly destroyed by exposure to air, heat, or light (Chow, 1964). Therefore, most vitamin supplementation is in the form of pyridoxine hydrochloride and analysis for pyridoxine activity by microbiological assay of diet ingredients probably measures pyridoxal phosphate and other intermediates as well.

2.2.3.2. Positive Functions

Pyridoxal phosphate is the coenzyme, codecarboxylase, for decarboxylation of amino acids. It is also involved in the transaminase systems, and 22 transaminases in animal tissues have been shown to require a distinct enzyme with pyridoxal phosphate as the coenzyme (Cammarata and Cohen, 1950). Pyridoxal phosphate has been shown to be the coenzyme for decarboxylation of 5-hydroxytryptophan with the consequential production of 5-hydroxytryptamine or serotonin (Weissbach *et al.,* 1959). Pyridoxal phosphate is also required for assisting desulfhydrase in converting cysteine to pyruvic acid. Porphyrin synthesis is also involved and pyridoxal phosphate is the cofactor for synthesis of γ-aminolevulinic acid. Many neurohormones require pyridoxal phosphate as a coenzyme in their synthesis and it is involved and is essential for tryptophan utilization and metabolism of glutamic acid, lysine, methionine, histidine, cysteine, and alanine. Pyridoxine is also involved in fat metabolism, especially of the essential fatty acids. It is involved in the synthesis of messenger RNA, which generates transfer of information at the site of polypeptide synthesis (Montjar *et al.,* 1965). The vitamers B_6 play a most important role in protein metabolism, and as a result, carnivorous fish have stringent requirements for pyridoxine in the diet and stores are rapidly exhausted (Hardy *et al.,* 1979).

2.2.3.3. Deficiency Syndrome

Signs of pyridoxine deficiency in fish are listed in Table 2.2. Since salmonids, ictalurids, and very young cyprinids are carnivorous, with protein requirements for young animals of between 40 and 50% of the ration, pyridoxine stores are rapidly exhausted when fish are held on pyridoxine-deficient rations. Acute deficiency signs occur after 14–21 days in salmon on a diet devoid of pyridoxine and the entire population dies in 28 days when fed a diet containing 50% or more protein in 12–15°C water. Since pyridoxine is involved in brain metabolism and in the homeostasis of serotonin, epileptic-type fits occur. Also, general nervous disorders, hyperirritability,

and alteration of the control of melanophore contraction occurs. Postmortem *rigor mortis* occurs very rapidly. Rapid and gasping breathing with flexing of the opercles is a common observation, and edema in the peritoneal cavity with colorless serous fluid occurs in some fish on some experimental treatments (Halver, 1953a, 1957; Coates and Halver, 1958). Salmon, trout, carp, and yellowtail exhibit premortem rigor a few hours before death, and when the deficiency has progressed this far, recovery is very unlikely unless fish are injected with pyridoxal phosphate. Tetany, nervous disorder, and a blue–green color occur in deficient catfish (Andrews and Murai, 1979). Japanese eels showed anorexia and convulsions in 3–4 weeks on deficient diets (Arai *et al.,* 1972). Alanine aminotransferase activity was related to vitamers B_6 in trout (Smith *et al.,* 1974; Jurss, 1978). Handling the animals generally induces more damage than vitamin administration corrects. Recovery of those fish still feeding is equally rapid and dramatic on administration of pyridoxine hydrochloride in the diet. Deficiency signs disappear within a day or two after the pyridoxine is replaced in the ration. Erythrocyte transaminase activity and plasma transaminase activity reflect the deficiency state (Hardy *et al.,* 1979; Cowey *et al.,* l981; Adron *et al.,* l978; Kissel *et al.,* 1981).

2.2.3.4. Requirements

Pyridoxine requirements for salmonids, cyprinids, and ictalurids are listed in Table 2.3. Trout requirements were obtained from analysis of meat–meal mixtures, growth response, and assay for maximum liver storage. Salmon requirements were assessed by feeding different increments of crystalline pyridoxine hydrochloride added to the vitamin test diet and measuring the growth response and maximum liver storage. Trout requirements appear to be lower than those of salmon but the experimental salmon were smaller fish and may have reflected the higher protein requirement for that size animal and water temperature. Requirements for other species used maximum growth or enzyme saturation levels.

2.2.3.5. Sources and Protection

Good sources of vitamer B_6 activity are yeast, cereal brans, cereal grains, cereal germ, egg yolk, liver, and glandular tissues. Pyridoxine compounds in phosphorylated form present in agricultural products are fairly stable but are labile to ultraviolet radiation. Dietary ingredients in open pans should be protected from exposure to sunlight. Some pyridoxal phosphate will be lost on exposure to air. Free forms of pyridoxal and pyridoxamine are rapidly destroyed by air, light, and heat when in a moist form such as in the preparation of moist diets. Pyridoxine hydrochloride supplementation is most desirable for preparation of custom or commercial fish diets because

of the tremendous role pyridoxine plays in maintaining normal protein metabolism during growth of carnivorous fish.

2.2.3.6. Antimetabolites and Inactivation

Antagonists may compete for reaction sites of the apoenzyme or may react with pyridoxal phosphate to form inactive compounds. Deoxypyridoxine is a potent B_6 antagonist because of competition for apoenzyme sites but is a useful agent to accelerate B_6 deficiency in experimental animals (Martin *et al.,* 1948). This same compound inhibits tyrosine decarboxylase (Beiler and Martin, 1947). Methoxypyridoxine is another antagonist and toxopyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine) produces liver damage in rats, inhibits glutamic acid decarboxylase, and causes convulsions (Nishizawa *et al.,* 1958). Isonicotinic acid hydrazide, used for the treatment of tuberculosis, is chemically related to pyridoxine and acts as a B_6 antagonist. Specific pyridoxal-5-phosphate antagonists were reviewed by Chow (1964).

2.2.3.7. Clinical Assessment

Plasma and erythrocyte transaminase activity has been shown to reflect the pyridoxine status of the animal. A high tryptophan load in the diet increases the vitamin B_6 requirement and misleads plasma transaminase activity measurements (Chow, 1964). Liver storage measured by microbiological assay was 5–6 μg of B_6 activity/g of fresh sea salmon liver, whereas fingerling salmon fed a 50% protein diet in fresh water had 2–3 μg/g of wet tissue. Assay of diet for the vitamers B_6 by microbiological methods gives a truer representation of the total B_6 activity including intermediates than is obtained in specific chemical determinations. A fluorescent lactone of pyridoxic acid can be prepared from the urine of humans by heating with strong acids, but this simple fluorometric analysis needs to be tested for fish metabolic wastes because of altered pathways for elimination of nitrogenous compounds by these aquatic animals. Alanine aminotransferase activity was related to vitamer B_6 intake in trout (Smith *et al.,* 1974; Jurss, 1978). Hardy *et al.* (1979) used erythrocyte glutamic-oxaloacetic transaminase (aspartate transaminase) activity to determine pyridoxine status in salmon.

2.2.4. Pantothenic Acid

A chick dermatitis was cured by Elvehjem and Koehn in 1935 with a factor containing β-alanine. The requirement for this factor was determined by Lepkovksy in 1936 and the active material was isolated and used by Jukes (1939) and by Woolley *et al.* (1939). Pantothenic acid was synthesized by Stiller *et al.* (1940). Pantothenol was shown to be the active factor by Pfaltz

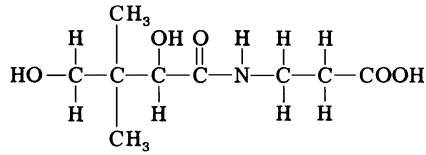


FIG. 2.6

Pantothenic acid.

in 1943 (Hein, 1964). Phillips *et al.* observed clubbed gills in trout fed pantothenic acid-deficient diets in 1945. Rucker *et al.* (1952) observed that salmon fed low-pantothenic acid diets developed clubbed gills.

2.2.4.1. Chemical Structure, Characteristics, and Analogs

Pantothenic acid may be considered a dihydroxydimethylbutyric acid bonded to β-alanine. The chemical formula is $C_9H_{12}O_5N$ and the structural formula for 2,4-dihydroxy-3,3-dimethylbutyryl-β-alanine is shown in Fig. 2.6. The free acid is a yellow, viscous oil and therefore the compound generally used in fish nutrition is the calcium salt. This salt is a white crystalline powder readily soluble in water and mild acid and almost-insoluble in fat solvents. It is stable to oxidizing and reducing agents and to autoclaving but is labile to dry heat, hot alkali, or hot acid. Pantothenol has almost as much activity as pantothenic acid for growth of chicks. Pantothenic acid acetate, benzoate, and diphosphate esters are biologically active for animals but not for lactic acid bacteria. The optical isomer L-pantothenic acid appears to be physiologically inert. Some organisms may utilize a portion of the molecule. Bacteria appeared to require only the dihydroxydimethylbutyric acid, and some yeasts utilize only β-alanine. Animals, however, need the entire pantothenic acid molecule or its reduced alcohol form to satisfy the vitamin requirements (Chow, 1964).

2.2.4.2. Positive Functions

Pantothenic acid is part of acetyl coenzyme A, which occurs in many enzymatic processes involving two-carbon compounds. It has been shown to be required by all animal species studied and by many microorganisms (Chow, 1964; West *et al.*, 1966). The acetyl coenzyme A system is involved in the acetylation of aromatic amines and choline; condensation reactions for the synthesis of acetate, fatty acids, and citrate; and the oxidation of pyruvate and acetaldehyde. And it is essential for the development of the central nervous system. The two-carbon fragment called "active acetate," or acetyl coenzyme A, is an essential intermediate in metabolism. It is involved in most acylation reactions in the body including acyl groups other than acetate such as succinate, benzoate, propionate, and butyrate. Pantothenic

acid is involved in adrenal function and the production of cholesterol. Coenzyme A is also involved in many other steps of intermediate metabolism of carbohydrates, fats, and proteins. It is obviously a key nutrient for normal physiology and metabolism of a growing fish.

2.2.4.3. Deficiency Syndrome

Deficiency signs for pantothenic acid are summarized in Table 2.2. Under standard test conditions with a deficient diet fed in 10–15°C water systems, salmon and trout exhaust pantothenic acid stores in 8–12 weeks. Yellowtail developed deficiency signs after only 10–14 days (Hosokawa 1989). Fish stop feeding and microscopic or hand lens examination of gill filament show proliferation of epithelial surface plus swelling and clubbing together of the filaments and lamellae. The surface of the gills is often covered with an exudate. Fish become prostrate or sluggish. The opercles are distended and the animals appear to have a "mumpy" appearance when viewed from above (Phillips *et al.*, 1945; Rucker *et al.*, 1952). Necrosis, scarring, and cellular atrophy of the tender gill elements occur and anemia develops after prolonged deficiency (Halver, 1953a,b, 1957). Dietary gill disease has been adequately described and correlated with pantothenic acid deficiency. The same type of symptom has been observed in salmon, trout, eel, carp (Ogino, 1967b; Steffens, 1969), yellowtail (Hasokawa, 1989), red sea beam (Yone and Fuji, 1974), and catfish (Dupree, 1966). Catfish develop eroded jaws, fins, and barbels (Murai and Andrews, 1979). After replacement of pantothenic acid in the diet, recovery is rapid for those fish still feeding and the typical clubbed gill disappears clinically after about 4 weeks on the recovery diet; however, evidence of necrosis and scarring are retained as the gill filaments and lamellae repair.

2.2.4.4. Requirements

Dietary requirements for salmon, trout, and other fish are listed in Table 2.3. Trout values were determined using the Wisconsin technique for maximum growth response or using the Cortland technique of assaying diet ingredients, measuring the growth response, and assaying for maximum liver storage for the different diet treatments. Salmon values were determined by using test diets supplemented with increments of calcium pantothenate, measuring the growth response, and assaying for maximum liver storage. Requirements for other species used intake versus growth or tissue saturation (NRC, 1993).

2.2.4.5. Sources and Protection

Good sources for pantothenic acid are cereal bran, yeast, liver, kidney, heart, spleen, and lung. Fish flesh is a relatively rich source, although the

content is only about 20% of the pantothenic acid found in animal glandular tissue. Royal jelly probably contains the greatest amount and must have some role in the transformation of bee larvae into queen bees, as it contains over 500 μg of pantothenol/g of dry weight (Pearson and Burgin, 1941). Pantothenic acid in the sodium or calcium salt form is relatively stable and can be incorporated into either moist or dry fish diets. Some loss is incurred during autoclaving and excessive heat should therefore be minimized during diet preparations. Because the free acid is labile to heat and also to acid and alkali, some loss can be expected during warm, moist, diet preparation or during warm, moist storage. Certain cereal brans may have pantothenic acid bound in a form unavailable to fish because of the low digestibility coefficients and so should be used sparingly as the pantothenic acid source for the diet. During pasteurization some loss may occur during preparation of wet fish or animal glandular tissue in moist diet formulas.

2.2.4.6. Antimetabolites and Inactivation

Because pantothenic acid affects the respiration of many types of cells, compounds such as 6-mercaptopurine, 2,6-diaminopurine, and 8-azaguanine, which inhibit growth of tumors, are antagonistic to pantothenic acid (Chow, 1964). Pantoyltaurine is an antimetabolite of pantothenic acid and has been used to accelerate deficiency syndromes in experimental animals (Winterbottom *et al.,* 1947). Many derivatives of this compound have been prepared and used for this purpose. Winterbottom *et al.* (1947) reported that methyl-ω-pantothenic acid is also an antagonist which interferes with the formation of acetyl coenzyme A and accelerates deficiency symptoms in animals. This compound inhibited sulfanilamide acetylation in pigeon liver homogenates but did not prevent citric formation (Dietrich and Shapiro, 1956). Pantothenic acid itself can be used to overcome the inhibitory effects of these antagonists. It has been shown to reverse the blocking of nuclear mitosis with 6-mercaptopurine in animals. A high pantothenic acid or coenzyme A intake is effective. The migratory urge of salmon can be inhibited by providing high levels of calcium pantothenate in the diet, but this effect is only transitory and salmon reverted to the migratory urge after about 3 weeks of a high pantothenic acid dietary intake (Burrows *et al.,* 1951).

2.2.4.7. Clinical Assessment

Assay of the pantothenic acid content in the diet may be misleading unless care is exercised in proper hydrolysis of the raw materials being assayed. Pantothenic acid is liberated only slowly by normal hydrolytic procedures and inadequate hydrolysis will result in low values. Complete hydrolysis with enzyme preparations will liberate all the pantothenic acid from biologically active material of glandular tissues, fish flesh, yeast, and bran. Coenzyme

A is present in cells of most biological material, which must be hydrolyzed adequately to liberate pantothenic acid, pantotheine, phosphopantothenic acid, phosphopantotheine, and bound coenzyme A (Brown, 1959). Liver storage for actively feeding sea salmon shows a pantothenic acid content of 18–20 μg/g of fresh tissue. Young chinook and coho salmon fingerlings reared in fresh water at 12–15°C showed a maximum liver storage of about 14–16 μg of pantothenic acid/g of fresh liver tissue. Load tests and acetylation reactions of sulfanilamide have been used in humans and other experimental animals but have not yet been extended for assessment of pantothenic acid status in fish.

2.2.5. Niacin

Nicotinic acid was synthesized by Huber and Weidel in 1873 (Hein, 1964) but was left on the shelf as an organic compound unrelated to the severe pellagra afflictions occurring throughout the world at that time. Sixty years later Warburg and Christian (1935) showed it to be present in coenzymes I and II, and 2 years thereafter Elvehjem *et al.* (1937) cured "black tongue" in dogs with the vitamin. Niacin was postulated to be part of factor H for fish in 1937 (Tunison *et al.,* 1943), but deficiency symptoms were not adequately described until reported in trout by McLaren *et al.* (1947a).

2.2.5.1. Chemical Structure, Characteristics, and Analogs

Niacin or nicotinic acid is pyridine-3-carboxylic acid, with the chemical formula $C_6H_5O_2N$ and the structural formula shown in Fig. 2.7. Niacin is the preferred nomenclature rather than nicotinic acid, the original name of the material synthesized in 1873 by Hubert and Euler. Nicotinic acid amide or niacinamide is the common form in which the vitamin is physiologically active. Niacin is a white, crystalline solid, soluble in water and alcohol and more soluble in alkali. It is stabile in the dry state and may be autoclaved for short periods without destruction. It is also stabile to heat in mineral acids and alkali. Niacin is both a carboxylic acid and an amine and forms quaternary ammonium compounds because of its basic nature. Acidic

Fig. 2.7

Niacin: nicotinic acid (*left*) and niacinamide (*right*).

characteristics include salt formation with alkali and reactional heavy-metal salts. Niacin can be esterified easily, then converted to amides. Niacinamide is a crystalline powder with the formula $C_6H_6N_2O$ and is soluble in water and ethanol, and the dry material is stable up to about 60°C. In aqueous solutions it is stable for short periods of autoclaving. It is the form of the vitamin normally found in niacinamide adenine dinucleotide phosphate (NADP). Niacin, niacinamide, NAD, and several derivatives of NAD and NADP have biological activity.

2.2.5.2. Positive Functions

The major functions of niacin in NAD and NADP are the removal of hydrogen from substrates and the transfer of hydrogen or electrons to another coenzyme in the hydrogen transport series. Most of these enzyme systems function by alternating between the oxidized and the reduced state of the coenzymes NAD–NADH and NADP–NADPH. These oxidation–reduction reactions are anaerobic, as, for example, when pyruvate acts as the hydrogen acceptor and lactate is formed. The second type of oxidation–reduction reaction is coupled to electron transport with subsequent oxidation of reduced NADH or NADPH, and these are aerobic reactions which function in respiration. Both NAD and NADP are involved in the synthesis of high-energy phosphate bonds which furnish energy for certain steps in glycolysis, in pyruvate metabolism, and in pentose synthesis. Niacin is also involved in lipid metabolism, amino acid and protein metabolism, and photosynthesis. An interrelationship between thiamin and niacin exists since both vitamins are involved in coenzyme systems of carbohydrate metabolism in energy-generating systems of intermediary metabolism where food material is oxidized to furnish heat for physiological functions, to maintain homeostasis, for body temperature in homeotherms, or to generate high-energy phosphate bonds for subsequent physiological reactions of the living organism. Over 100 pyridine-linked dehydrogenases function in normal metabolism as hydrogen acceptors in energy-yielding or biosynthetic pathways. A good review of niacin biochemistry has been presented by Goldsmith (1964).

2.2.5.3. Deficiency Syndrome

The deficiency signs in fish are listed in Table 2.2. Stores of niacin are exhausted more slowly under experimental conditions than stores of some of the other vitamins, resulting in less defined and more slowly developing symptoms. Elvehjem cured black tongue in dogs by administration of niacin in 1937 (Elvehjem *et al.,* 1937) and a history of pellagra-like symptoms in primates of ancient record was elegantly described by Casal over 200 years ago (Harris, 1919). Niacin deficiencies in fish were experimentally induced in the late 1940s and early 1950s by using basal diets which had a low niacin

content. Loss of appetite and poor food conversion were the first signs noted; then fish turned dark and went off feed. On continued exposure to the deficient regimen, lesions in the colon appeared, erratic motion was observed, edema of the stomach and colon appeared, and muscle spasms occurred while fish were apparently resting (McLaren *et al.,* 1947a; Wolf, 1951; Halver, 1953a,b, 1957). A predisposition to sunburn in fish confined in the open in shallow ponds or raceways was described (DeLong *et al.,* 1958b). Carp showed a congestion of the skin with subcutaneous hemorrhages (Aoe *et al.,* 1967a). Eels showed similar skin lesions and abnormal swimming (Arai *et al.,* 1972). Catfish showed skin lesions, deformed jaws, and anemia (Andrews and Murai, 1978). Common symptoms of niacin deficiency in most fish studied were muscular weakness and spasms, coupled with poor growth and poor food conversion.

2.2.5.4. Requirements

Niacin requirements in young fish tested experimentally are listed in Table 2.3. Brook and brown trout requirements were calculated by feeding meat–meal mixtures which were assayed for niacin content and then noting the growth response and calculating the point of maximum liver storage at the end of the experimental period. Rainbow trout and salmon requirements were determined by feeding test diets containing different increments of niacin and then measuring growth and the point of maximum liver storage at the end of the experimental period. Salmon requirements appear to be approximately twice those of trout and may reflect differences in metabolism or an imbalance of nutrients in the test diets. In homeotherms on a balanced test ration the niacin requirement is generally estimated to be about 10 times the thiamin requirement. These rations generally contain considerable carbohydrate material to furnish energy to maintain body temperature. In fish the requirement appears to be 20 to 30 times the thiamin needs determined for the same test conditions and test rations. This difference may be due to the low carbohydrate content of young fish diets and the higher protein content of these rations. A well-established conversion of tryptophan to niacin has been reported by Nishizuka and Hayaishi (1963) for mammalian liver. This conversion may account for the slow development of niacin deficiency syndrome in fish. However, after 10–14 weeks on diets devoid of niacin, deficiency symptoms did occur in several species of fish. The symptoms were reduced by replacement of niacin in the ration even when high-protein diets containing an excess of tryptophan were fed. Conversion of tryptophan to niacin is limited in fish and niacin must be fed to prevent deficiency and impaired growth. However, too much niacin inhibits growth (Poston, 1969a; Poston and Lorenzo, 1973; Poston and Combs, 1980).

2.2.5.5. Sources and Protection

Niacin is found in most animal and plant tissues. Rich sources are yeast, liver, kidney, heart, legumes, and green vegetables. Wheat contains more niacin than corn and the vitamin is also found in milk and egg products. The vitamin is very stable, as it is generally found in coenzyme form in raw materials. Niacin added to the diet as a supplement remains relatively unaltered during diet manufacture, processing, and storage.

2.2.5.6. Antimetabolites and Inactivation

Pyridine-3-sulfonic acid and 3-acetylpyridine are compounds structurally related to niacin and are antimetabolites for this vitamin in animals and in microorganisms. Additional niacin can overcome the antimetabolite effect. A niacin deficiency symptom in rats may be induced by 6-aminoniacinamide. The symptom is reversed by the addition of 10 times more niacinamide than antimetabolite (Goldsmith, 1964). Thioacetamide and phenothiazine have been reported to inhibit niacin function in fish and thus cause a predisposition to sunburn and skin lesions (Rucker, 1957). Similar results occurred in deficient rainbow trout (Poston and Wolfe 1985).

2.2.5.7. Clinical Assessment

Liver storage levels of actively feeding sea salmon show 70–80 μg of niacin/g of wet liver tissue. About half of this amount is present in fingerling salmon raised in 12–15°C water environments and fed test rations containing 40–50% protein and niacin supplements of 500–750 mg/kg dry diet. Urinary metabolites of niacin have been measured in other animals on a standard niacin load in test rations containing a standard tryptophan load. The technique is well developed to measure the N^1-methyl derivative in mammalian urine (Handley and Bond, 1948). These data have not been reported for fish but metabolism chambers are available for collecting branchial and urinary wastes from large fish intubated with different dietary material (Lotlikar *et al.,* 1967; Smith, 1971).

2.2.6. Biotin

Egg white "injury" in rats was described by Bateman (1916) and by Boas (1927). Neural disturbances from this injury were reported by Findlay and Stern in 1929, and the active material was called "coenzyme R" by Allison *et al.* (1933) and "vitamin H" by György in 1939. Biotin was isolated by Kogl and Tormis (1936) and the functions were defined for the active material by György in 1940. Biotin was synthesized by du Vigneaud (1942) and by Harris *et al.* (1943). Biotin was once thought to be part of factor H for fish.

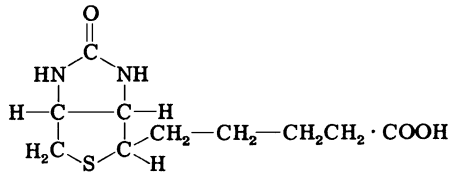


Fig. 2.8

Biotin.

Blue slime patch disease due to biotin deficiency in trout was reported by Phillips *et al.* (1945) and McLaren *et al.* (1947a).

2.2.6.1. Chemical Structure, Characteristics, and Analogs

δ-Biotin, hexahydro-2-oxo-l-thieno-3,4-imidazole-4-valeric acid, has the chemical formula $C_{10}H_{16}O_3N_2S$ and the structural formula shown in Fig. 2.8. It is a monocarboxylic acid slightly soluble in water and alcohol and insoluble in fat solvents. Salts of the acid are soluble in water. Aqueous solutions or the dry material are stable at 100°C and to light. The vitamin is destroyed by acids and alkalis and by oxidizing agents such as peroxides and permanganate. Biocytin is a bound form of biotin isolated from yeast, plant, and animal tissues (Wright *et al.,* 1954). Other bound forms of the vitamin can generally be liberated by peptic digestion. Oxybiotin has partial vitamin activity but oxybiotin sulfonic acid and other analogs are antimetabolites inhibiting the growth of bacteria. This inhibition can be overcome by additional biotin and therefore must be due to inhibition of incorporation of the biotin into coenzymes (West *et al.,* 1966). Avidin, a protein found in raw egg white, binds biotin and makes it unavailable to fish and other animals. This binding is irreversible in raw material, but heating to denature the protein makes the bound biotin available again to the fish. Biocytin or ε-biotinyllysine (the ε-amino group of lysine and the carboxyl of biotin being combined in a peptide bond) is hydrolyzed by the enzyme biotinase, making the protein-bound biotin available.

2.2.6.2. Positive Functions

Biotin is required in several specific carboxylation and decarboxylation reactions, reviewed by Lardy and Peanasky (1953), Vagelos (1964), and Knappe (1970). Biotin is part of the coenzyme of several carboxylating enzymes fixing CO_2, such as propionyl coenzyme A in the formation of propionic acid, acetyl-CoA carboxylase, and pyruvate carboxylase. Carboxylase fixation of CO_2 to form methylmalonyl coenzyme A is involved in the carboxylation and decarboxylation of tricarboxylic acids. Biotin is also involved

in purine synthesis and is the coenzyme of malonyl coenzyme A involved in elongation of fatty acids (Waite and Wakil, 1966). It is involved in general lipid synthesis in animals and in the deamination and biosynthesis of citrulline. It is also involved in the conversion of unsaturated fatty acids to the stable cis form in the synthesis of biologically active fatty acids.

2.2.6.3. Deficiency Syndrome

Some signs of biotin deficiency in salmonids are skin disorders, muscle atrophy, lesions in the colon, loss of appetite, and spastic convulsions. Hematology discloses fragmentation of erythrocytes. Poor growth is a common symptom and has been reported for salmonids (McLaren *et al.,* 1947a; Phillips *et al.,* 1949; Wolf, 1951; Halver, 1953a,b, 1957), common carp (Ogino *et al.,* 1970b), goldfish (*Carassius auratus*) (Tomiyama and Ohba, 1967), channel catfish (Lovell and Buston, 1984), and eel (Arai *et al.,* 1972). Blue slime patch disease in brook trout deficient in biotin appears to be typical for this species and is described in detail by Phillips *et al.* (1949). Fish reared in 10–15°C water exhaust biotin stores in 8–12 weeks and the first signs are anorexia, poor food conversion, and general listlessness before the more acute deficiency symptoms become detectable (Woodward and Frigg, 1989). Liver acetyl coenzyme A carboxylase and pyruvate carboxylase are reduced (Poston and McCartney, 1974), and degenerate acinar cells occur in the pancreas, with deposition of glycogen in the kidney tubules (Poston and Page, 1980). Catfish developed light skin and lower pyruvate carboxylase activity (Robinson and Lovell, 1978).

2.2.6.4. Requirements

The biotin requirements for young salmon, trout, carp, goldfish, and eel raised under experimental conditions appear to be about the same. Brown trout appeared to require nearly twice as much biotin in the diet as do brook or rainbow trout (Phillips *et al.,* 1949). The requirements are listed in Table 2.3 and were determined for trout by feeding different meat–meal mixtures containing various levels of biotin and assaying for liver storage. The requirements for salmon were determined by feeding test diets with different increments of biotin added. The requirements for carp were likewise determined by the use of test diets and added biotin. Similar techniques were used for other fish species.

2.2.6.5. Sources and Protection

Rich sources of biotin are liver, kidney, yeast, milk products, and egg yolks. Nut meats contain good supplies of biotin isotels. The diet should be protected from strong oxidizing agents or conditions which promote oxidation of ingredients. Raw egg white should not be incorporated into moist

fish diets. Cooking will inactivate the avidin, which irreversibly binds biotin. Natural ingredients used to manufacture most fish diets generally contain sufficient biotin for normal growth of salmonids and ictalurids (Casteldine *et al.,* 1978; Lovell and Buston, l984; NRC, 1993).

2.2.6.6. Antimetabolites and Inactivation

Raw egg white has already been discussed; it irreversibly binds biotin and makes it unavailable to young fish. Many biotin homologs with different side-chain lengths inhibit the growth of bacteria. Oxybiotin sulfonic acid inhibits biotin and generates deficiency symptoms. Oxybiotin can form derivatives which compete for biotin sites during the formation of coenzymes. Oxybiotin, a chemically synthesized compound, has about the same biological activity as natural δ-biotin.

2.2.6.7. Clinical Assessment

Liver pyruvate carboxylase activity reflects the biotin status in fish (Robinson and Lovell, 1978). Measurement of urinary excretion of biotin in animals is not a good clinical method, as biotin is synthesized by several organisms in the gut. Biotin is one of the most expensive vitamins to add to fish rations. Actively feeding sea salmon have liver biotin concentrations of 10–12 μg/g of wet liver tissue. The concentrations in the liver of young salmon fingerlings fed test diets containing an excess of biotin in fresh water were between 6 and 8 μg of the vitamin/g of tissue. Fish with these levels of biotin in the liver are probably of a sound biotin nutritional status.

2.2.7. Folic Acid (Folacin)

A megaloblastic anemia was induced in monkeys by McCarrison in 1921 (Hein, 1964). An active extract was isolated and used to cure anemia by Day *et al.* (1938) and vitamin M and vitamin B_c were reported by Stokstad (1943). Folic acid was synthesized by Pfiffner *et al.* in 1946 and was soon used in fish diets to try to cure purified diet anemia. Tunison *et al.* (1943), Phillips *et al.* (1947), and McLaren *et al.* (1947a) all worked with folic acid as the antianemic factor in 1945–1947. Halver and Norris tested it in salmon in 1949 and Wolf (1951) incorporated it into his test diets for trout in 1950.

2.2.7.1. Chemical Structure, Characteristics, and Analogs

Folic acid, pteroylglutamic acid, or folacin has the chemical formula $C_{19}H_{19}N_7O_6$ and the structural formula shown in Fig. 2.9. Folic acid crystallizes into yellow spear-shaped leaflets which are soluble in water and dilute alcohol. It can be precipitated with heavy-metal salts. In acid it is

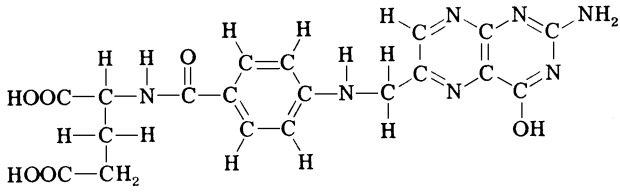


FIG. 2.9

Folic acid.

easily destroyed by heat and deteriorates when exposed to sunlight or during prolonged storage. Several analogs have biological activity including pteroic acid, rhizopterin, folinic acid, xanthopterin, and several formyltetrahydropteroylglutamic acid derivatives. These have closely allied ring structures and many have been isolated as derivatives in various animals or microbiological preparations. One simple form, xanthopterin, present in the pigments of insects, is shown in Fig. 2.10 and is of special interest because of early work with this compound as antianemic factor H for fish (Simmons and Norris, 1941).

2.2.7.2. Positive Functions

Folic acid is required for normal blood cell formation and is involved as a coenzyme in one-carbon transfer mechanisms (Huennekens *et al.,* 1957, 1958; Nakao and Greenberg, 1958). In the presence of ascorbic acid, folic acid is transformed into the active 5-formyl-5,6,7,8-tetrahydropteroylglutameric acid. Folic acid is involved in many one-carbon metabolism systems such as serine and glycine interconversion, methionine–homocysteine synthesis, histidine synthesis, and pyrimidine synthesis for bases of the nucleoticles DNA and RNA (Hartman and Buchanan, 1959). Several coenzyme forms of the active vitamin have been isolated. A good general discussion is given by West *et al.* (1966) and Pike and Brown (1975). Folic acid is involved in the conversion of megaloblastic bone marrow to the nomaloblastic type.

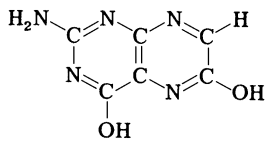


FIG. 2.10

Xanthopterin.

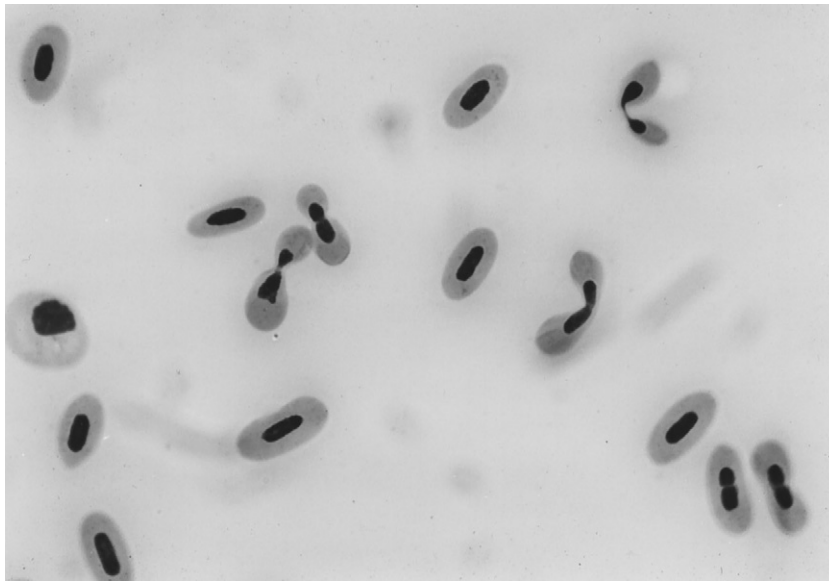


FIG. 2.11

Blood smear of folic acid-deficient coho salmon. Note the senile cells and divided nucleus of erythrocytes.

It plays a role in blood glucose regulation and improves cell membrane function and hatchability of eggs.

2.2.7.3. Deficiency Syndrome

Macrocytic normochromic anemia occurs in several experimental animals, including fish, fed diets devoid of the vitamers folacin (Phillips *et al.,* 1963; Aoe *et al.,* 1967c; Smith, 1968; Smith and Halver, 1969). Increasing numbers of senile cells are observed as the deficiency progresses until only a few old and degenerating cells are found in the blood of deficient fish. Anterior kidney imprints disclose only adult cells and no proforms present. Figures 2.11 and 2.12 show blood smears from deficient coho salmon and kidney imprints from these same fish. In comparison, Fig. 2.13 shows a blood smear of fish 28 days after folic acid was replaced in the ration and Fig. 2.14 shows an anterior kidney imprint with many immature cells and proforms present. Other signs observed have been poor growth, anorexia, general anemia, lethargy, fragile fins, dark skin pigmentation, and infarction of spleen in various species of fish (Arai *et al.,* 1972; John and Mahajan, 1979;

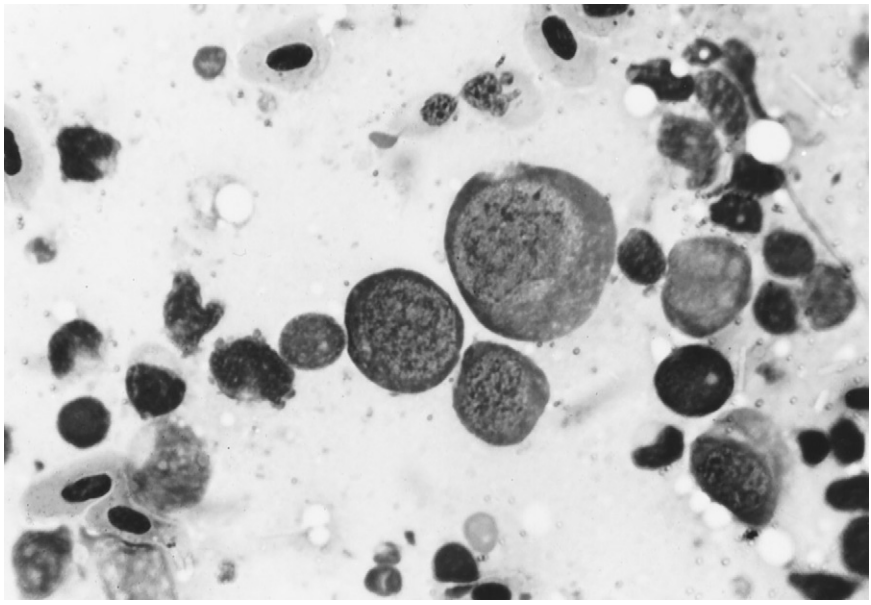


FIG. 2.12

Anterior kidney imprint of folic acid-deficient coho salmon. Note the senile cells, divided nucleus, and absence of immature or proforms of erythrocytes.

Hosokaiwa 1989). Duncan and Lovell (1991) reported deficiency-induced reduced growth and increased sensitivity to bacterial infection in catfish.

2.2.7.4. Requirements

Folic acid requirements based on feeding meat–meal mixtures or test diets plus crystalline compounds, measuring the growth response and food conversion, observing for anemia, and determining the maximum liver storage of the vitamin are listed in Table 2.3. The requirement seems to be about the same for trout and salmon. Marginal macrocytic anemias occur in fish fed diets containing marginal amounts of folacin. Individual fish ingesting adequate amounts of the vitamin have a low variation in total erythrocyte counts. Combined deficiency of folic acid and vitamin B_{12} accelerates the onset of anemia.

2.2.7.5. Sources and Protection

Yeast, green vegetables, liver, kidney, glandular tissue, fish tissue, and fish viscera are good sources of folic acid. Insects contain xanthopterin, which has folic acid activity and the same nuclear structure as folic acid. At one

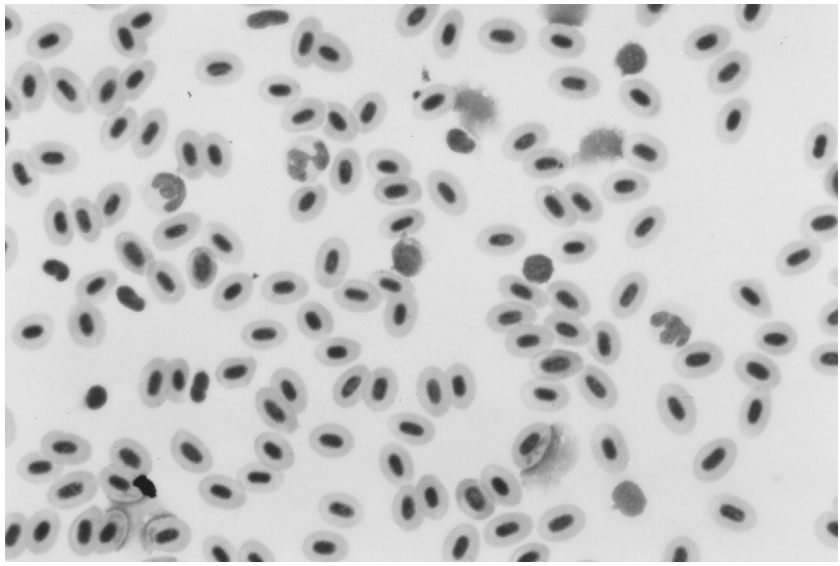


FIG. 2.13

Blood smear from a normal coho salmon. Note the variety of erythrocyte types, including immature forms, present.

time the yellow pigment of xanthopterin was identified as the fish antianemic factor H, but subsequent experiments showed only partial activity and that folic acid itself was a much more potent antimacrocytic anemia factor. Probably, insects do contribute to the folacin requirement of wild fish, but in scientific fish husbandry other agricultural or industrial products form more reliable sources. Some folic acid may be synthesized by the gut bacteria, especially in some warm-water fish species (Kashiwada *et al.,* 1971). Activity is lost during extended storage and when material is exposed to sunlight. Therefore, dry diet materials should be carefully protected during manufacture and moist diet rations should be carefully preserved. Both types of fish diets should be fed soon after manufacture to assure the original folic acid activity.

2.2.7.6. Antimetabolites and Inactivation

One antagonist for folacin is 4-aminopteroylglutamic acid or aminopterin. This material, when incorporated in the diet of guinea pigs and rats, induces anemia and leukopenia and has been used to treat leukemia in man (Chow, 1964). Amethopterin (4-amino-N^{10}-methylpteroylglutamic acid) also can be used to induce deficiency by suppressing 4-amino coenzymes,

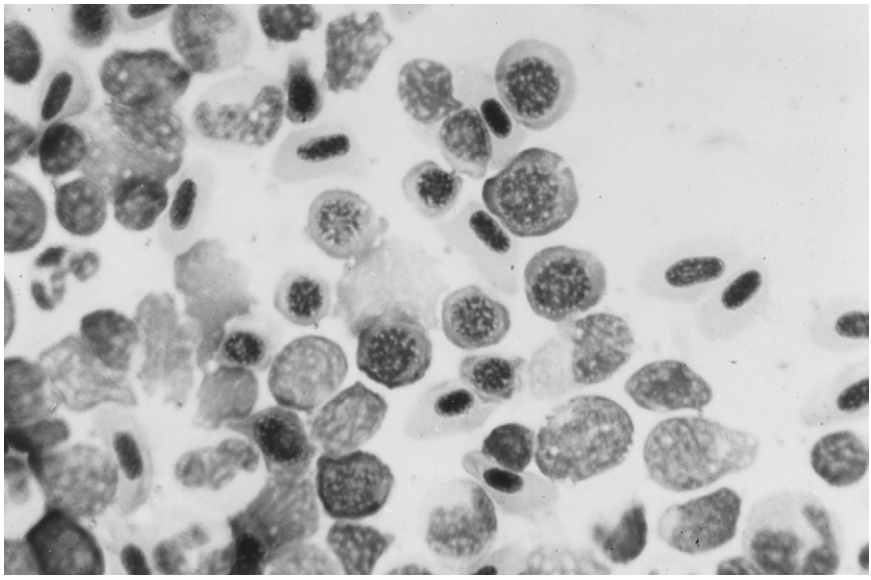


FIG. 2.14

Head kidney imprint of a normal coho salmon. Note the variety of cells present and proforms of erythrocytes during the different states of development.

with resultant poor purine, pyrimidine, and nucleic acid production. Likewise, amethopterin inhibits nucleic acid synthesis and macrocytic anemia eventually occurs.

2.2.7.7. Clinical Assessment

Hematology is used as a simple clinical tool to assess hemopoiesis in fish. Anterior kidney imprints easily disclose a normal distribution of immature cells and proforms undergoing reticulosis. Actively feeding sea salmon and young salmon fingerlings fed diets rich in folacin show a liver storage of 3–4 μg of folic acid/g of wet tissue. Microbiological assay is preferred for assessment of total vitamers for folacin in dietary raw materials because the total biological activity it measures includes all the various coenzyme forms and folic acid analogs. Assessment of the dietary level of the vitamers folacin and intake of fish feed is important for intensive cold-water fish husbandry. In pond culture techniques where the diet is supplementary in nature and fish eat aquatic and terrestrial insects, algae, and other food, folic acid in the supplement is not as critical as when the animals must depend entirely on the supplement. As folic acid is labile

in storage, excessive amounts are generally added during feed manufacture in anticipation of improper or long-term storage. However, prudent fish husbandry dictates rapid use of manufactured rations with minimum storage and/or additional supplementation with folic acid. Routine periodic hematology of the fish assures proper nutritional status for maximum production and sound health. The author has noted in several series of experiments that when fish diseases occur through inadvertent contamination of the water supply, those groups of fish partially or completely deficient in folic acid are the first lots to show acute disease symptoms. Therefore, folic acid must also play an important role in resistance to disease. Experiments testing this hypothesis should yield important dividends to improve fish husbandry.

2.2.8. Vitamin B_{12}

The antipernicious anemia factor was isolated and crystallized nearly simultaneously by Rickes *et al.* (1948) and by Smith (1948). A tentative chemical formula for the active APF (animal protein factor) was established by Todd and co-workers in 1955 (Hein, 1964). One milligram of vitamin B_{12} was made available by Dr. Major of Merck and Co. to test antianemic action in salmon in the late fall of 1949, and Halver and Horris injected anemic salmon with crystalline B_{12} alone and in combination with folic acid and xanthopterin. Positive hemopoiesis occurred within a few days after vitamin B_{12} plus folic acid was injected, and the salmon showed a rapid recovery from the anemia. In 1950 Wolf (1951) added both vitamin B_{12} and folic acid to supply factor H in his trout test diet.

2.2.8.1. Chemical Structure, Characteristics, and Analogs

Vitamin B_{12} or cyanocobalamin has a molecular weight of ~1500 and the approximate chemical formula $C_{63}H_{88}O_{14}PCo$, depending on the state of hydration (Smith, 1960). The approximate structural formula is shown in Fig. 2.15. The molecule has a planar group and a nucleotide group lying nearly at right angles to one another (Chow, 1964). Cyanocobalamin is a red crystalline compound which was isolated almost simultaneously by investigators in England and in the United States. This cobalt-containing vitamin has a net charge of 1 and has a cyano group linked to the central cobalt molecule which can be replaced with various other small groups to form hydroxocobalamin, nitrocobalamin, thiocyanocobalamin, and chlorocobalamin. The crystalline material or aqueous solution of vitamin B_{12} is stabile to mild heat in neutral solutions but is rapidly destroyed by heating in dilute acid or alkali. Crude concentrates are more unstabile and lose activity rapidly. The compound is similar to the porphyrins in spatial configuration,

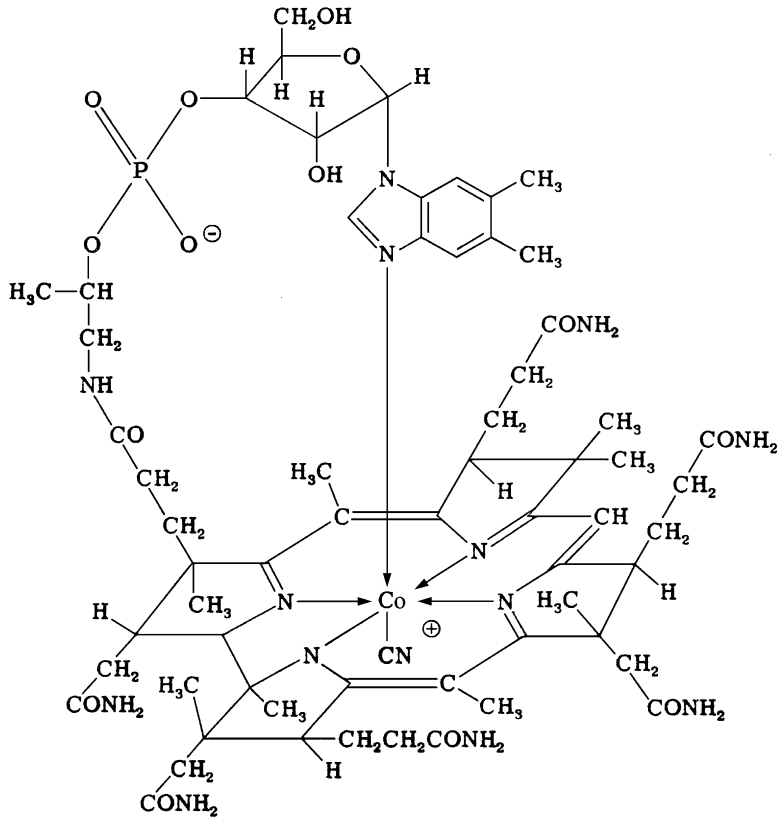


FIG. 2.15

Vitamin B_{12}, depicting the planar structure and central cobalt atom.

with a central cobalt atom linked to four reduced pyrrole rings which form a large macro ring (Hein, 1964). Several other vitamers B_{12} have been isolated with a good biological activity for animals or bacteria (Chow, 1964).

2.2.8.2. Positive Functions

Cyanocobalamin is involved with folic acid in hemopoiesis. It is required for growth by many microorganisms and is a growth factor for many animals (Hunter *et al.,* 1949; Hartman *et al.,* 1949; Ott *et al.,* 1948; Johnson and Neumann, 1949). The animal protein factor present in fish meal and other animal meals was not correlated with fish antianemic factor H until crystalline vitamin B_{12} was injected into anemic chinook salmon fingerlings in 1949 and positive hemopoiesis was observed (Halver, 1953a, 1969).

A coenzyme incorporating vitamin B_{12} is involved in the reversible isomerization of methylmalonyl coenzyme A to succinyl coenzyme A and in the isomerization of methyl aspartate to glutamate. Cyanocobalamin is involved in the coenzyme for the methylation of homocystine to form methionine. It is also involved in several other one-carbon metabolism reactions and in the synthesis of labile methyl compounds. One vitamin B_{12}-containing coenzyme acts in methylation of the purine ring during thymine synthesis. Vitamin B_{12} is also involved in cholesterol metabolism, in purine and pyrimidine biosynthesis, and in the metabolism of glycols (Chow, 1964). In addition, it is involved in fatty acids with an odd number of carbon chains and in the recycling of tetrahydrofolic acid (NRC, 1983).

2.2.8.3. Deficiency Syndrome

Deficiency signs in young pigs, chicks, and rats are abnormal blood elements, poor growth, porphyrin whiskers, scaly feet, and pernicious anemia (West *et al.,* 1966). An intrinsic factor is necessary for good absorption of the vitamin from the gut. This factor is a low molecular weight mucoprotein which normally occurs in gastric juice, especially in hog gut mucosa (Abeles and Lee, 1961; Landboe-Christensen and Plum, 1948). Pernicious anemia which results without gastric intrinsic factor or vitamin B_{12} is characterized by abnormal hematology. This same picture occurs in blood smears in fish deficient in vitamin B_{12}. Erythrocytes are fragmented, with many aberrant forms present. Hemoglobin determination is erratic between fish, and erythrocyte counts have a range extending from frank anemia to a near-normal blood pattern. Cyanocobalamin stores in fish tissues are slowly exhausted, and only after 12–16 weeks of testing do the symptoms appear in deficient salmon populations. Poor appetite, poor growth, poor food conversion, and some dark pigmentation can be observed before frank anemia is detected (Halver, 1957). Chinook or coho salmon reared on diets devoid of vitamin B_{12}, but with adequate sources of folic acid, show typical microcytic hypochromic anemia, with fragmented erythrocytes and many immature forms present in both blood smears and erythrocyte counting chambers.

2.2.8.4. Requirements

Difficulties in assaying for the vitamers B_{12} in diets, the long induction period for anemias to develop, and the preparation and assay problems in assessment of liver storage have limited quantitative requirement determinations to young chinook salmon fingerlings raised on stringently controlled test diets in special water systems of a low microorganism count. The figures listed in Table 2.3 are the best tentative requirements which could be calculated from the crystalline vitamin B_{12} intake observed, absence of microcytic pernicial anemia, normal hematology at the end of the 16-week

feeding period, and maximum liver storage of vitamers B_{12} determined by *Lactobacillus leichmannii* microbiological assay.

2.2.8.5. Sources and Protection

Rich sources of animal protein factor or vitamers B_{12} are fish meal, fish viscera, liver, kidney, glandular tissues, and slaughterhouse waste meat. Some vitamin B_{12} is synthesized by gut bacteria in fish (Kashiwada *et al.* 1970; Sugata, 1990, 1991) and may contribute to the B_{12} available for metabolism by the animal. This phenomenon may provide justification for supplementation of fish diets with trace amounts of cobalt (Limsuwan and Lovell, 1981). Intestinal bacterial synthesis of B_{12} was sufficient to supply the B_{12} requirements of tilapia (*Tilapia nilotica*) (Lovell and Limsuwan, 1982). Since vitamin B_{12} is labile on storage, and is easily destroyed by heating in mild acid solutions, care must be exercised in preparing diet containing flesh or meat scraps poorly stored at a low pH and subsequently pasteurized or sterilized. Likewise, storage conditions should be cold and of a short duration before the diet is used to assure maximum retention of vitamin B_{12} activity in the diet when consumed.

2.2.8.6. Antimetabolites and Inactivation

Hydroxocobalamin, chlorocobalamin, nitrocobalamin, and other derivatives of the cyanide radical attached to the cobalt atom in vitamin B_{12} have varying degrees of activity, but mild *in vitro* treatment with cyanide converts these analogs back into cyanocobalamin (Kaczka *et al.,* 1950). The vitamin B_{12} coenzymes are very unstable in light, and exposure to direct sunlight for a few minutes results in complete decompositions of the coenzyme. Also, dilute acid solutions increase sensitivity, and purine-containing analogs of B_{12} coenzymes are very labile in dilute acid solution (Chow, 1964). Therefore, B_{12} preparations for experimental work should be carefully preserved in acotinic glass, in neutral solutions, until their use in experiments. Likewise, moist diet preparations should be protected from light and maintained at a neutral pH rather than in acid or alkali pH ranges to preserve as much vitamin B_{12} activity as possible.

2.2.8.7. Clinical Assessment

Generalized anemia with fragmentation of erythrocytes and extremely varying hemoglobin erythrocyte counts indicate possible B_{12} deficiency. A prompt response in individual fish is obtained by injecting B_{12} alone or in combination with folic acid at the ratio 1 part vitamin B_{12} to 100 parts folic acid. Hematology in fish with vitamin B_{12} deficiency is characterized by these varied blood cell types, whereas folic acid deficiency shows only senile cells, some with pycnotic nuclei, characteristic of macrocytic normochromic

anemia. Therefore, characterization of the anemia is critical to separate symptoms of one antianemic factor from those of the other. A combination of the two vitamins is McCay's fish antianemic factor H.

2.2.9. Ascorbic Acid

Experimental work on curing scurvy with fruit juice was described by Lind in 1753, but nearly 200 years elapsed before the exact chemical compound responsible for reducing the symptoms was defined. Vitamin C was named by Drummond (1920). Glen King was studying the vitamin in 1922 (Halver, J. L. 2001) L-Glucuronic acid was isolated by Szent-György in 1928. Crystalline vitamin C was isolated and demonstrated to be the antiscorbutic vitamin by King and Waugh (1932; Waugh and King, 1932) and was named "ascorbic acid" by Szent-György and Haworth (1933), and L-dihydroascorbic acid was synthesized by Reichstein *et al.* (1933) in that same year. McCay and Tunison reported scoliosis in brook trout fed formalin-preserved meat in 1934, and McLaren *et al.* (1947a) observed hemorrhages in trout fed rations low in ascorbic acid. Nearly 20 years then elapsed before Kitamura *et al.* (1965) demonstrated a critical need in trout for vitamin C, and 4 years later the need for L-ascorbic acid was demonstrated in salmon by Halver *et al.* (1969). Fish were thus only recently added to the list of animals requiring L-ascorbic acid in the diet. A comprehensive review of ascorbic acid in aquatic animals was compiled by Dabrowski (2001b).

2.2.9.1. Chemical Structure, Characteristics, and Analogs

Ascorbic acid and its inactive analog dehydroascorbic acid have the formula $C_6H_8O_6$ and the structural formulas shown in simple form in Fig. 2.16. The reduced or active form is a white, odorless, crystalline compound, soluble in water but insoluble in fat solvents. Dihydroascorbic acid is easily and rapidly oxidized to dehydroascorbic acid, which is much less active biologically than the reduced form. Ascorbic acid readily forms salts and is labile to

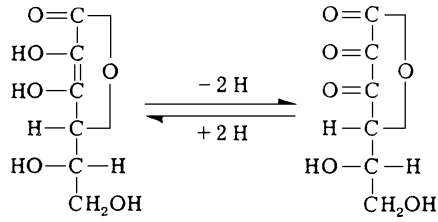


FIG. 2.16

Vitamin C: L-ascorbic acid (*left*) and oxidized form or dehydroascorbic acid (*right*).

free oxygen. Reduced ascorbic acid is very stable in acid solutions because of the preservation of the lactone ring, but in alkaline solutions hydrolysis occurs rapidly and vitamin activity is lost. Copper and heavy-metal ions enhance oxidation, and reduction back from the oxidized to the reduced form can be accomplished in the laboratory. This reaction also proceeds readily in the body, with the vital reducing agents glutathione and NADPH involved. Vitamin C is very heat labile and prone to atmospheric oxidation, especially in the presence of copper, iron, or several other metallic catalysts. The reduced form is the most biologically active form but several derivatives or salts may be formed which have varying degrees of ascorbate activity (Woodruff, 1964; WHO, 1970). One derivative, L-ascorbate-2-sulfate (vitamin C2S), is a heat-stable form synthesized by salmonids from excess dietary L-ascorbic acid (vitamin C_1) and used as a tissue storage form of this vitamin (Fig. 2.17) (Halver, 1986). It is also found in brine shrimp cysts (*Artemia* sp.) and in many other animal tissues and is resistant to oxidation when in the sulfated state (Tucker and Halver, 1984). A chemically synthesized form, L-ascorbate-2-phosphate (C2P), is readily used by fish as a vitamin C source. Other stabile forms of derivatives with other electron-dense groups coupled on the 2-position of ascorbic acid have been synthesized and are used in feed manufacture.

2.2.9.2. Positive Functions

L-Ascorbic acid acts as a biological reducing agent for hydrogen transport. It is involved in many enzyme systems for hydroxylation, i.e., hydroxylation of tryptophan, tyrosine, and proline. It is involved in detoxification of aromatic drugs and also acts in the production of adrenal steroids (WHO, 1970). Ascorbic acid is necessary for the formation of collagen and normal cartilage

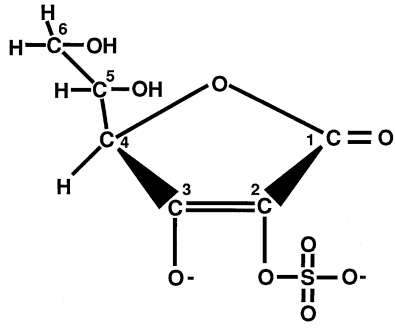


FIG. 2.17

Vitamin C2S: L-ascorbate-2-sulfate.

as well as normal tooth formation, bone formation, bone repair, and wound healing (Knox and Goswami, 1961; Gould, 1960). Ascorbic acid plays a role synergistically with vitamin E in the maintenance of intracellular antioxidants and free radical traps. It acts synergistically with vitamin E and selenium to maintain the activity of glutathione peroxidase and superoxide dismutase. The conversion of folic acid to folanic acid requires vitamin C for the active coenzyme form (Wolbach and Home, 1926; Woodruff, 1964). Ascorbic acid is involved in the formation of chondroitin sulfate fractions and intercellular ground substances and is capable of forming sulfate derivatives with very stable chemical characteristics (West *et al.,* 1966; Tolbert *et al.,* 1975; Andrews and Crawford, 1982). Intubated labeled ascorbic acid is rapidly mobilized and fixed in deficient fish in areas of rapid collagen synthesis and becomes concentrated in the thick collagen of the skin and in cartilagenous bones. Also, pituitary and adrenal glands of the anterior kidney of partially deficient fish concentrate intubated vitamin C. Ascorbic acid is also involved in the maturation of erythrocytes for maintenance of normal blood hematology (Johnson *et al.,* 1971). Stored ascorbate-2-sulfate (C2S) can be readily converted into L-ascorbic acid (C_1) by the enzyme C_2 sulfatase to provide tissue metabolism demands for C_1. This enzyme system operates under C_1 feedback control to maintain circulating levels of C_1 in the tissues (Benitez and Halver, 1982). Either C2S or C-2-phosphate can be used to replete Atlantic salmon (*Salmo salar*) and reduce fish scurvy symptoms (Halver and Hardy, 1994). The various phosphate derivatives are also excellent C sources and are the most common forms currently used, as phosphate is readily hydrolyzed off by digestive phosphatases, generating C_1 for use (Dabrowski, 2001a).

2.2.9.3. Deficiency Syndrome

Scurvy, with impaired collagen formation, perifollicular hemorrhages, loose teeth, and poor osteoid formation, anemia, and edema have been reported in other animals (WHO, 1970). Deficiency signs in fish are generally related to impaired collagen formation. Fish soon show hyperplasia of collagen and cartilage, then scoliosis, lordosis, internal hemorrhage, resorbed opercles, and abnormal support cartilage in gills, spine, and fins, with hyperplasia of the jaw and snout (Halver *et al.,* 1969). The same symptoms have been observed in trout, salmon, yellowtail, carp, guppies (*Poecilia reticulata*), catfish, snakehead, tilapia, minnows, mullet, char, and other fish (Kitamura *et al.,* 1965; Poston, 1967; Lovell, 1973; Stickney *et al.,* 1984; Sakaguchi *et al.,* 1969; Yone and Tujii, 1974; Mahajan and Agrowl, 1979; Halver, 1979; Thomas *et al.,* 1985; Tucker and Halver, 1986). Histologically, hypertrophy of the adrenal tissue and hemorrhages at the bases of fins have been observed in coho salmon. Deficiency signs cease to develop and new growth becomes normal on replacement of ascorbic acid in the ration. An anemia eventually

develops in extremely deficient fish and extreme scoliosis and lordosis do not repair but are walled off with new growth around the afflicted areas of the spine when ascorbic acid is added to the deficient ration (Kitamura *et al.,* 1965). Reduced bone collagen was also shown in catfish (Wilson and Poe, 1973; Lim and Lovell, 1978) and in common carp and roach (Dabrowski *et al.,* 1988, 1989).

2.2.9.4. Requirements

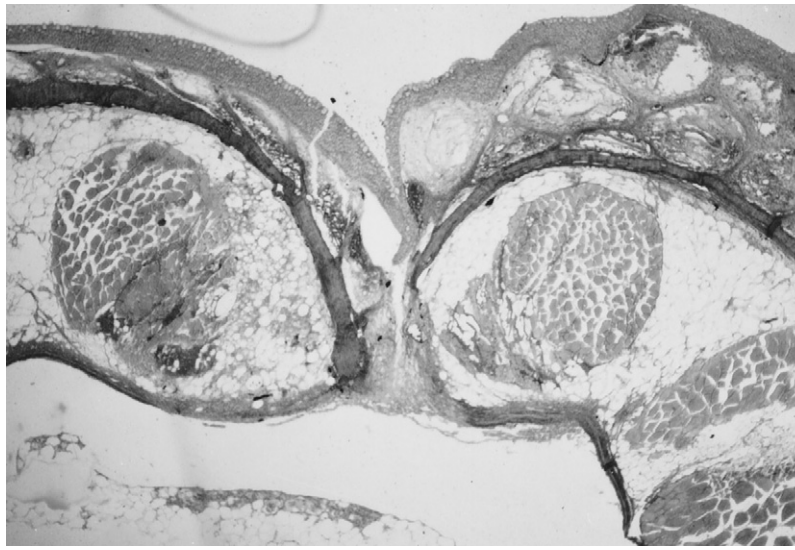
Rainbow trout have been most studied with a variety of test diets and different ascorbic acid intakes. These fish have a varied requirement, depending on the criteria used to measure the need. Reasonable blood and anterior kidney storage levels were obtained with an intake of about 100 mg of vitamin C/kg of dry ration in 10, 12, or 15°C water systems. When wound repair experiments were initiated, however, or when fish were exposed to other stress, then the requirements doubled or tripled. When severe abdominal or intramuscular wounds were inflicted, young fish needed at least 500 mg of active ascorbate for tissue repair comparable to that of control fish receiving 1 g or more of ascorbate in the diet/kg of dry diet. Coho salmon appear to need about half of these requirements for adequate tissue levels and for maximum severe wound repair rates (Halver, *et al.*, 1969). This phenomenon is illustrated in Table 2.4, reporting growth response and tissue repair for rainbow trout and coho salmon, and in Figs. 2.18–2.22, showing wound repair rates in typical fish fed 50, 100, 200, 400, or 1000 mg of reduced ascorbic acid/kg of diet 3 weeks after wounds were inflicted. The requirement for ascorbic acid

Table 2.4

Growth and Tissue Ascorbate[a]

	Trout			Salmon		
		Ascorbate concentrate[b]			Ascorbate concentrate[b]	
C diet treatment (mg/100g)	Average weight at 24 weeks (g)	Blood (μg/g)	Kidney (μg/g)	Average weight at 24 weeks (g)	Blood (μg/g)	Kidney (μg/g)
0	2.4	—[c]	—[c]	5.0	22.3 ± 2.2	89
5	9.6	34.4 ± 2.9	125	6.0	30.5 ± 1.2	132
10	10.6	34.6 ± 1.3	137	5.7	35.8 ± 1.6	265
20	10.1	38.8 ± 3.3	132	6.1	34.2 ± 2.3	183
40	10.2	46.8 ± 6.2	162	6.3	33.7 ± 2.0	225
100	10.8	51.0±4.6	247	6.0	37.8 ± 2.3	321

[a] From Halver *et al.* (1969).
[b] Average of five samples for blood (±SD) and two for head kidney tissue.
[c] No fish available for assay



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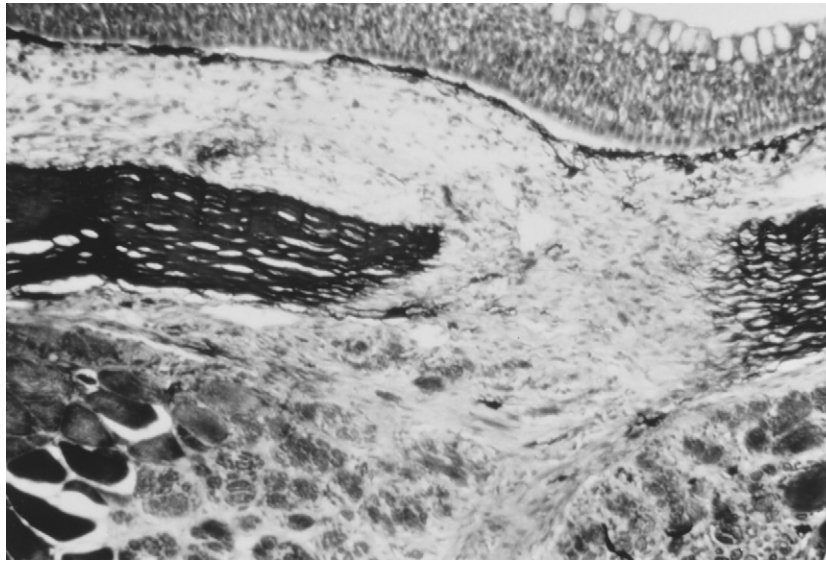
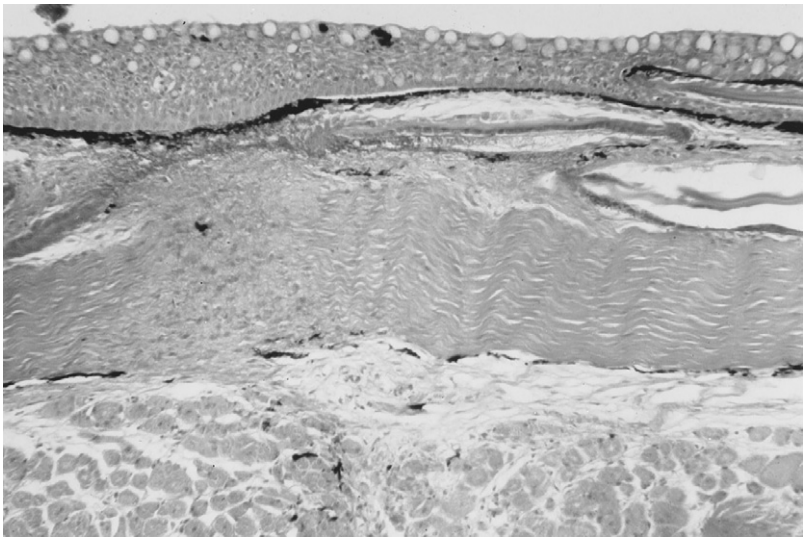


2.19

FIG. 2.18–2.22

Typical sections through abdominal tissue wounds showing repair after 3 weeks in rainbow trout fed 50 (Fig. 2.18), 100 (Fig. 2.19), 200 (Fig. 2.20), 400 (Fig. 2.21), or 1000 (Fig. 2.22) mg of L-ascorbic acid/kg of diet, respectively. Rate of wound repair was directly related to vitamin C content of diet.



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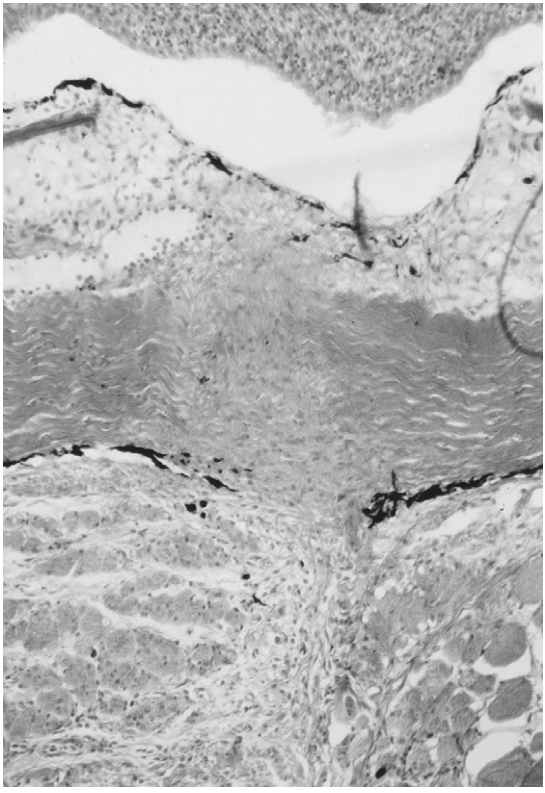

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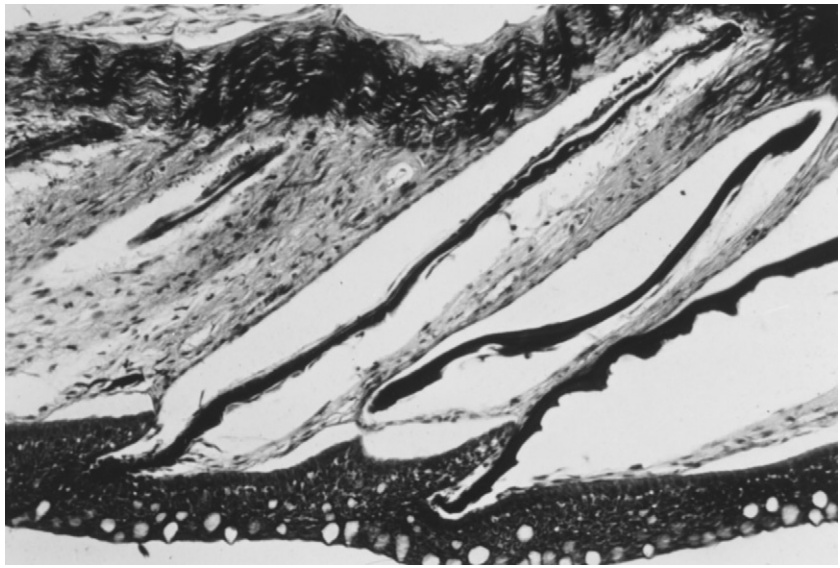

2.22

must therefore be related to the stress, to the growth rate, to the size of the animal, and to the other nutrients present in the diet. A compromise value of about 200 mg of ascorbic acid/kg diet for trout and salmon raised in freshwater systems between 10 and 15°C would ensure reasonable tissue storage levels and furnish some excess for mild stress conditions and for ascorbic acid loss from the diet through oxidation during feed preparation and subsequent storage before the food is fed. Large carp can synthesize some ascorbate and the requirement for this species may be dependent on the size of the fish and environment in which reared (Ikeda and Sato, 1964). Fish also need more vitamers C when exposed to infectious diseases. Navarre and Halver (1989) have shown that rainbow trout exposed to known concentrations of *Vibrio anguillarum* by either injection or dipping were more resistant to infection with elevated vitamin C intake. Improved resistance to a viral disease and diminished mortality in rainbow trout were reported when elevated levels of L-ascorbate-2-polyphosphate were fed in the diet (Anggawati-Satyabudhy *et al.,* 1989). Improved immunoresistance to a viral disease in trout was also demonstrated when ascorbate-2-phosphate was increased in the diet (Angggawati-Satyabudhy *et al.,* 1989). Reproduction may also increase the demands for vitamin C (Soliman *et al.,* 1986; Sandnes *et al.,* 1984). In constrast, sturgeons *Acipenser,* have gulonolactone dehydrogenase present and can synthesize ascorbic acid from glucose; therefore they have little dependence on dietary sources for vitamin C (Dabrowski, 2001b).

2.2.9.5. Sources and Protection

Ascorbic acid is widely distributed in nature, with citrus fruits, cabbage, liver, and kidney tissue good sources of the vitamin. High levels of vitamin C are found in glandular fish tissue and appreciable quantities are still present in fish meals made from whole fish. Probably, the addition of synthetic industrial material to the ration, should be considered, however, to assure adequate intake of this important vitamin for normal growth, tissue repair, and sound physiological function of fish. Fresh insects and fish tissues contain reasonable amounts of the vitamin, and certain dried fruits such as lingonberries and cranberries, which have a high benzoic acid level to protect the vitamin C content, are exotic but rich sources of the vitamin. When ascorbic acid is used, the food must be protected from aerobic oxidation and any moist feed must be carefully protected from oxidizing agents, from air, and from copper, iron, and other metals which catalyze the oxidation of ascorbic acid into the biologically inactive form. Several heat- and oxidation-stable forms of ascorbate are available for use as dietary sources for fish diets; the ascorbate form is the most stable but may not be used as readily as the phosphate forms by some fish species. C2P is currently the most available economic form generally used in diet formlations but the phosphate can be hydrolyzed easily by the many phosphatases present in many fish diet ingredients and the liberated ascorbic acid may then be oxidized and lost as a vitamin C source. Esters on the C-6 position are partially stable, but more stable forms are synthesized, with electron-dense moieties on the C-2 position of the ascorbic ring. Decreasing stability is of the order C2S, C2-monophosphate, C2-diphosphate, C2-triphosphate, C2-glycoside, C6-palmitate, and ascorbic acid.

2.2.9.6. Antimetabolites and Inactivation

D-Ascorbic acid, the optical isomer of the active form, has no activity and competes for sites of several enzyme reactions. 6-Deoxy-L-ascorbic acid has very low activity, and L-glucoascorbic acid very little activity. These can be expected to be involved in chemical reactions because of their close similarity to the parent compound. A low tryptophan content in the diet appears to increase the demand for ascorbic acid (West *et al.,* 1966). This other nutrient is certainly not an antimetabolite of the oxidation reduction or hydrogen transport vitamin but may increase the demand for vitamin C during detoxification and elimination of unneeded amino acid. Dehydroascorbic acid has a good activity and is involved in some metabolism reactions. As mentioned above, L-dihydroascorbic acid (C_1) is extremely labile to oxidation, especially so in the presence of copper, iron, or several other metals which catalyze atmospheric oxidation into the biologically

inactive form. L-Ascorbate-2-sulfate (C2S) is resistant to oxidation and is also heat stable under neutral or mild acid conditions.

2.2.9.7. Clinical Assessment

Assessing the ascorbic acid status of experimental animals is normally attempted by tissue ascorbate analysis. Most of the assays used previously measure the total ascorbate and not the biologically active dihydroascorbic acid (Woodruff, 1964). Thus they are consequently fraught with errors and misconception regarding the true vitamin C status. In fish tissues, blood and liver do not adequately reflect the ascorbic acid intake and status, but the anterior kidney, which contains adrenal tissue, is a fairly representative tissue storage site for the vitamin (Halver *et al.,* 1969). Stress rapidly reduces the ascorbic acid content of this tissue, with concurrent production of adrenal steroids (Wedemeyer, 1969). Conversely, dietary intake is reflected up to the point of four- or fivefold storage from deficient levels, and only massive dietary intake appears to elevate tissue storage further (Halver *et al.,* 1969). Examination of fragile support cartilage in the gill filaments under a low magnification will detect early hypovitaminosis C before clinically acute symptoms become noticeable. Vertebral collagen levels can be used to measure the vitamin C status in trout (Sato *et al.,* 1978) and in catfish (El Naggar and Lovell, 1991). One clinical analysis to assess the vitamin C status in trout and salmon uses the anterior kidney, with samples selected from the junction of the two wings forward, in which tissues are blotted free of blood with filter paper and the total ascorbate is assayed by one of the improved quick methods to determine the total ascorbate in this typical storage area for vitamin C in fish (Halver *et al.,* 1969). Another assay can measure C_1 in blood (Hilton *et al.,* 1978), but the best assay of tissues and feed samples should measure both C_1 and C2S using high-performance liquid chromatography (HPLC) techniques with a tandem dual column to separate and assay C_1 and C_2 quantitatively (Felton and Halver, 1987). Other techniques can be used to separate and assay levels of C_1, C2S, and C2P in diet ingredients (Felton and Halver, 1989; Felton *et al.,* 1994). A simpler assay for "total C" is by hydrolyzing of the tissues or feed components in strong acid (e.g., 10% trichloroacetic acid), which liberates most of the ascorbate derivatives, and then measuring the resultant ascorbic acid present. The enigmas encountered in assaying for vitamers C have been reported recently (Halver, 2001).

2.2.10. Inositol

Muscle "sugar" was discovered by Scherer in 1850 and was characterized by Maquenne (1900) in 1887. Woolley showed it to be an alopecia-preventing factor for mice (1940), and the stereo configuration of the

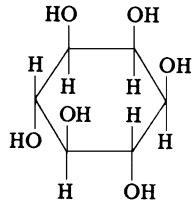

FIG. 2.23

Inositol, depicting the *myo*-inositol form of the vitamin.

active factor, *myo*-inositol, was proved by Posternak (1936) and by Dangschat (1942). McLaren *et al.* (1947a) observed poor growth and poor food passage in inositol-deficient trout, and these symptoms were confirmed in salmon by Halver (1953a,b) and in carp by Aoe and Masuda (1967).

2.2.10.1. Chemical Structure, Characteristics, and Analogs

Seven optically inactive and two optically active isomers of hexahydroxycyclohexane can exist. One of the optically active forms, *meso*-inositol or *myo*-inositol, has the biological activity. The chemical formula is $C_6H_{12}O_6$, and the structural formula, with the hydroxyl groups in positions 1, 2, 3, and 5 in one plane and positions 4 and 6 in the other plane, is shown in Fig. 2.23. *myo*-Inositol or *meso*-inositol is a white crystalline powder soluble in water and insoluble in alcohol and ether. The material can be synthesized but is easily isolated from biological material in free or combined forms (Anderson and Wallis, 1948; Weidlein, 1954). The mixed calcium–magnesium salt of the hexophosphate is phytin. Isomers have little biological activity but do compete in chemical reactions.

2.2.10.2. Positive Functions

myo-Inositol is a structural component in living tissues. It has lipotropic action, preventing accumulation of cholesterol in one type of fatty liver disease, and is involved with choline in homeostasis of normal lipid metabolism (West *et al.,* 1966). It is a growth-promoting substance for microorganisms and prevents alopecia in mice (Wolley, 1940). In addition, it is an emergency carbohydrate source in muscle and is a major structural component in the phospholipid structures in animal tissues (Stetten and Stetten, 1946). Its primary function appears to serve as a structural element, with the six hydroxy groups available for esterification or for acid salt formation to form an integral portion of cell membranes. The stereo configuration of these cell membrane elements probably plays a major role in cell membrane permeability to various ions and molecules (West *et al.,* 1966). Phosphatidylinositol

was shown to be involved in signal transduction in several metabolic processes (Mathews and van Holde, 1990; NRC, 1993).

2.2.10.3. Deficiency Syndrome

Poor growth, increased gastric emptying time, edema, a dark color, and distended stomachs are symptoms observed in salmon, trout, carp, red sea bream, and catfish held for long periods on inositol-deficient test rations (Halver, 1970; Yone *et al.*, 1971). A spectacle eye condition described for rats (Wolley, 1940) has not been observed under the experimental conditions used in fish studies. The major deficiency sign is inefficiency in digestion and food utilization and concomitant poor growth, leading to a population of fish with distended abdomens. Some loss of activity of cholinesterase and aminotransferases has been reported in trout, red sea bream, eel, parrot fish, and yellowtail (NRC, 1981, 1993).

2.2.10.4. Requirements

Inositol needs have been measured in salmon and carp and the requirement is high for maximum growth and maximum liver storage. Whether liver storage is an accurate criterion to determine requirements is debatable since inositol intake was compared with maximum growth rate and diet conversion to develop a tentative requirement for young fish for this "muscle" sugar. Intestinal bacteria synthesis and *de novo* synthesis of inositol in the liver of channel catfish were found (Burtle, 1981). Supplemental inositol improved growth and skin lesions in carp (Aoe and Masuda, 1967).

2.2.10.5. Sources and Protection

myo-Inositol occurs ubiquitously in large amounts wherever biological tissue is found. Wheat germ, dried peas, and beans are rich sources. Brain, heart, and glandular tissues are very good sources of biologically active inositol. Citrus fruit pulp and dried yeast also contain inositol. The compound is stable, so normal diet preparation and storage techniques should assure adequate intake for young growing fish. Some synthesis has been reported in carp intestine (Aoe and Masuda, 1967) and in channel catfish (Burtle and Lovell, 1989).

2.2.10.6. Antimetabolites and Inactivation

There are seven optically inactive and one optically active but biologically inactive stereoisomers of inositol. Because inositol is synthesized in the biologically active form by many microorganisms in the gut, only chemically synthesized inactive isomers added to diets would appear in sufficient amounts to interfere with the metabolism of inositol for growth and normal physiological function. Because of the spatial configuration of the

biologically active form and the need to fit these forms into tissue stereobiochemical structure, biologically inactive forms do not compete for critical sites in metabolism. However, the active cis–trans isomer does compete and introduces errors in the structural configuration of essential components. Methyl derivatives and mono-, di-, and triphosphoric acid esters occur naturally. Salts of the hexaphosphate or phytin make the bound inositol partially unavailable to the animal (Weidlein, 1954; West *et al.,* 1966; Spinelli, 1979).

2.2.10.7. Clinical Assessment

Tissue analysis for inositol status has not been successfully employed in animal nutrition except for the measurement of free inositol in seminal plasma in animal sires (Hartree, 1957). Bull plasma contained over 500 mg% of the material in healthy animals of a good breeding status. The seminal plasma of bulls, rabbits, rams, and stallions ran lower in exhausted animals and during recovery back to a good breeding status. The presence of these very high quantities of free inositol has not been adequately explained. Assessment in fish has been based on a lack of deficiency signs coupled with the most efficient food conversion. Actively feeding sea salmon show 1–1.5 mg of inositol/g of fresh liver tissue and young fingerlings raised in fresh water at 10–15°C contained 600–700 mg/g of liver tissue. An alternate, better assessment may be based on a standard muscle section or whole-carcass analysis for free or bound inositol. Projection of inositol intake from normal fish diet ingredients should indicate an excess of this particular vitamin.

2.2.11. Choline

Methylation as a basic metabolic process was postulated by Hofmeister (1894). Methyl transfer was shown *in vivo* by Thompson (1917), and the interrelationships among choline, methionine, and homocystine were shown by du Vigneaud in 1939–1942 (Rosenberg, 1945). Trout fed low-choline rations developed hemorrhagic kidneys according to McLaren *et al.* (1947a) and salmon showed an aversion to food in choline-deficient diet experiments Halver (1953a,b).

2.2.11.1. Chemical Structure, Characteristics, and Analogs

Choline has the chemical formula $C_5H_{15}NO_2$. It readily forms salts with the structural formula shown in Fig. 2.24. Choline is a very strong organic base and forms many derivatives widely distributed in animal and vegetable tissue. One derivative, acetylcholine, is involved in transmission of nerve impulses across synapses. Choline is very hydroscopic and very soluble in

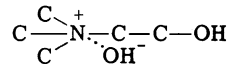

FIG. 2.24

Choline.

water and is stable to heat in acid solutions but decomposes in alkaline solutions. Choline reacts with many chemical compounds, as it is a strong base.

2.2.11.2. Positive Functions

Choline acts as a methyl donor for methylation of tissue intermediates. It is a lipotropic and antihemorrhagic factor preventing the development of fatty livers. It is involved in the synthesis of phospholipids and in fat transport. Acetylcholine transmits the excitory state across the ganglionic synapsis and neuromuscular junctions (Griffith and Nye, 1954; West *et al.,* 1966). Choline is a component of phospholipids in the fragile cellular membranes of tissue. Choline is essential for growth and good food conversion in fish.

2.2.11.3. Deficiency Syndrome

Deficiency signs involve poor growth and poor food conversion, with impaired fat metabolism. Hemorrhagic kidneys and intestines have been reported in trout (McLaren *et al.,* 1947a) and increased gastric emptying time has been observed in salmon (Halver, 1957, 1970). Similar deficiency signs were observed in catfish (Dupree, 1966), carp (Ogino *et al.,* 1970a), eel (Arai *et al.,* 1972), and lake trout (Ketola, 1976).

2.2.11.4. Requirements

The requirements for salmon are listed in Table 2.3. The requirements listed were determined by feeding increments of choline in test diets to young fish in a carefully controlled environmental system, observing the growth response, and assessing the requirement by the maximum growth and food conversion at the minimum intake which would promote maximum liver storage of choline. At the end of the 12-week experimental period, both chinook salmon and coho salmon appear to require about the same dietary intake of choline. The requirement of carp was reported to be about 100 mg/kg body weight/day (Ogino *et al.,* 1970a) to prevent fatty liver development in young fish. Rumsey (1991) suggested that about 50% of the choline requirement of trout could be met by betaine.

2.2.11.5. Sources and Protection

Rich sources of choline are wheat germ, soybean and other bean meals, brain, and heart tissue. Choline hydrochloride, a common supplementary form used in fish diet preparations, reacts with α-tocopherol and vitamin K preparations—probably inactivating these vitamins when mixed into diet preparations. Therefore, choline should be added in a water carrier and the fat-soluble vitamins added in an oil carrier to prevent reaction when in direct contact at high concentrations with this strong base.

2.2.11.6. Antimetabolites and Inactivation

The choline status of fish can be estimated by assay of the choline content of the dietary ingredients and the absence of deficiency signs. Maximum liver storage may not be the best criterion to determine choline nutritional status but has been used to assess the tentative requirement listed for two species of salmon.

2.2.12. *p*-Aminobenzoic Acid

2.2.12.1. Chemical Structure, Characteristics, and Analogs

p-Aminobenzoic acid has the chemical formula $C_8H_7O_2N$ and the structural formula shown in Fig. 2.25. This compound is a substituted benzoic acid, and isomers with the amino group in the ortho, meta, or para position occur. Various derivatives of the carboxyl group can be made. *p*-Aminobenzoic acid is a white, crystalline powder which is water soluble and heat and light stable in aqueous and mild alkaline solution (West *et al.,* 1966).

2.2.12.2. Positive Functions

p-Aminobenzoic acid is a growth-promoting vitamin for microorganisms and a high intake has been shown to counteract the antimetabolite effect of sulfonamides in bacterial culture (Wagner and Folkers, 1964). No positive function or deficiency signs have been observed in fish and no requirements

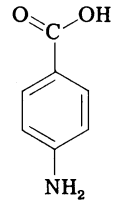

Fig. 2.25

p-Aminobenzoic acid.

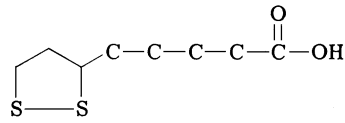

Fig. 2.26

Lipoic acid.

have been determined for this compound except for microorganisms. The vitamin status of *p*-aminobenzoic acid is probably confined to microorganisms, where it is utilized in the synthesis of folacin compounds. A common antimetabolite of *p*-aminobenzoic acid is sulfonilamide and other sulfa compounds which are toxic to microorganisms, forming the basis for sulfa therapy for bacterial infection.

2.2.13. Lipoic Acid

2.2.13.1. Chemical Structure, Characteristics, and Analogs

Lipoic acid has the chemical formula $C_8H_{12}O_2S_2$ and the structural formula shown in Fig. 2.26. Lipoic acid is both fat soluble and water soluble. Several derivatives of the carboxylic acid group have been identified.

2.2.13.2. Positive Functions

Lipoic acid functions as a coenzyme in α-keto acid decarboxylation. It was discovered independently in several laboratories during the period 1945–1950 and shown to be an essential component of multienzyme units. Its functions have been reviewed by Wagner and Folkers (1964). It is an extremely active biological catalyst. It has been called the pyruvate oxidation or pyruvate decarboxylation factor, as it is involved in pyruvate oxidative decarboxylation. The multienzyme unit also includes thiamin pyrophosphate, coenzyme A, and flavin adenine dinucleotide. Therefore, it is closely associated with thiamin in many oxidative decarboxylations of α-keto acids (Lehninger, 1977). Glandular tissues are good sources of vitamers of lipoic acid. No requirements have been determined for fish.

2.3 The Fat-Soluble Vitamins

The fat-soluble vitamins A, D, E, and K each occur in different chemical forms having physiological activity. Isotels for these vitamers are well known but only one or two of the more common and well-recognized forms can be included for discussion. The fat-soluble vitamins differ from the

water-soluble vitamins in their accumulative action. Little evidence has been recorded for hypervitaminosis with the water-soluble vitamins since these compounds are rapidly metabolized and excreted when the intake exceeds the liver or tissue storage capacity, but hypervitaminosis is a common occurrence in fish and other animals when large quantities of any one of the fat-soluble vitamins are ingested. Sometimes these hypervitaminosis symptoms mimic hypovitaminosis signs, as in the case of the vitamers A and D. The toxicity symptoms observed when excess vitamin E or K is ingested are more discrete. Fish rations often include large quantities of fish meal or fish viscera and are often enriched with fish oils to increase the caloric density or content of polyunsaturated fatty acids of the ration. In these cases, excessive intake of the fat-soluble vitamins is often encountered.

2.3.1. The Vitamers A

A fat-soluble rat growth-promoting factor was described by Hopkins (1912) and by Osborne and Mendel in the early 1900s (1914). McCollum and Simmonds, (1917) cured eye disease, xerophthalmia, with this material. The chemical structure of vitamin A and its relationship to β-carotene were shown by Von Euler *et al.* (1928). The active vitamin A was synthesized by Fuson and Christ (1936) and by Kuhn and Morris (1937). The interrelationships between retinene and retinene$_2$, corresponding to vitamers A$_1$ and A$_2$, were defined by Morton *et al.* (1947), Koehn (1948), and Olson (1961, 1964). Fish oil was shown to be a rich source of vitamers A and to contain vitamin A and neovitamin A at a 2 : 1 ratio. No need for vitamin A in fish was reported until Halver observed xerophthalmia and cataracts in fish fed vitamin A-deficient diets in 1958 and Nigashi *et al.* (1960) reported that vitamin A had a growth-promoting effect in eels. Hypervitaminosis A was reported to occur in salmon by Burrows *et al.* (1952) and in trout by Poston *et al.* (1966). Requirement of vitamers A was reported for carp (Aoe *et al.*, 1968), catfish (Dupree, 1970), tilapia (Katsuyana and Maatusno, 1988), and yellowtail (Hosokawa, 1989).

2.3.1.1. Chemical Structure, Characteristics, and Analogs

Vitamin A$_1$ (retinol) has the chemical formula $C_{20}H_{29}OH$ and vitamin A$_2$ (retinol$_2$) has the formula $C_{20}H_{27}OH$. The structural formula for these fat-soluble alcohols is shown in Fig. 2.27. The relationship of the vitamin A alcohols to naturally occurring β carotene containing two symmetrical betaionone rings is shown in Fig. 2.28. Retinene, the aldehyde form of vitamin A, has been isolated from the retina of dark-adapted eyes and is involved in vision in dim light. Retinoic acid, which is the oxidized form of vitamin A alcohol, has been shown to have some vitamin A activity. Olson

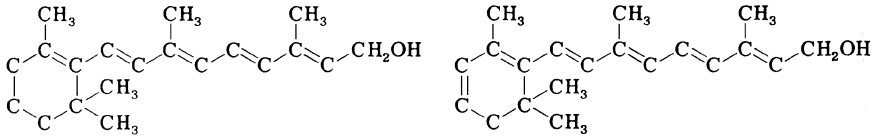

FIG. 2.27

Vitamers A_1 and A_2. *Left:* All-*trans*-vitamin A_1 (retinol$_1$). *Right:* All-*trans*-vitamin A_2 (retinol$_2$).

(1964) has reviewed the biosynthesis and metabolism of carotinoids and vitamers A. Vitamin A_1 is found in saltwater fish, whereas vitamin A_2 is more abundant in freshwater fish. Braekkan *et al.* (1969) have shown interconversion of one form to the other in living fish tissues. Oxidative conversion of retinol to A_2 has been shown in trout and tilapia, and vitamin A_2 can be generated from carotene, canthaxanthin, and other carotinoids (Katsugawa and Matsumoto 1988). Fish oils contain vitamin A as free alcohols or esters. Vitamin A alcohol occurs as a light-colored viscous oil which is heat labile and subject to air oxidation. β-Carotene occurs as an orange, crystalline compound which is more stable to heat and oxidation. The vitamers A are water insoluble but are soluble in fat and organic solvent (Dam and Sondergaard, 1964).

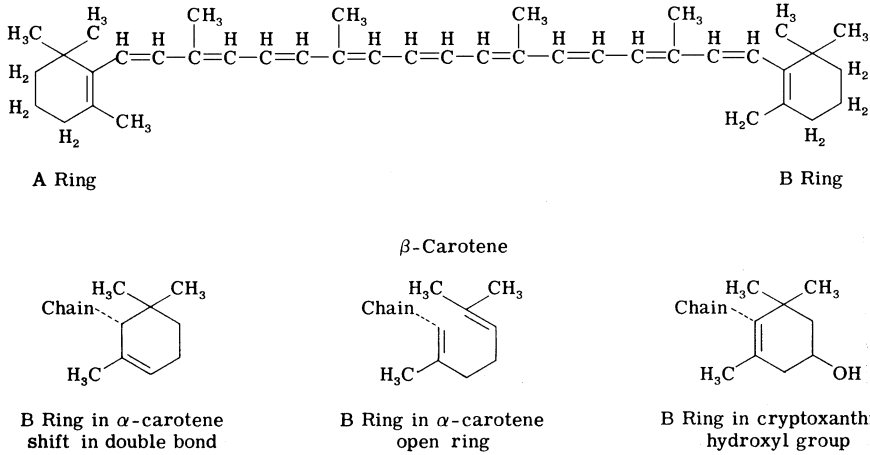

FIG. 2.28

β-Carotene and altered B-ring structures in α-carotene, γ-carotene, and cryptoxanthin.

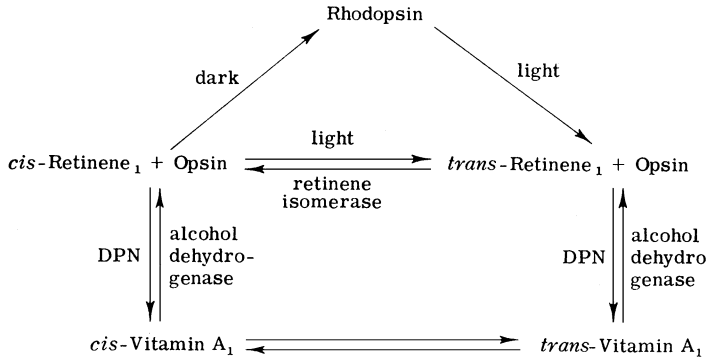

FIG. 2.29

The rhodopsin cycle.

2.3.1.2. Positive Functions

Vitamin A is essential in maintaining epithelial cells. McCollum and Davis (1913) and Osborne and Mendel (1914) first showed that cod liver oil contained a substance which promoted growth, and in 1917, McCollum and Simmonds showed that the substance prevented xerophthalmia in rats. Many changes in organ epithelia, including the respiratory tract, the tongue, and tissues of the mouth, eye, liver, and kidney, have been described by Wolbach and Bessey (1942) and by Follis (1958). Atrophy of the epithelium and formation of stratified keratinizing tissue occur. Vitamin A is a stimulus for new cell growth and aids in maintaining resistance to infection. It increases longevity under various conditions of senility in mammals. Vitamin A and retinene are essential for normal vision (Dam and Sondergaard, 1964; West *et al.,* 1966). Vitamin A is required in vertebrates for the regeneration of light-sensitive rhodopsin in the retina (Blomhoff *et al.,* 1992). Fish have variable abilities to hydrolyze β-carotene into retinol (Poston *et al.,* 1976; Katsugama and Matsumo, 1988). The interrelationship of *cis*- and *trans*-vitamin A in the rhodopsin cycle is shown in Fig. 2.29.

2.3.1.3. Syndrome of Deficiency or Excess

Hypovitaminosis A is characterized by poor growth, poor vision, keratinization of epithelial tissue, xerophthalmia, night blindness, hemorrhage in the anterior chamber of the eye, hemorrhage at the base of the fins, and abnormal bone formation (Dam and Sondergaard, 1964; West *et al.,* 1966; Aoe *et al.,* 1968; Kitamura *et al.,* 1967; Dupree, 1966; Poston *et al.,* 1977; Hasokuma, 1989). Nerve degeneration has been reported in pigs, chickens, rats, rabbits, and ducks but only occasionally observed in fish after long

periods of deficiency. Hypervitaminosis A has been described in fish (Burrows *et al.*, 1952; Halver, 1970; Poston *et al.*, 1966; Poston and Levington, 1969) and other animals and involved enlargement of the liver and spleen, abnormal growth, skin lesions, epithelial keratinization, hyperplasia of head cartilage, and abnormal bone formation, resulting in ankylosis and fusion of vertebrae. Hypervitaminosis A is reflected in a very high liver oil vitamin A content and elevated serum alkaline phosphatase. Removal of excess vitamin A from the diet promotes rapid recovery.

2.3.1.4. Requirements

Dietary requirements for vitamin A alcohol are listed in Table 2.3. Jones *et al.* (1966) have shown a requirement for growth in fish held in light, but not in darkness, and Dupree (1966) has reported similar problems in determining requirements in catfish. Therefore, the requirement for maximum growth and reproduction is related to exposure to light and reflects observations in other animals that near-normal growth will occur with a very low vitamin A intake in protected environments where fish are not exposed to stress, infection, and ultraviolet radiation.

2.3.1.5. Sources and Protection

Cod liver oil is one typical standard reference oil, which contains relatively small amounts of vitamin A, whereas black sea bass, swordfish, and ling cod oils contain 100-fold more. Whale liver oil contains kitol, which has little or no biological activity until heated above 200°C (Embree and Shantz, 1943). Then one molecule of biologically active vitamin A is generated per molecule of whale kitol. This biologically inactive kitol may be deposited in the whale as a defense mechanism against hypervitaminosis A during excessive vitamin A intake. A possibility of hypervitaminosis A occurs when tuna, shark, or ling cod viscera are used in the preparation of moist diets. Synthetic vitamin A preparations, such as vitamin A palmitate, are available and are often used to supplement rations low in fish meal, fish viscera, or carotenes. Some fish species seem to be able to utilize β-carotene as a vitamin A source (Morton and Creed, 1939), whereas others are unable to split the β-carotene molecule and vitamin A must be added to the diet in the retinol, retinene, or retinoic acid form (Neilands, 1947; Poston, 1969b; Poston *et al.*, 1976). Several carotinoids can be converted into vitamin A in the liver of fish (Katsuyama and Matsumo, 1988).

2.3.1.6. Clinical Assessment

Vitamin A status can best be assessed by the absence of deficiency signs and assay of liver oil for vitamin A content. Assay for blood or plasma

vitamin A levels in other animals has not shown vitamin A status. The simple Carr-Price vitamin A determination in liver oil indicates storage and the information can be used to give the vitamin A status of fish.

2.3.2. The Vitamers D

Rickets was induced with test diets by Hopkins (1906), and Mellanby (1919) cured the disease in dogs by adding cod liver oil to the ration. Steenbock and Black (1924) showed that ultraviolet light was involved in antirachitic function, and provitamin D was identified as ergosterol by Windaus and Hess (1927) and by Rosenheirn and Webster (1927). Crystalline vitamin D was isolated by Angus *et al.* (1931) and activated 7-dehydrocholesterol was isolated by Windaus *et al.* (1936). One early report by Jewell *et al.* (1933) on fish diet tests mentioned the need of catfish and goldfish for vitamin D (cod liver oil) in the diet, but the test diets used probably were deficient in other vitamins or growth factors which the crude cod liver oil furnished. Hypervitaminosis D has been shown to elevate alkaline phosphatase in trout and salmon.

2.3.2.1. Chemical Structure, Characteristics, and Analogs

Several biologically active forms of vitamin D occur and the chemical structure of one, vitamin D_2 or ergocalciferol, with the chemical formula $C_{28}H_{44}O$, is shown in Fig. 2.30. Vitamin D_3 or activated 7-dehydrocholesterol has the chemical formula $C_{27}H_{44}O$ and contains a more simplified, unsaturated eight-carbon side chain. Vitamin D (cholecalciferol) is formed in most animal tissue by rupture of one of the ring bonds of 7-dehydrocholesterol

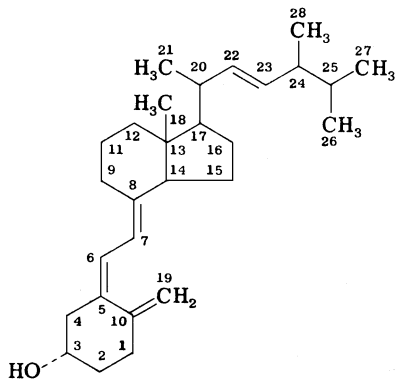

FIG. 2.30

Vitamin D_2.

when exposed in the skin to ultraviolet radiation. Cholecalciferol is a white, crystalline compound soluble in fat and organic solvents and is stable to heat and oxidation in mild alkali or acid solutions (Dam and Sondergaard, 1964). Several derivatives from substitution in the rings or of functional groups in the side chain are isomers of the precursors or isotels of the vitamin and have variable biological activity in different animals. These have not been tested for maintaining homeostasis of calcium and phosphate in fish.

2.3.2.2. Positive Functions

Vitamin D_3 functions as a precursor of 1,25-dihydrocholecalciferol, which stimulates the absorption of calcium from the intestine. Vitamin D is essential for maintaining homeostasis of calcium and inorganic phosphate. Vitamin D is involved in alkaline phosphatase activity, promotes intestinal absorption of calcium, and influences the action of parathyroid hormone on bone (Dam and Sondergaard, 1964; West *et al.,* 1966). Lovelace and Podoliak (1956) have shown that fish may sequester calcium from water through the gill membrane; thus the major function of vitamin D for other animals may not be necessary to satisfy calcium requirements for fish except when the animals are reared in low-calcium waters. Cholecalciferol has been reported to be three times more effective than ergocalciferol in supplying the vitamin D needs of trout and catfish (Barnett *et al.,* 1982; Andrews *et al.,* 1980).

2.3.2.3. Syndrome of Deficiency or Excess

Hypovitaminosis D for fish has been described (Barnett *et al.,* 1979). Rickets and abnormal bone formation, described in detail for animals, have been observed in fish fed low-vitamin D diets in low-calcium waters. Impaired calcium homeostasis, with tetany in white muscle and structural changes in muscle fibers, has been reported (George *et al.,* 1979). Increased plasma triiodothyronine has been observed (Leatherland *et al.,* 1980). Hypervitaminosis D has also been reported. Poston (1969b) has shown that brook trout fed large doses of vitamin D show impaired growth, lethargy, and a dark coloration. High intake of vitamin D mobilizes phosphorus and calcium from the bone and tissues and may result in fragile bones, poor growth, and poor appetite related to the nausea described in humans afflicted with hypervitaminosis D (Dam and Sondergaard, 1964; West *et al.,* 1966). Arterial and kidney lesions reported for rats (Gillman and Gilbert, 1956) and dogs (Herzfeld *et al.,* 1956) have not been described histologically for fish, and hypercalcemia in blood plasma has not been described for fish on high-vitamin D diets. This area needs to be explored because of the potential for hypervitaminosis of D in fish fed diets containing various fish viscera which might contain large amount of the vitamers D. Tuna liver oil may contain, for example, 100 to 1000 times as much active vitamin D as cod liver oil.

2.3.2.4. Requirements

Requirements of fish for vitamin D are listed in Table 2.3 but little work has been done in this area using highly purified diets and controlled experiments with young growing fish. No demonstration of synthesis of vitamers D in fish reared on diets devoid of vitamin D or vitamin D precursors has been reported. Therefore, the actual requirement of vitamin D for maintenance of homeostasis of calcium–phosphorus levels in the young growing animal has not been adequately investigated and the true vitamin D requirements of young fish have only been suggested (NRC, 1981, 1993).

2.3.2.5. Sources and Protection

The vitamin D requirements of many animals can be met by exposure of the skin to sunlight, which activates cholesterol derivatives by ring structure rupture. This subject is well reviewed by Dam and Sondergaard (1964) and by Wagner and Folkers (1964). Since the vitamin is fat soluble and accumulates in lipid stores, fish liver oil is a rich source of the material. The content varies tremendously in liver oil, however, with values of about 25 IU/g present in soup fin shark liver oil and over 200,000 IU/g in albacore tuna liver oil (West *et al.,* 1966). Cod liver oil contains from 100 to 500 IU/g and animal liver contains some vitamin D. One international unit (IU) is equal to 0.025 μg of crystalline vitamin D_2.

2.3.2.6. Clinical Assessment

Absorption maxima in the ultraviolet region can be used to detect provitamins D in the nonsaponifiable fraction of oils. Concentrated preparations of vitamin D can be assayed by the Carr–Price antimony trichloride reaction when assay is necessary to determine biologically active material in liver oil of fish on different treatments. The chick assay may not apply to fish liver storage levels because the biologically active form for fish has been determined to be cholecalciferol (Barnett *et al.,* 1982). Clinical assessment for hypervitaminosis D must rely on crude methods for determination of pro- and active vitamin D in liver oil samples by the Carr–Price reaction or by measuring absorption in the UV spectrum.

2.3.3. The Vitamers E

The existence of an antisterility vitamin was postulated by Evans and Bishop (1922). This factor was named "vitamin E" by Sure (1924). The active tocopherol was isolated, characterized, and synthesized by Karrer *et al.* (1938).

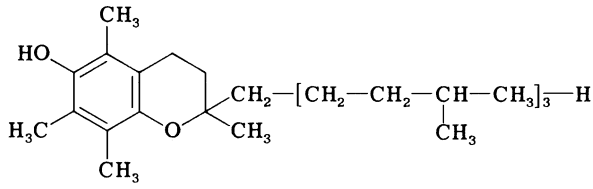

FIG. 2.31

α-Tocopherol(5,7,8-trimethyltocol).

2.3.3.1. Chemical Structure, Characteristics, and Analogs

The vitamers E are compounds known as tocopherols and are derivatives of tocol, which has a saturated side chain, or of tocotrienol, which contains three unsaturated carbon–carbon bonds in the side chain. One of the most important tocopherols, α-tocopherol (5,7,8-trimethyltocol), has the chemical formula $C_{29}H_{50}O_2$ and the structural formula shown in Fig. 2.31. Eight naturally occurring tocopherol derivatives have been isolated and all belong to the D series. Synthetic α-tocopherol is a racemic DL-α-tocopherol mixture (Dam and Sondergaard, 1964; West *et al.,* 1966). The derivatives of tocol or of tocotrienol are named α-, β-, γ-, δ-, ϵ-, η-, ζ_1-, and ζ_2-tocopherol as shown in Table 2.5. The pure tocopherols are fat-soluble oils which are capable of esterification to form crystalline compounds. The tocopherols are stable to heat and acids in the absence of oxygen but are rapidly oxidized in the absence of oxygen, peroxides, or other oxidizing agents (Dam and Sondergaard, 1964). The tocopherols are sensitive to ultraviolet light and are excellent antioxidants in the free form, whereas the tocopherol esters are poor *in vitro* antioxidants. The esters are more stable and are commonly used as dietary supplements—anticipating hydrolysis in the gut and absorption of the free alcohol to act as an active intra- and intercellular antioxidant.

Table 2.5

The Tocopherols

α-Tocopherol	5,7,8-Trimethyltocol
β-Tocopherol	5,8-Dimethyltocol
γ-Tocopherol	7,8-Dimethyltocol
ζ_2-Tocopherol	5,7-Dimethyltocol
η-Tocopherol	7-Methyltocol
δ-Tocopherol	8-Methyltocol
ϵ-Tocopherol	5,8-Dimethyltocotrienol
ζ_1-Tocopherol	5,7,8-Trimethyltocotrienol

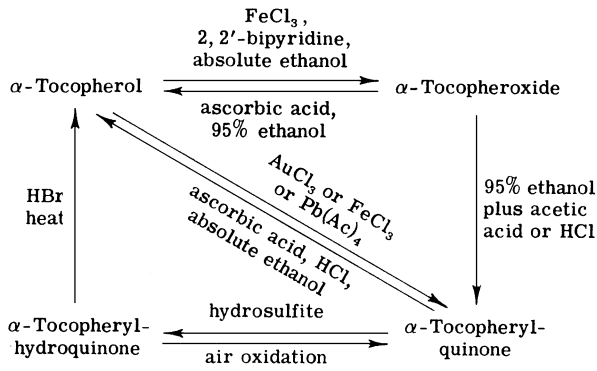

FIG. 2.32

Oxidation–reductiion of α-tocopherol.

Ethyl derivatives on the aromatic ring are also active. Oxidation products of α-tocopherol can be reduced with hydrosulfite to α-tocopherylhydroquinone or, in the presence of ascorbic acid, to α-tocopherol (Dam and Sondergaard, 1964). The oxidation–reduction of tocopherylquinones is shown in Fig. 2.32.

2.3.3.2. Positive Functions

The tocopherols act as inter- and intracellular antioxidants to maintain homeostasis of labile metabolites in the cell and tissue plasma. As physiological antioxidants, these usually protect oxidizable vitamins and labile unsaturated fatty acids. Vitamin E functions together with selenium and ascorbic acid in the enzymes glutathione peroxidase and superoxide dismutase to stop the chain reactions of polyunsaturated fatty acid peroxidation (Lehninger, 1977). Vitamers E are involved in encephalomalacia in chicks (Pappenheimer and Goettsch, 1931), erythrocyte hemolysis in several animals (György and Rose, 1948; Horwitt *et al.,* 1963; Woodall *et al.,* 1964), and steatitis in mink, pigs, and farm animals (Dam and Sondergaard, 1964). The tocopherols prevent exudative diathesis in chicks, white muscle disease in fish (Poston *et al.,* 1976), and dietary liver necrosis in rats. Vitamers E are involved with selenium and with vitamin C for normal reproductive activity and are involved in the prevention of nutritional muscular dystrophy in chick, yellowtail (Sakaguchi and Hamaguchi, 1969), and carp (Hashimoto *et al.,* 1966). The tocopherols act as free radical traps to stop the chain reaction during peroxide formation and stabilize unsaturated carbon bonds or polyunsaturated fatty acids and other long-chain labile compounds (Dam and Granados, 1945; Dam *et al.,* 1952; Tappel and Zalkin, 1960). Vitamin E

in its antioxidant capacity is involved in the maintenance of normal permeability of capillaries and heart muscle. It was first shown to be involved in prevention of sterility and fetal resorption in rats. It may likewise be involved in embryo membrane permeability and hatchability of fish eggs.

2.3.3.3. Syndrome of Deficiency or Excess

Deficiency signs in fish are listed in Table 2.2. One of the first signs for fish fed normal amounts of polyunsaturated fatty acids is erythrocyte fragility, followed closely by anemia, ascites, xerophthalmia, poor growth, poor food conversion, epicarditis, and ceroid deposits in spleen and liver. Muscle dystrophy and xerophthalmia have been described in yellowtail and carp (Sakaguchi and Hamaguchi, 1969; Hashimoto *et al.,* 1966). Impaired erythropoiesis, fragmentation of erythrocytes, marked susceptibility to stress of handling, ascites, and exudative diathesis have been reported in deficient salmon and trout. Similar signs of deficiency have been observed in warmwater and marine species fed diets low in vitamers E and high in polyunsaturated fatty acid oils (Dupree, 1988; Murai and Andrews, 1974; Lovell *et al.,* 1984; Cowey *et al.,* 1981, 1983; Toyoda, 1985). Several nonspecific cell degenerative conditions have been described in several species of fish fed large quantities of polyunsaturated fatty acids with inadequate tocopherol in the ration. Hypervitaminosis E involves poor growth, toxic liver reaction, and death (Watanabe *et al.,* 1970; Poston, 1971).

2.3.3.4. Requirements

Requirements of fish for vitamin E are listed in Table 2.3. The exact requirement of fish for α-tocopherol, the vitamin E form used in these test diet experiments, may depend on the amount and type of polyunsaturated fatty acids in the oil components of the ration (Woodall *et al.,* 1964; Watanabe *et al.,* 1970). Polyunsaturated labile fish oils may invoke an increased requirement for intracellular antioxidants (Hashimoto *et al.,* 1966). Also, the amount of tocopherol needed as a supplement to the ration will depend on the form of the vitamer used, the method of diet preparation, and the storage conditions under which the rations are held before feeding. The physiological requirements of the species tested are listed and were determined with carefully protected test diets prepared immediately before feeding and containing relatively low amounts of polyunsaturated fatty acids.

2.3.3.5. Sources and Protection

Wheat germ oil, soybean oil, and corn oil are rich sources of tocopherols. Synthetic α-tocopherol in the esterified acetate or phosphate form is commonly used as a diet supplement. These esters are much more stable than

the free form, which is rapidly lost by air oxidation or in the presence of labile compounds such as polyunsaturated fish oils. Wheat, corn, or bean oils are very stable and, when incorporated into the diet, tend to stabilize the labile fatty acids present as well. An interrelationship among vitamins E, C, and A is involved in the protection of the labile vitamin A molecule (Dam and Granados, 1945; Dam *et al.,* 1952). Therefore, it is essential to prepare, store, and feed fish rations containing quantities of labile fish oils (Sinnhuber, 1969; Stansby, 1967) in the minimum time to prevent loss of tocopherol content and subsequent rapid destruction of vitamins E, C, and A. The addition of *in vitro* antioxidants such as BHA (butylhydroxyanisole) and BHT (butylhydroxytoluene) or ethoxyquin tends to protect fats and other labile compounds in the ration from oxidation, but these antioxidants have little vitamin E activity as the physiological intracellular antioxidant for the growing fish (see Hardy and Barrows, Chapter 9).

2.3.3.6. Clinical Assessment

The erythrocyte fragility test indicates the physiological state of the fish (Woodall *et al.,* 1964). The absence of histologically detectable ceroid in liver and spleen from representative samples in the population is a good clue to the presence of adequate amounts of physiological antioxidants in the fish (Wood and Yasutake, 1956). A barbituric acid test for oxidation of components in the ration or a peroxide number test for peroxidation of diet components has not been applied to fish tissues as a clinical tool for assessment of nutrition state except when liver oils become saturated with very labile polyunsaturated fatty acids such as when feeding squid or saury oils (Hashimoto *et al.,* 1966; Watanabe *et al.,* 1970). These assays are good indicators of the state of potential oxidation and the oxidized state of the finished ration, but the absence of deficiency signs and a normal erythrocyte fragility are better clinical tests. Cowey *et al.* (1981) found that ascorbic acid-stimulated lipid peroxidation in liver mitochondria and microsomes of trout reflected the α-tocopherol status of the animal. Peroxide hemolysis of fish erythrocytes also may determine vitamin E deficiency (Hung *et al.,* 1981). Analysis for tocopherol is difficult, time-consuming, and applicable only in critical research experiment situations.

2.3.4. The Vitamers K

The name of vitamin K (for "koagulation") was proposed by Dam (1935). Dam *et al.* (1939) and McKee *et al.* (1939) isolated the vitamin from alfalfa and from fish meal in 1939, and it was synthesized by Almquist and Klose (1939), McKee *et al.* (1939), and Fieser (1939).

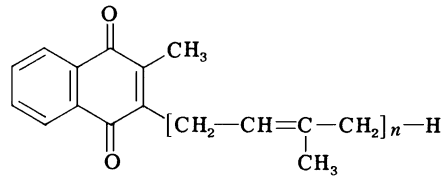

FIG. 2.33

Vitamin K_2 series: *n* may equal 6, 7, or 9 isoprene units.

2.3.4.1. Chemical Structure, Characteristics, and Analogs

Vitamin K is 2-methyl-3-phytyl-1,4-naphthoquinone, with the chemical formula $C_{31}H_{46}O_2$ and the structural formula shown in Fig. 2.33. The vitamers K_2 contain six, seven, or nine isoprene units in the side chain, which varies from 30 to 45 carbon atoms. Many isotels of vitamin K have been identified in animal tissues, plant tissues, and microorganisms. The structures of phthiocol, 2-methylhydroxy-3-phytyl-1,4-naphthoquinone, and menadione (K_3), 2-methyl-1,4-naphthoquinone, are shown in Fig. 2.34. These are fat-soluble, fairly stable compounds but, as methylnaphthoquinones, are labile to oxidation and exposure to ultraviolet radiation. Menadione is very reactive and is subject in aqueous media to chemical interaction and the formation of compounds and complexes which may interfere with physiological activity (Dam and Sondergaard, 1964).

2.3.4.2. Positive Functions

Vitamin K is involved in the synthesis of messenger RNA in the synthesis of blood-clotting proteins—prothrombin, plasma thromboplastin, proconvertin, and at least one other factor. A simplified scheme of blood coagulation is shown in Fig. 2.35. Substituted forms of vitamin K are strongly bacteriostatic and may serve as an alternate defense mechanism for bacterial infections. Vitamin K is involved with vitamins A and E and ascorbic

FIG. 2.34

Active naphthoquinones: phthiocol (*left*) and menadione (*right*).

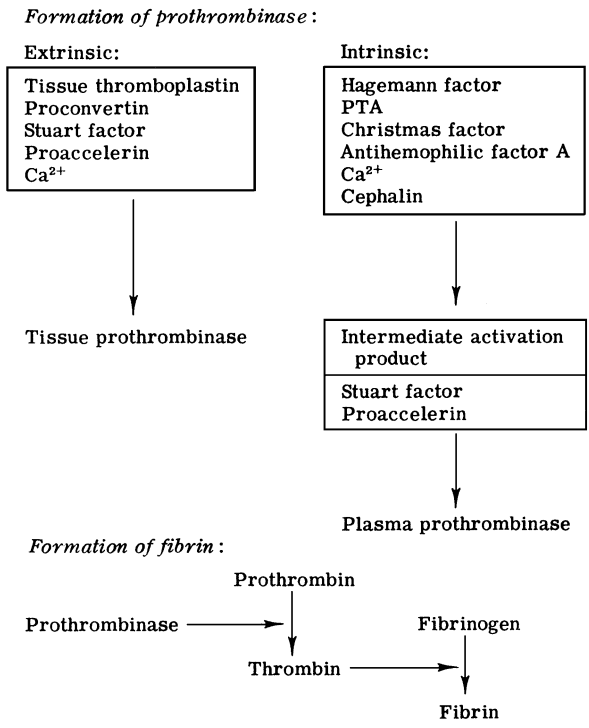

FIG. 2.35

Blood coagulation scheme. A simplified version of processes involved in blood coagulation—both extrinsic and intrinsic factors are involved in the production of prothrombinase to initiate the hydrolysis of prothrombin to activate blood clotting.

acid in homeostasis of physiologically active vitamins A and E (Dam and Sondergaard, 1964). Vitamin K may be involved in coenzyme Q-type compounds, which function between flavoproteins and cytochromes in electron transport mechanisms (West *et al.,* 1966). Vitamin K is a cofactor for carboxylation of glutamyl residues to α-carboxylglutamic acid precursors of blood-clotting proteins (Suttie, 1980). The primary role of vitamin K is to maintain a fast normal blood-clotting rate, which is very important to fish, living in a water environment.

2.3.4.3. Syndrome of Deficiency or Excess

A summary of the deficiency signs is listed in Table 2.2. The prothrombin time in salmon fed diets devoid of vitamers K was increased three to five times, and during prolonged deficiency states, anemia and hemorrhagic

areas appeared in the gills, eyes, and vascular tissues. Increased blood-clotting time has also been reported for other fish reared on diets with a low vitamin K content (Dupree, 1966; Poston, 1964). Interrelationships with other vitamins have not been documented in fish experiments and the primary deficiency signs remain slow blood clotting and hemorrhage, severe anemia, and death in wounded fish. Hemorrhagic areas often appear in fragile tissues such as the gills. Intake of vitamers K at 2000–3000 mg/kg diet can be tolerated by trout (Poston, 1971), but higher levels may cause liver toxicity and death.

2.3.4.4. Requirements

Tests for qualitative requirements of fish for vitamin K have been completed (Phillips *et al.,* 1963). The deficiency signs occur at 10 to 14 weeks of testing when good diets are used with positive control of the vitamin K content. Quantitative requirement studies have been completed on some fish and the vitamin K requirements of rapidly growing young fish, especially those held at a high population density under intensive fish husbandry techniques, are listed in Table 2.3.

2.3.4.5. Sources and Protection

Vitamin K sources are green, leafy vegetables. Alfalfa leaves are one of the best sources of vitamers K. Low levels are found in soybeans and animal liver (Isler and Wiss, 1959). Synthetic menadione is a good supplement for adequate vitamin K intake. The vitamin K content of ground alfalfa is fairly stable but the synthetic material should be protected from exposure to ultraviolet light and to excessive oxidizing or reducing conditions. The use of dry, rapidly cured alfalfa is essential to minimize formation of the physiological antagonist dicumarol. The diet should be kept dry, prepared with minimum exposure to air oxidation, and fed as soon as practicable after manufacture to minimize vitamin K loss through storage, interreaction, and oxidative destruction. Menadione sodium bisulfite and menadione dimethylpyrimidol bisulfite are commonly used as K supplements in fish diets (Adams, 1978) (see Hardy and Barrows, Chapter 9).

2.3.4.6. Antimetabolites and Inactivation

Dicumarol, 3,3′-methylenebis(4-hydroxycoumarin), and warfarin are shown in Fig. 2.36. Dicumarol was isolated from spoiled sweet clover hay and shown to prevent the normal function of vitamin K in maintenance of normal blood-clotting times (Link, 1959). Early work was with cattle and hogs but was soon extended to rats, other experimental animals, and humans. Dicumarol is an anticoagulant and has been used to prevent thrombosis in animals and humans. It is not an antimetabolite competing for

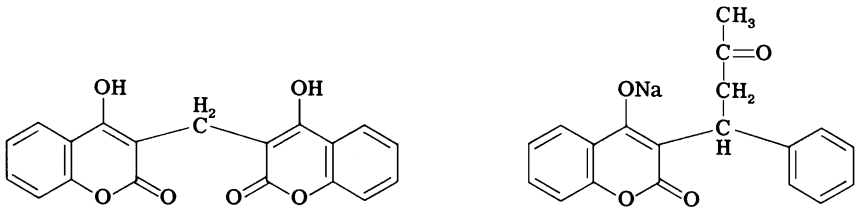

FIG. 2.36

Dicoumarol and warfin: note the similarity of the first half of the molecular structures.

vitamin K sites but plays another role in preventing normal blood clotting. Vitamin K counteracts the dicumarol effect and the results are quantitative with intake, allowing dicumarol to be administered to adjust the blood-clotting time in special situations, according to the vitamin K status of the animal, and then counteracting the dicumarol effect by the administration of more vitamin K (Mushett and Seeler, 1947). Another anticoagulant, warfarin, is a common rat poison which has 5 to 10 times the anticoagulant activity of dicumarol (Shapiro and Ciferri, 1957). Warfarin's effect can also be reversed by the administration of vitamin K. Both compounds are general antagonists to vitamin K.

2.3.4.7. Clinical Assessment

The vitamin K status of fish is determined by determining the blood-clotting time (Casillas *et al.,* 1975). Chemical methods for determination of menadione to assess the content of active metabolite after storage can use the 2,4-dinitrophenylhydrazone derivative and measure the content spectrophotometrically. The standard Dam and Sondergaard chick assay (1953) will measure total biologically active vitamers K in dietary raw materials but this type of technique has not been applied to vitamin K activity in the liver or tissues of young growing fish.

2.4 Other Factors

2.4.1. More Animal Protein Factors

Each year more forms of animal protein factor (vitamins B_{12}, B_{13}, etc.) have been either isolated or postulated as being present in meat, fish, yeast, or other biological materials used as diet components for experimental animals. Whether these factors are specific vitamins or a particular form which increases the utilization or function of one of the other vitamins or one of the other major nutrient components in the ration has not been defined.

Whenever purified test rations are used to rear fish under controlled experimental conditions, these rations contain all the known demonstrated nutrients, but these fish populations fail to grow as well as those fed moist diets consisting of fish tissue, glandular tissue, animal tissue, dextrinized starch, and fish oils. Generally, about a 10% difference in growth is observed between the best purified diets and practical moist or dry rations. These growth response differences are most common when protein is used as the major dietary component and may be due to increased activation of proteolytic enzymes, with concomitant orderly hydrolysis and absorption of amino acids at or near the rate used for tissue protein synthesis in the growing animals. The fish meal or animal protein components may contain as yet unisolated and undefined new animal protein factors.

2.4.2. Citrovorum Factor

Szent-György and Ruszynyák (1936) reported "vitamin P" in pepper, citrus fruit, and paprika, which improved capillary fragility in guinea pigs on a flavin-free diet containing adequate ascorbic acid (Zacho, 1939). Some capillary hemorrhage and a characteristic wrist stiffness developed in guinea pigs and was remedied by material present in the rinds of citrus fruits (Bourne, 1943). Vitamin P activity is found in rutin isolated from buckwheat and in esculin from chestnuts. Structural formulas for these are shown in Fig. 2.37. Other citrovorum factors are concentrated in citrus fruit pulp and rind, but requirements for fish have not been demonstrated.

2.4.3. Factors in Cell Permeability

Unknown factors exist which influence osmoregulation and cell membrane permeability. These may be especially important in fish during transition from fresh water to a saltwater environment, where the water flow

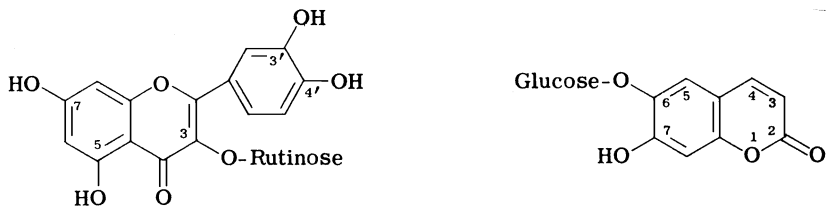

FIG. 2.37

Vitamers P: rutin (*left*) and esculin (*right*). In certain animals, these compounds may have vitamin-like activity alone or in combination with other vitamin factors.

through exposed tissues is reversed and the kidneys alternatively excrete much dilute urine or become quiescent, depending on the external environment of the fish. An interrelationship among vitamins E, C, K, and A in respiration has been mentioned but the exact role of these and other factors such as selenium, zinc, copper, and sodium chloride has not been defined. Both pituitary function and thyroid function are associated during smoltification with rapid development of gill secretory cells, which maintain salt and water balance in the new hypertonic environment. Much work needs to be done in this area, and fish may be important experimental animals in which to study cell permeability because of the unique environmental demands on these animals for survival.

2.4.4. Coenzyme Activation Factors

Interrelationships among thiamin, zinc, cocarboxylase activity, trace amounts of copper, several oxidation–reduction systems, iodine, iodinated amino acids, and metabolic rate should be mentioned. These are examples which show the interrelationship in the viable animal system of vitamins, coenzymes, and mineral ions. The structural stereo configuration of several proteins, for example, insulin, is determined in part by the zinc present. Another example is the planar structure of vitamin B_{12}relative to the central cobalt atom. Most of the water-soluble vitamins act as coenzymes for enzyme functions, which often require trace amounts of certain mineral ions for activation. This is another area where fish could be used as study animals and model systems to develop comparative biochemistry experiments to define the general nature of certain basic biological processes in cell physiology and intermediary metabolism.

2.5 Anemias and Hemapoiesis

Several types of nutritional anemias occur which may be due to several nutritional deficiencies. Unfortunately, most anemias are multiple deficiencies of essential nutrients and the fish clinician must carefully observe the characteristics of the anemia involved to obtain clues as to the nutritional cause.

2.5.1. Megaloblastic Anemias

Deficiencies in folic acid, ascorbic acid, and iron can cause megaloblastic anemia, with characteristic macrocytic, senile erythrocytes in the circulating blood. An adequate description of folic acid, macrocytic, and normochromic

anemia has been reported by Smith (1968; Smith and Halver, 1969) and forms a reference for the cytology of this type of hematology. Feeding folic acid and xanthopterin, increasing the ascorbic acid content, and assuring adequate ferrous ion intake will result in a rapid recovery, with anterior kidney imprints and blood smears showing rapid regeneration of the pro forms of the erythrocytes. Many immature forms will be seen within a few days after administration of the missing nutrients.

2.5.2. Pernicious Anemias

The more common type of anemia encountered in fish is similar to the pernicious anemias described in other experimental animals. This disease can be caused by inadequate intake of vitamin B_{12}, which is necessary for the conversion of 5-methyltetrahydrofolate back into the active folic acid pool. The net result is the decreased synthesis of deoxyribonucleic acid, which is necessary for the synthesis and development of new red blood cell pro forms. A hog mucosal factor improves the absorption of vitamin B_{12} severalfold, and adequate B_{12} is necessary for proper folic acid function in the synthesis and maturation of proerythrocyte forms. Little is known about the role of the gastric mucosal factor in the absorption of vitamin B_{12} in fish tissues, but it is important and may determine adequate absorption of dietary vitamin B_{12} in rats, in other experimental animals, and in humans.

The clinical characteristic of pernicious anemias is the presence of distorted blood cells and cellular debris in the plasma. Inadequate amounts of vitamin E to maintain blood cell membrane integrity also result in a pernicious type of anemia, with many abnormal blood cells present. This is also commonly seen when diets rich in polyunsaturated fatty acids, but with inadequate *in vitro* antioxidants and an inadequate vitamin E content, are fed to salmonids for 12–20 weeks. Locker-burned food generally means oxidized, polymerized fatty acids, ceroid in the liver and spleen, and few, if any, active tocopherols left in the ration (Wood and Yasutake, 1956). Pernicious-type anemia is one of the first symptoms observed in fish fed these storage-oxidized products. This can be differentiated from animal protein factor anemia by the failure to respond to either vitamin B_{12} or a water-soluble vitamin supplement. Other pernicious-type anemias can occur from deficiencies of biotin, pantothenic acid, and niacin. The history of fish nutrition is replete with many vitamin combinations which were reported to have factor H activity (McCay and Dilley, 1927). Anemias resulting from these are general breakdowns in nucleic acid synthesis, and subsequent protein synthesis, with resultant hematology and cytology indicative of pernicious anemia.

2.5.3. Hemapoiesis

The most convenient tool for the fish nutritionist to assess clinical status is hematology. The first general sign of abnormal hematology is impaired hemapoiesis. The fish clinician must realize that many factors are involved, including vitamins, minerals, amino acids, antagonist hormones, and physiological stimulations. Folic acid, vitamin B_{12}, and ascorbic acid nutritional state are obvious determinants. Also, limiting trace minerals such as iron, copper, and manganese and limiting amino acids in the diet result in abnormal growth by interfering with the rate of protein structure. Fish erythrocytes are enucleated and need proper nucleic acid synthesis for normal erythropoiesis. Erythropoiesis-inhibiting agents such as dicumarol, warfarin, and phenothyazine should be considered. Finally, pituitary and adrenal hormones, which stimulate protein synthesis, and thyroid hormones, which regulate metabolic activity and protein synthesis, play dominant roles in blood cell formation. Repeated extraction of quantities of blood for clinical experiments through in-dwelling cannulas will stimulate erythropoiesis, which will be reflected in the blood cytology and anterior kidney imprints (Ashley and Smith, 1963). The tremendous physiological change involved in the transition of anadromous fish from a hypo- to a hypertonic environment appears to increase their sensitivity to hemorrhage and probably also has effects on normal hemapoiesis (Zaugg and McLain, 1969; Zaugg, 1970).

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3

Amino Acids and Proteins

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3.1 Introduction

Proteins are the major organic material in fish tissue, making up about 65 to 75% of the total on a dry-weight basis. Fish consume protein to obtain amino acids. The protein is digested or hydrolyzed and releases free amino acids, which are absorbed from the intestinal tract and distributed by the blood to the organs and tissues. These amino acids are used by the various tissues to synthesize new protein. A regular intake of protein or amino acids is required because amino acids are used continually by the fish, either to build new proteins (as during growth and reproduction) or to replace existing proteins (maintenance). Inadequate protein in the diet results in a reduction or cessation of growth and a loss of weight due to withdrawal of protein from less vital tissues to maintain the functions of more vital tissues. On the other hand, if too much protein is supplied in the diet, only part of it will be used to make new proteins, and the remainder will be converted to energy.

The first definitive studies on protein and amino acid nutrition of fish were conducted by Halver and co-workers in the late 1950s and early 1960s in chinook salmon (*Oncorhynchus tshawytscha*). The initial amino acid test diets were formulated based on the amino acid content of chicken whole egg protein, chinook salmon egg protein, and chinook yolk sac fry protein (Halver, 1957). The amino acid test diet formulated based on the amino acid content of chicken whole egg protein gave the best growth and feed efficiency, and was therefore adopted as the amino acid test diet. This diet was used to determine the qualitative amino acid requirements of the chinook salmon (Halver *et al.,* 1957). The gross protein requirement of chinook salmon was determined by feeding test diets containing a mixture of casein, gelatin, and crystalline amino acids to simulate the amino acid content of whole egg protein (DeLong *et al.,* 1958). Subsequent experiments utilizing test diets containing a mixture of casein, gelatin, and crystalline amino acids to form an amino acid pattern of 40% whole egg protein were used to determine the quantitative amino acid requirements of the 10 indispensable amino acids for the chinook salmon (Halver *et al.,* 1958; DeLong *et al.,* 1962; Chance *et al.,* 1964; Halver, 1965). These initial pioneering studies by Halver and colleagues have served as the basic model for many subsequent studies on the amino acid and protein nutriture of several fish species.

3.2 Protein Requirements

3.2.1. Gross Requirements

3.2.1.1. Finfish

Fish, like other animals, do not have a true protein requirement but have a requirement for a well-balanced mixture of essential or indispensable and nonessential or dispensable amino acids. Numerous investigators have utilized various semipurified and purified diets to estimate the protein requirements of fish. The estimated protein requirements of several species of juvenile fish are summarized in Table 3.1. Most of these values have been estimated from dose–response curves, yielding the minimum amount of dietary protein which resulted in maximum growth. Some of these requirement values appear to have been overestimated because of inadequate consideration of one or more of the following dietary factors: (a) the energy concentration of the diet, (b) the amino acid composition of the dietary protein, and (c) the digestibility of the dietary protein.

The optimal dietary protein level for fish, as well as other animals, is influenced by the optimal dietary protein-to-energy balance, the amino acid composition and digestibility of the test protein(s), and the amount of nonprotein energy sources in the test diet. Excess energy in the test diet may limit consumption, as it has been suggested that fish, like other animals, eat to meet their energy requirement (see Chapter 1, by Bureau *et al.*). Most investigators state that they have used isoenergetic diets to determine the protein requirements, however, as the metabolizable energy of the various ingredients has not been determined for most fish, these workers have used various estimated physiological fuel values in expressing the protein requirement in relation to the dietary energy level. The influence of changes in dietary energy on protein utilization, as well as the sparing effects of dietary lipid and carbohydrate on dietary protein, has been discussed elsewhere (Wilson, 1989).

The data in Table 3.1 indicate that the protein requirements of fish are much higher (two to four times) than those of other vertebrates. This observation has led certain investigators, including me, to suggest that the efficiency of protein utilization is lower in fish than in other animals. Tacon and Cowey (1985) first noted that the dietary protein requirements of fish are not that dissimilar from those of other vertebrates when expressed relative to feed intake (grams of protein per kilogram of body weight per day) and live weight gain (grams of protein per kilogram of live weight gain). Bowen (1987) compared several parameters relating protein intake to growth of fish and other vertebrates and found very little difference in

Table 3.1

Estimated Protein Requirements of Juvenile Fish

Species	Protein source	Estimated requirement (%)	Reference
Asian sea bass (*Lates calcarifer*)	Casein, gelatin	45	Boonyaratpalin (1991)
Atlantic halibut (*Hippoglossus hippoglossus*)	Fish meal	51	Helland and Grisdale-Helland (1998)
Atlantic salmon (*Salmo salar*)	Fish meal	55	Grisdale-Helland and Helland (1997)
Blue tilapia (*Oreochromis aureus*)	Casein, egg albumin	34	Winfree and Stickney (1981)
Brown trout (*Salmo trutta*)	Casein, fish meal, FPC[a]	53	Arzel *et al.* (1995)
Channel catfish (*Ictalurus punctatus*)	Whole egg protein	32–36	Garling and Wilson (1976)
Chinook salmon (*Oncorhynchus tshawytscha*)	Casein, gelatin, amino acids	40	DeLong *et al.* (1958)
Coho salmon (*Oncorhynchus kisutch*)	Casein	40	Zeitoun *et al.* (1974)
Common carp (*Cyprinus carpio*)	Casein	38 31	Ogino and Saito (1970) Takeuchi *et al.* (1979)
Estuary grouper (*Epinephelus salmoides*)	Tuna muscle meal	40–50	Teng *et al.* (1978)
European eel (*Anguilla anguilla*)	Fish meal	40	de la Higuera *et al.* (1989)
European sea bass (*Dicentrarchus labrax*)	Fish meal	50	Hidalgo and Alliot (1988)
Florida pompano (*Trachinotus carolinus*)	Fish meal, soy meal	45	Lazo *et al.* (1998)
Gilthead bream (*Sparus aurata*)	Casein, FPC, amino acids	40	Sabaut and Luquet (1973)
Golden shiner (*Notemigonus crysoleucas*)	Fish meal, casein	29	Lochmann and Phillips (1994)
Goldfish (*Carassius auratus*)	Fish meal, casein	29	Lochmann and Phillips (1994)

(*continues*)

Table 3.1 *(Continued)*

Estimated Protein Requirements of Juvenile Fish

Species	Protein source	Estimated requirement (%)	Reference
Grass carp (*Ctenopharygodon idella*)	Casein	41–43	Dabrowski (1977)
Hybrid striped bass (*Morone chrysops* × *M. saxatilis*)	Fish meal, casein	35	Nematipour *et al.* (1992)
Japanese eel (*Anguilla japonica*)	Casein, amino acids	44.5	Nose and Arai (1972)
Largemouth bass (*Micropterus salmoides*)	Casein, FPC	40	Anderson *et al.* (1981)
Milkfish (*Chanos chanos*)	Casein	40	Lim *et al.* (1979)
Mozambique tilapia (*Oreochromis mossambicus*)	White fish meal	40	Jauncey (1982)
Nile tilapia (*Oreochromis niloticus*)	Casein	30	Wang *et al.* (1985)
Plaice (*Pleuronectes platessa*)	Cod muscle	50	Cowey *et al.* (1972)
Puffer fish (*Fugu rubripes*)	Casein	50	Kanazawa *et al.* (1980)
Rainbow trout (*Oncorhynchus mykiss*)	Casein, gelatin	40	Zeitoun *et al.* (1973)
Red drum (*Sciaenops ocellatus*)	Fish meal, casein	35–45	Daniels and Robinson (1986)
Red sea bream (*Pagrus major*)	Casein	55	Yone (1976)
Smallmouth bass (*Micropterus dolomieui*)	Casein, FPC	45	Anderson *et al.* (1981)
Snakehead (*Canna micropeltes*)	Fish meal	52	Wee and Tacon (1982)
Sockeye salmon (*Oncorhynchus nerka*)	Casein, gelatin, amino acids	45	Halver *et al.* (1964)
Striped bass (*Morone saxatilis*)	Fish meal, SP[b]	47	Millikin (1983)
Yellow perch *(Perca flavescens)*	Casein, gelatin, amino acids	35	Brown *et al.* (1996)

(*continues*)

Table 3.1 *(Continued)*

Estimated Protein Requirements of Juvenile Fish

Species	Protein source	Estimated requirement (%)	Reference
Yellowtail (*Seriola quinqueradiata*)	Sand eel, fish meal	55	Takeda *et al.* (1975)
Zacco barbata	Fish meal	32	Shyong *et al.* (1998)
Zillii's tilapia (*Tilapia zillii*)	Casein	35	Mazid *et al.* (1978)

[a] Fish protein concentrate.
[b] Soy proteinate.

protein utilization among the species compared (Table 3.2). The data used to make this comparison included median values from 18 studies of fish and 8 studies of other vertebrates including calves, chickens, lamb, swine, and white rats. The only parameters that differed significantly were the level of protein in the diet required for maximum growth and the feed conversion efficiency. When the protein requirement data were recalculated to correct for differences in relative protein intake and growth rates, as suggested by Tacon and Cowey (1985), the resulting data were very similar for fish and other vertebrates. This indicates that the efficiency of protein utilization is very similar among the species compared.

Table 3.2

Parameters Relating Protein Intake to Growth of Fish and Other Vertebrates[a]

Parameter	Fish	Other vertebrates
Specific growth rate	2.765	2.445
Protein in diet (%)	40.3	20.0
Protein intake at maximum growth (mg protein ingested/g body wt/day)	16.5	12.0
Protein retention efficiency [100 × (g protein retained/g protein ingested)]	31.0	29.0
Protein growth efficiency (g growth/g protein ingested)	1.945	1.965
Feed conversion efficiency (g growth/g diet ingested)	0.78	0.26

[a] Data from Bowen (1987).

Table 3.3

Optimum Dietary Protein Levels for Crustacean

Species	Protein source	Optimum level (%)	Reference
Homarus americanus	Casein, gluten, shrimp meal	31	D'Abramo *et al.* (1981)
Homarus gammarus	Fish and crustacean meals	35	Lucien-Brun *et al.* (1985)
Macrobrachium rosenbergii	Soybean, tuna, shrimp meal	>35	Balazs and Ross (1976)
Metapenaeus monoceros	Casein	55	Kanazawa *et al.* (1981)
Palaemon serratus	Fish meal, shrimp meal	40	Forster and Beard (1973)
Penaeus durarum	Soybean meal	28–30	Sick and Andrews (1973)
Penaeus indicus	Prawn meal	43	Colvin (1976)
Penaeus japonicus	Shrimp meal	40	Balazs *et al.* (1973)
	Casein, egg albumin	54	Deshimaru and Kuroki (1974)
	Squid meal	60	Deshimaru and Shigeno (1972)
	Casein, egg albumin	52–57	Deshimaru and Yone (1978)
Penaeus merguiensis	*Mytilus edulis* meal	34–42	Sedgwick (1979)
Penaeus monodon	Casein, fish meal	46	Lee (1971)
Penaeus setiferus	Fish meal	28–32	Andrews *et al.* (1972)

3.2.1.2. Crustacea

Like finfish, most crustacea studied to date have rather high protein requirements, ranging from 30 to 60% of the dry diet (Table 3.3). Here, again, some of these values appear to be overestimated, for some of the same reasons as suggested for comparable values estimated for finfish. In addition, crustacean nutritional studies are complicated by the difficulty of producing water-stable formulated diets which resist leaching due to delayed consumption by the test organism. Some organisms also shred their food particle prior to ingestion, which may enhance leaching and make food consumption measurements very difficult. Guillaume (1997) has recently summarized the protein and amino acid needs of crustacea and discussed the various problems associated with determining protein and amino acid requirements in these organisms.

3.2.2. Factors Affecting Requirements

3.2.2.1. Size and Age

Generally, the protein requirements of fish decrease with increasing size and age. For example, the optimal dietary protein level for very young

salmonids is 45 to 50% of the diet, while juveniles require 40% and yearlings require about 35% dietary protein (Hilton and Slinger, 1981; Hardy, 1989). Similarly, channel catfish fry require about 40% protein, whereas fingerlings require 30 to 35% protein and larger fish (>110 g) require 25 to 35% protein (Page and Andrews, 1973; Wilson, 1991). The protein requirement for tilapia fry is about 50% of the diet, which decreases to about 35% as fish increase to 30 g in weight. Larger fish require only 25 to 35% dietary protein, depending on the rearing conditions (Lim, 1989).

3.2.2.2. Water Temperature

Changes in water temperature have been reported to alter the protein requirement of some fish, whereas they do not appear to affect other fish. For example, chinook salmon were found to require 40% protein at 8°C and 55% protein at 15°C (DeLong *et al.,* 1958). Similarly, striped bass were found to require 47% protein at 20°C and about 55% protein at 24°C (Millikin, 1982, 1983). However, when rainbow trout were fed practical diets containing 35, 40, and 45% crude protein at temperatures ranging from 9 to 18°C, no differences in protein requirement could be ascertained [National Research Council (NRC), 1981]. In general, the growth rate and feed intake increase as the water temperature increases, thus it is generally felt that a change in water temperature affects feed intake much more than the protein requirement.

3.2.3. Maintenance Requirements

The maintenance protein requirement of an animal is defined as the protein intake required to maintain nitrogen equilibrium. An animal is in nitrogen equilibrium when nitrogen intake is equal to nitrogen excretion and no change in body weight occurs. Animal cells are characterized by being in a dynamic steady state, in that their various components are constantly undergoing degradation and resynthesis. Therefore, sufficient amino acids must be supplied to maintain the body composition. Amino acids are withdrawn from body pools for synthesis of proteins, nucleic acids, and lesser components of cells and are removed by degradation through oxidative pathways. The replacement of this supply of amino acids therefore represents the absolute minimum requirement for amino acids in the diet or the maintenance protein requirement.

3.2.3.1. Methodology

Two type of methods can be used to estimate or determine the protein requirement for maintenance. The first or direct method involves measuring

the endogenous nitrogen excretion as the combined fecal, urinary, and branchial losses. The fish are either maintained without food, fed a protein-free diet, or fed a low-protein diet. The protein requirement for maintenance is then calculated based on the endogenous nitrogen excretion data by taking into account the digestibility and biological value of the test protein. The second or indirect method is much simpler and the most convenient method to use for fish. In this case, nitrogen retention can be measured by the difference between nitrogen consumed and nitrogen retained by the fish at the end of the experimental period. These data can also be combined with growth data obtained by feeding an increasing ration size and obtaining the nitrogen or protein intake which results in zero growth (Luquet and Kaushik, 1981).

3.2.3.2. Estimated Maintenance Requirements

Only a limited number of studies have been reported on the maintenance requirements of protein in fish. Ogino and Chen (1973) obtained a maintenance requirement of 0.95 g protein/kg body weight/day for common carp fed casein as the sole source of protein. Gatlin *et al.* (1986) reported the maintenance requirement for channel catfish to be 1.3 g protein/kg body weight/day based on growth rates of fish fed increasing rations from 0 to 5% of the body weight/day of diets containing either 25 or 35% crude protein from a casein–gelatin mixture. The requirement value was about 1.0 g protein/kg body weight/day based on protein retention data for the above growth studies. Somewhat higher values, 1.5 to 2.5 and 2.6 g digestible protein/kg body weight/day, have been reported for red drum (McGoogan and Gatlin, 1998) and rainbow trout (Kaushik and Gomes, 1988), respectively.

3.3 Qualitative Amino Acid Requirements

The first successful amino acid test diet for fish was developed by Halver (1957). He developed his initial test diet based on previous amino acid test diets used in determining the amino acid requirements of young albino rats. Halver (1957) compared test diets containing 70% crystalline L-amino acids formulated based on the amino acid patterns of whole chicken egg protein, chinook salmon egg protein, and chinook yolk sac fry protein. The test diet based on whole chicken egg protein gave the best growth and feed efficiency for chinook salmon for a 12-week period. Therefore, this test diet was used to determine the qualitative amino acid needs of chinook salmon (Halver *et al.*, 1957). These workers determined the essentiality of the

18 common protein amino acids by comparing the relative growth rates of chinook salmon fed the basal and the specific amino acid-deficient diets over a 10-week period. The results indicated that the following 10 amino acids were indispensable for chinook salmon: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. All other species that have been studied to date have been shown to require the same 10 amino acids.

3.4 Quantitative Amino Acid Requirements

3.4.1. Methodology

3.4.1.1. Amino Acid Test Diets

Most investigators have used the method developed by Halver and co-workers (Mertz, 1972) to determine the quantitative amino acid requirements of fish. This procedure involves feeding graded levels of one amino acid at a time in a test diet containing either all crystalline amino acids or a mixture of casein, gelatin, and amino acids formulated so that the amino acid profile is identical to that of whole chicken egg protein except for the amino acid being tested. This procedure has been used successfully with several species, however, the amino acid test diets must be neutralized with sodium hydroxide for utilization by carp (Nose *et al.,* 1974) and channel catfish (Wilson *et al.,* 1977).

Other investigators have used semipurified and practical diets supplemented with crystalline amino acids to estimate the amino acid requirements of certain fish. The semipurified diets have usually included an imbalanced protein as the major source of the dietary amino acids, e.g., zein (Kaushik, 1979) or corn gluten (Halver *et al.,* 1958; Ketola, 1983), which are deficient in certain amino acids. Practical-type diets utilize normal feed ingredients to furnish the bulk of the amino acids. These may be formulated with a fixed amount of intact protein, and the remaining protein equivalent is made up of crystalline amino acids (Luquet and Sabaut, 1974; Jackson and Capper, 1982; Walton *et al.,* 1984a). The various problems inherent in using these types of diets to assess the amino acid requirements of fish have been discussed elsewhere (Wilson, 1985).

3.4.1.2. Growth Studies

Most of the amino acid requirement values have been estimated based on the conventional growth response curve or Almquist plot. Replicate groups of fish are fed diets containing graded levels of the test amino acid until measurable differences appear in the weight gain of the test fish. A linear

increase in weight gain is normally observed with increasing amino acid intake up to a break point corresponding to the requirement of the specific amino acid, at which the weight gain levels off or plateaus.

Various methods have been used to estimate or calculate the break point corresponding to the requirement value based on the weight gain data. The requirement values for chinook salmon (reviewed by Mertz, 1972), common carp, and Japanese eel (Nose, 1979) were estimated using an Almquist plot without the aid of any statistical analysis, whereas others have used regression analysis to generate the Almquist plot (Harding *et al.,* 1977; Akiyama *et al.,* 1985a). Wilson *et al.* (1980) used the continuous broken-line model developed by Robbins *et al.* (1979) to estimate the requirement values. Santiago and Lovell (1988) used both the broken-line model and quadratic regression analysis to estimate the requirement values for Nile tilapia based on weight gain data. Quadratic regression analysis resulted in the lowest error term for estimating the requirement values, whereas the broken-line model yielded the lowest error term for only three requirement values. Most of the requirement values that have been reported within the last 10 years have been estimated based on the broken-line model.

3.4.1.3. Serum or Tissue Amino Acid Studies

Some investigators have found a high correlation of either serum or blood and muscle free amino acid levels with dietary amino acid intake in fish. The hypothesis is that the serum or tissue content of the amino acid should remain low until the requirement for the amino acid is met and then increase to high levels when excessive amounts of the amino acid are fed. This technique has proven useful in confirming the amino acid requirements in only a few cases. For example, of the 10 indispensable amino acid requirement studies in the channel catfish, only the serum lysine (Wilson *et al.,* 1977), threonine (Wilson *et al.,* 1978), histidine (Wilson *et al.,* 1980), and methionine (Harding *et al.,* 1977) data were useful in confirming the requirement values estimated based on weight gain data. Serum methionine data on sea bass (Thebault *et al.,* 1985) and serum lysine of hybrid striped bass (Griffin *et al.,* 1992) have been used to confirm the requirement values for these species. Blood and muscle arginine concentrations were found to increase gradually in rainbow trout fed increasing levels of arginine and were not useful for assessing the arginine requirement of this species (Kaushik, 1979). Walton *et al.* (1984b) were unable to use blood tryptophan levels to confirm the tryptophan requirement of rainbow trout. Of the 10 amino acids required by Nile tilapia, Santiago and Lovell (1988) were able to use only the muscle free lysine, threonine, and isoleucine concentrations to confirm the requirement values for these amino acids based on growth studies.

3.4.1.4. Amino Acid Oxidation Studies

This technique is based on the general hypothesis that when an amino acid is limiting or deficient in the diet, the major portion will be utilized for protein synthesis, and little well be oxidized to carbon dioxide, whereas when the quantity of an amino acid is supplied in excess, and is thus not a limiting factor for protein synthesis, more of the amino acid will be oxidized. The intake level which produces a marked increase in amino acid oxidation should then be a direct indicator of the requirement value for that specific amino acid.

This technique has been evaluated in rainbow trout with only limited success. Walton *et al.* (1984a) were successful in using this technique to confirm the lysine requirement of rainbow trout based on weight gain data. Following the growth study, three fish from each dietary treatment were injected intraperitoneally with a tracer dose of [U-^{14}C]lysine and the respired carbon dioxide was collected over a 20-hr period. The level of [^{14}C]carbon dioxide produced was used as a direct measurement of the rate of oxidation of lysine in the fish. The level of oxidation observed was very low in fish fed low dietary levels of lysine, somewhat higher in fish fed intermediate dietary levels, and much higher in fish fed higher levels of dietary lysine. The breakpoint of the dose–response curve indicated a dietary requirement of 20 g lysine/kg diet, which was in close agreement with the value of 19 g lysine/kg diet obtained from growth data. Similarly, Anderson *et al.* (1993) were able to use the lysine oxidation approach to confirm the requirement based on growth data in Atlantic salmon. In a study involving tryptophan, Walton *et al.* (1984b) found that the requirement value based on oxidation data was lower, 2.0 versus 2.5 g/kg diet, than the value based on weight gain data. These workers concluded that the oxidation technique is not suitable for use in the absence of growth data because of its lack of precision in determining requirement values from graphical plots.

Kim *et al.* (1992c) were unsuccessful in using phenylalanine oxidation rates to evaluate the phenylalanine requirement of rainbow trout. In their study, fish were fed diets containing varying levels of phenylalanine plus L-[1-^{14}C]phenylalanine for 10 to 20 days. The expired $^{14}CO_2$ increased gradually with increasing levels of phenylalanine in the diet, without any apparent break point. These workers concluded that this technique is probably not appropriate for determining amino acid requirements in fish.

3.4.2. Arginine Requirements

The arginine requirement values for fish are summarized in Table 3.4. Salmon have the highest requirement, about 6% of dietary protein, whereas

Table 3.4

Arginine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	4.1	Growth studies	Lall *et al.* (1994)
	5.0–5.1	Growth studies	Berge *et al.* (1997)
	4.6	Ideal protein	Rollin (1999)
Catla	4.8	Growth studies	Ravi and Devaraj (1991)
Channel catfish	4.3	Growth studies	Robinson *et al.* (1981)
Chinook salmon	6.0	Growth studies	Klein and Halver (1970)
Chum salmon	6.5	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	3.6	Ideal protein	Unprasert (1994)
Coho salmon	5.8	Growth studies	Klein and Halver (1970)
	3.2	Growth studies	Arai and Ogata (1993)
	4.9–5.5	Growth studies	Luzzana *et al.* (1998)
Common carp	4.3	Growth studies	Nose (1979)
	3.8	Protein accretion	Ogino (1980)
European sea bass	4.6	A/E ratios[b]	Kaushik (1998)
Gilthead bream	5.0	Growth studies	Luquet and Sabaut (1974)
	5.4	A/E ratios	Kaushik (1998)
Hybrid striped bass	4.4	Growth studies	Griffin *et al.* (1994a)
Japanese eel	4.5	Growth studies	Arai (Nose, 1979)
Japanese flounder	3.4	A/E ratios	Forster and Ogata (1998)
Milkfish	5.3	Growth studies	Borlongan (1991)
Mozambique tilapia	4.0	Growth studies	Jackson and Capper (1982)
Nile tilapia	4.2	Growth studies	Santiago and Lovell (1988)
Rainbow trout	3.3	Growth studies	Kaushik (1979)
	3.5	Protein accretion	Ogino (1980)
	3.5	Growth studies	Rodehutscord *et al.* (1995)
	3.5–4.2	Growth studies	Chiu *et al.* (1988)
	3.8	Growth studies	Forster (1993)
	3.6–4.0	Growth studies	Walton *et al.* (1986)
	4.0	Growth studies	Kim *et al.* (1992b)
	4.1	Growth studies	Pack *et al.* (1995)
	4.7	Growth studies	Cho *et al.* (1992)
	5.4–5.9	Growth studies	Ketola (1983)
Red drum	3.7	A/E ratios	Moon and Gatlin (1991)
Red sea bream	3.5	A/E ratios	Forster and Ogata (1998)
Rohu	2.9	Growth studies	Khan and Jafri (1993)
Turbot	4.8	A/E ratios	Kaushik (1998)
Wels	3.4	Growth studies	Toth (1986)
White sturgeon	4.8	Protein accretion	Ng and Hung (1995)
Yellow perch	4.3	Growth studies	Twibell and Brown (1997)

[a] Requirements are expressed as percentage of protein.

[b] (Indispensable amino acid content/total indispensable amino acid content including cysteine and tyrosine) × 1000.

the other species require about 4 to 5% of protein. The requirement value of about 4% of protein for rainbow trout appears to be the most reasonable, however, values ranging from 3.3 to 5.9% have been reported. The estimated requirement values appear to be a little lower than those obtained by growth studies.

3.4.3. Histidine Requirements

The histidine requirements of fish are presented in Table 3.5. Excellent agreement has been found among the species studied, with a range of 1.5 to 2.5% of protein for the requirement values. Wilson *et al.* (1980) were able to confirm the requirement value by the serum free histidine pattern in channel catfish. There was a significant increase in the serum free histidine concentration up to the dietary requirement as determined based on growth data and then the serum histidine remained constant at a higher dietary intake.

Table 3.5

Histidine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	1.8	Ideal protein	Rollin (1999)
Catla	2.5	Growth studies	Ravi and Devaraj (1991)
Channel catfish	1.5	Growth studies	Wilson *et al.* (1980)
Chinook salmon	1.8	Growth studies	Klein and Halver (1970)
Chum salmon	1.6	Growth studies	Akiyama *et al.* (1985a)
	1.6	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	1.2	Ideal protein	Unprasert (1994)
Coho salmon	1.8	Growth studies	Klein and Halver (1970)
	0.9	Growth studies	Arai and Ogata (1993)
Common carp	2.1	Growth studies	Nose (1979)
	1.4	Protein accretion	Ogino (1980)
European sea bass	1.6	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	1.7	A/E ratios	Kaushik (1998)
Japanese eel	2.1	Growth studies	Arai (Nose, 1979)
Japanese flounder	1.3	A/E ratios	Forster and Ogata (1998)
Milkfish	2.0	Growth studies	Borlongan and Coloso (1993)
Nile tilapia	1.7	Growth studies	Santiago and Lovell (1988)
Rainbow trout	1.6	Protein accretion	Ogino (1980)
Red drum	1.7	A/E ratios	Moon and Gatlin (1991)
Red sea bream	1.4	A/E ratios	Forster and Ogata (1998)
Turbot	1.5	A/E ratios	Kaushik (1998)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.

3.4.4. Branched-Chain Amino Acid Requirements

3.4.4.1. Isoleucine Requirements

The isoleucine requirements of fish are presented in Table 3.6. The requirement appears to be about 2.2 to 3% of protein for those species studied except for the Japanese eel and milkfish, which have higher requirement values.

Wilson *et al.* (1980) determined the effects of dietary isoleucine on serum free isoleucine, leucine, and valine in channel catfish. Even though the serum isoleucine increased somewhat with increasing isoleucine intake, these data did not confirm the requirement as determined based on growth data. The serum free leucine and valine concentrations appeared to parallel the serum free isoleucine concentrations. A relative high mortality rate was observed in fish fed the isoleucine-deficient diet.

Table 3.6

Isoleucine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	3.2	Ideal protein	Rollin (1999)
Catla	2.4	Growth studies	Ravi and Devaraj (1991)
Channel catfish	2.6	Growth studies	Wilson *et al.* (1980)
Chinook salmon	2.2	Growth studies	Chance *et al.* (1964)
Chum salmon	2.4	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	2.0	Ideal protein	Unprasert (1994)
Coho salmon	1.2	Growth studies	Arai and Ogata (1993)
Common carp	2.5	Growth studies	Nose (1979)
	2.3	Protein accretion	Ogino (1980)
European sea bass	2.6	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	2.6	A/E ratios	Kaushik (1998)
Japanese eel	4.0	Growth studies	Arai (Nose, 1979)
Japanese flounder	2.0	A/E ratios	Forster and Ogata (1998)
Lake trout	2.0–2.6[c]	Growth studies	Hughes *et al.* (1983)
Milkfish	4.0	Growth studies	Borlongan and Coloso (1993)
Nile tilapia	3.1	Growth studies	Santiago and Lovell (1988)
Rainbow trout	2.4	Protein accretion	Ogino (1980)
Red drum	2.9	A/E ratios	Moon and Gatlin (1991)
Red sea bream	2.2	A/E ratios	Forster and Ogata (1998)
Turbot	2.6	A/E ratios	Kaushik (1998)
White sturgeon	3.0	A/E ratios	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.
[c] These values were recalculated based on the calculated nitrogen content of the test diets.

3.4.4.2. Leucine Requirements

The leucine requirement values are presented in Table 3.7. The requirements values agree quite well, ranging from 3.3 to 3.9% of protein, except for the higher values of a little more than 5% of protein reported for the Japanese eel and milkfish. In general, the estimated values based on A/E ratios [(indispensable amino acid content/total indispensable amino acid content including cysteine and tyrosine) × 1000] and protein accretion are somewhat higher than those found based on growth studies.

Wilson *et al.* (1980) reported that the serum free leucine level in channel catfish remained constant regardless of the dietary leucine intake. There was, however, a marked effect of dietary leucine on the serum free isoleucine and valine levels. There was about a sixfold increase in serum free isoleucine and valine concentrations at the 0.7% dietary leucine level compared

Table 3.7

Leucine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	5.2	Ideal protein	Rollin (1999)
Catla	3.7	Growth studies	Ravi and Devaraj (1991)
Channel catfish	3.5	Growth studies	Wilson *et al.* (1980)
Chinook salmon	3.9	Growth studies	Chance *et al.* (1964)
Chum salmon	3.8	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	3.5	Growth studies	Unprasert (1994)
Coho salmon	3.4	Growth studies	Arai and Ogata (1993)
Common carp	3.3	Growth studies	Nose (1979)
	4.1	Protein accretion	Ogino (1980)
European sea bass	4.3	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	4.5	A/E ratios	Kaushik (1998)
Japanese eel	5.3	Growth studies	Arai (Nose, 1979)
Japanese flounder	3.9	A/E ratios	Forster and Ogata (1998)
Lake trout	3.5–4.6[c]	Growth studies	Hughes *et al.* (1983)
Milkfish	5.1	Growth studies	Borlongan and Coloso (1993)
Nile tilapia	2.8–3.6	Growth studies	Santiago and Lovell (1988)
Rainbow trout	4.4	Protein accretion	Ogino (1980)
Red drum	4.7	A/E ratios	Moon and Gatlin (1991)
Red sea bream	4.2	A/E ratios	Forster and Ogata (1998)
Turbot	4.6	A/E ratios	Kaushik (1998)
White sturgeon	4.3	Protein accretion	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.
[c] These values were recalculated based on the calculated nitrogen content of the test diets.

to the 0.6% leucine level. These elevated levels of isoleucine and valine did not return to the baseline levels until a dietary level of 1.2% or above was fed. This observation was interpreted to indicate that leucine may facilitate the tissue uptake of branched-chain amino acids and/or their intracellular metabolism.

3.4.4.3. Valine Requirements

The valine requirement values of fish are presented in Table 3.8. Reasonable agreement exists among the values reported for the species studied, indicating that the requirement ranges from about 2.5 to 4% of protein. Studies on the effect of valine intake on serum valine levels in channel catfish showed that they responded in a manner similar to that described for isoleucine (Wilson *et al.*, 1980).

Table 3.8

Valine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	3.9	Ideal protein	Rollin (1999)
Catla	3.6	Growth studies	Ravi and Devaraj (1991)
Channel catfish	3.0	Growth studies	Wilson *et al.* (1980)
Chinook salmon	3.2	Growth studies	Chance *et al.* (1964)
Chum salmon	3.0	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	2.4	Ideal protein	Unprasert (1994)
Coho salmon	2.2	Growth studies	Arai and Ogata (1993)
Common carp	3.6	Growth studies	Nose (1979)
	2.9	Protein accretion	Ogino (1980)
European sea bass	2.9	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	3.0	A/E ratios	Kaushik (1998)
Japanese eel	4.0	Growth studies	Arai (Nose, 1979)
Japanese flounder	2.5	A/E ratios	Forster and Ogata (1998)
Lake trout	2.6–3.3[c]	Growth studies	Hughes *et al.* (1983)
Milkfish	3.6	Growth studies	Borlongan and Coloso (1993)
Nile tilapia	2.8	Growth studies	Santiago and Lovell (1988)
Rainbow trout	3.1	Protein accretion	Ogino (1980)
Red drum	3.1	A/E ratios	Moon and Gatlin (1991)
Red sea bream	2.5	A/E ratios	Forster and Ogata (1998)
Turbot	2.9	A/E ratios	Kaushik (1998)
White sturgeon	3.3	Protein accretion	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.
[c] These values were recalculated based on the calculated nitrogen content of the test diets.

3.4.4.4. Interactions

Some differences appear to exist in the apparent isoleucine–leucine–valine interactions among different fish. Chance *et al.* (1964) found that the isoleucine requirement in chinook salmon increased slightly with increasing levels of dietary leucine. This effect was not observed in either the common carp (Nose, 1979) or the channel catfish (Robinson *et al.,* 1984). Nose (1979) did, however, observe reduced growth rates in carp fed high dietary isoleucine levels during a leucine requirement study. This reduced growth rate was not observed when the leucine requirement study was repeated at a lower isoleucine level. Robinson *et al.* (1984) concluded that a nutritional interrelationship does exist among the branched-chain amino acids in the channel catfish but the interaction does not appear to be as severe as has been observed in certain other animals.

3.4.5. Lysine Requirements

The lysine requirement values for fish are summarized in Table 3.9. In general, lysine appears to be the first limiting amino acid in feedstuffs commonly used in formulating feeds for warmwater fish (Robinson *et al.,* 1980b) and perhaps other fish as well. Therefore, more requirement values have been reported for this amino acid. The requirement appears to range from 4 to 5% of protein for most fish. The values of 5.7 for common carp and 6.2 for catla, an Indian major carp, may indicate that carps have a higher lysine requirement than other fish. The value of 6.1 for rainbow trout appears to be out of line, as two other investigators have reported much lower values. The estimated values are very similar to those obtained by growth studies.

Serum free lysine levels were useful in confirming the lysine requirement in channel catfish originally determined at 24% crude protein (Wilson *et al.,* 1977); however, serum free lysine levels provided little indication of the lysine requirement when reevaluated at a 30% crude protein level (Robinson *et al.,* 1980b) Walton *et al.* (1984a) observed a good agreement between the lysine requirement values determined by growth studies and those determined by amino acid oxidation studies in rainbow trout.

3.4.5.1. Arginine – Lysine Interactions

A dietary lysine–arginine antagonism has been well documented in certain animals. Based on growth studies, this antagonism has not been demonstrated in channel catfish (Robinson *et al.,* 1981), blue tilapia (Liou, 1989), rainbow trout (Kim *et al.,* 1992b), hybrid striped bass (Griffin *et al.,* 1994a), or yellow perch (Twibell and Brown, 1997). Kaushik and Fauconneau (1984) have presented some biochemical evidence indicating that some metabolic

Table 3.9

Lysine Requirements

Fish	Requirement[a]	Based on	Reference
African catfish	5.7	Growth studies	Fagbenro *et al.* (1998b)
Atlantic salmon	4.0	Growth studies	Anderson *et al.* (1993)
	3.2–3.6	Growth studies	Berge *et al.* (1998)
	6.1	Growth studies	Rollin (1999)
Blue tilapia	4.3	Growth studies	Liou (1989)
Catla	6.2	Growth studies	Ravi and Devaraj (1991)
Channel catfish	5.1	Growth studies	Wilson *et al.* (1977)
	5.0	Growth studies	Robinson *et al.* (1980b)
Chinook salmon	5.0	Growth studies	Halver *et al.* (1958)
Chum salmon	4.8	Growth studies	Akiyama *et al.* (1985a)
	5.0	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	4.8	Growth studies	Unprasert (1994)
Coho salmon	3.8	Growth studies	Arai and Ogata (1993)
Common carp	5.7	Growth studies	Nose (1979)
	5.3	Protein accretion	Ogino (1980)
European sea bass	4.8	Growth studies	Tibaldi and Lanari (1991)
Gilthead sea bream	5.0	Growth studies	Luquet and Sabaut (1974)
Hybrid striped bass	4.0	Growth studies	Griffin *et al.* (1992)
	4.0	Growth studies	Keembiyehetty and Gatlin (1992)
Japanese eel	5.3	Growth studies	Arai (Nose, 1979)
Japanese flounder	4.6	Growth studies	Forster and Ogata (1998)
Milkfish	4.0	Growth studies	Borlongan and Benitez (1990)
Mozambique tilapia	4.1	Growth studies	Jackson and Capper (1982)
Nile tilapia	5.1	Growth studies	Santiago and Lovell (1988)
Rainbow trout	3.7	Growth studies	Kim *et al.* (1992b)
	4.2	Growth studies	Walton *et al.* (1984a)
	4.2	Growth studies	Pfeffer *et al.* (1992)
	5.3	Protein accretion	Ogino (1980)
	6.1	Growth studies	Ketola (1983)
Red drum	4.4	Growth studies	Craig and Gatlin (1992)
	5.7	Growth studies	Brown *et al.* (1988)
	5.7	A/E ratios[b]	Moon and Gatlin (1991)
Red sea bream	4.4	Growth studies	Forster and Ogata (1998)
Rohu	5.9	Growth studies	Khan and Jafri (1993)
	5.7	Growth studies	Murthy and Varghese (1997)
Turbot	5.0	A/E ratios	Kaushik (1998)
White sturgeon	5.4	Protein accretion	Ng and Hung (1995)
Yellowtail	4.1	Growth studies	Ruchimat *et al.* (1997b)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.

antagonism may exist between lysine and arginine in rainbow trout. These workers found that an increasing dietary lysine intake affected plasma arginine and urea levels and ammonia excretion. These changes were found to be due to a decrease in the relative rate of arginine degradation as the level of dietary lysine increased. Similarly, some evidence has been presented which indicates that some metabolic interactions occur between arginine and lysine when elevated levels of either are fed to Atlantic salmon (Berge *et al.,* 1997, 1998).

3.4.6. Sulfur Amino Acid Requirements

Methionine and cysteine are classified as sulfur-containing amino acids. Adequate amounts of both methionine and cysteine are needed for proper protein synthesis and other physiological functions of the fish. Cysteine is considered dispensable because it can be synthesized by the fish from the indispensable amino acid methionine. When methionine is fed without cysteine, a portion of the methionine is used for protein synthesis, and a portion is converted to cysteine for incorporation into protein. If cysteine is included in the diet, it reduces the amount of dietary methionine needed. Thus, to determine the total sulfur amino acid requirement (methionine plus cysteine), the dietary requirement for methionine is determined either in the absence of cysteine or with test diets containing very low levels of cysteine.

3.4.6.1. Methionine Requirements

The methionine or total sulfur amino acid requirement values are presented in Table 3.10. It appears that most fish have a requirement value of about 2 to 3% of protein, whereas catla, chinook salmon, and gilthead sea bream appear to require higher levels of methionine.

Rainbow trout appear to be unique in that methionine deficiency results in bilateral cataracts (Poston *et al.,* 1977). These workers observed cataracts in rainbow trout fed diets containing isolated soybean protein. The cataracts were prevented by supplementing the diet with methionine. Cataracts have also been observed in methionine-deficient rainbow trout by Walton *et al.* (1982), Rumsey *et al.* (1983), and Cowey *et al.* (1992). This deficiency sign has been reported in Arctic charr and actually used as the basis for establishing the methionine requirement in this species (Simmons *et al.,* 1999). Sulfur amino acid-deficient hybrid striped bass did not develop cataracts, however, when they were fed diets containing their requirement at a methionine-to-cysteine ratio of 40:60, they developed bilateral cataracts within 3 weeks and experienced mass mortality after 4 weeks (Keembiyehetty and Gatlin, 1993).

Table 3.10

Methionine or Total Sulfur Amino Acid Requirement

Fish	Requirement[a]	Based on	Reference
African catfish	3.2 (0)[b]	Growth studies	Fagbenro *et al.* (1998a)
Arctic charr	1.8 (0)	Growth studies	Simmons *et al.* (1999)
	2.7 (0)	Lens pathology	Simmons *et al.* (1999)
Asian sea bass	2.9 (0)	Growth studies	Coloso *et al.* (1999)
Atlantic salmon	3.1	Ideal protein	Rollin (1999)
Blue tilapia	2.8 (0)	Growth studies	Liou (1989)
Catla	3.6 (0)	Growth studies	Ravi and Devaraj (1991)
Channel catfish	2.3 (0)	Growth studies	Harding *et al.* (1977)
Chinook salmon	4.0 (1)	Growth studies	Halver *et al.* (1959)
Chum salmon	3.0 (1.2)	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	2.4 (0)	Growth studies	Unprasert (1994)
Coho salmon	2.7 (0)	Growth studies	Arai and Ogata (1993)
Common carp	3.1 (0)	Growth studies	Nose (1979)
	2.1 (0)	Growth studies	Schwarz *et al.* (1998)
	1.6 (0.8)	Protein accretion	Ogino (1980)
European sea bass	2.0 (1)	Growth studies	Thebault *et al.* (1985)
	2.3	A/E ratios[c]	Kaushik (1998)
Gilthead sea bream	4.0	Growth studies	Luquet and Sabaut (1974)
	2.4	A/E ratios	Kaushik (1998)
Hybrid striped bass	2.9 (0.13)	Growth studies	Keembiyehetty and Gatlin (1993)
	2.1 (0)	Growth studies	Griffin *et al.* (1994b)
Japanese eel	3.2 (0)	Growth studies	Arai (Nose, 1979)
Japanese flounder	1.9	A/E ratios	Forster and Ogata (1998)
Milkfish	2.5 (0.8)	Growth studies	Borlongan and Coloso (1993)
Mozambique tilapia	3.2 (0.7)	Growth studies	Jackson and Capper (1982)
Nile tilapia	3.2 (0.5)	Growth studies	Santiago and Lovell (1988)
Rainbow trout	2.2 (0)	Growth studies	Walton *et al.* (1982)
	2.3 (0)	Growth studies	Kim *et al.* (1992a)
	1.9 (0.2)	Growth studies	Cowey *et al.* (1992)
	2.4 (0.2)	Lens pathology	Cowey *et al.* (1992)
	3.0 (0.3)	Growth studies	Rumsey *et al.* (1983)
	1.8 (0.9)	Protein accretion	Ogino (1980)
Red drum	3.0 (0.12)	Growth studies	Moon and Gatlin (1991)
Red sea bream	2.2	A/E ratios	Forster and Ogata (1998)
Rohu	2.6 (1)	Growth studies	Khan and Jafri (1993)
Turbot	2.7	A/E ratios	Kaushik (1998)
White sturgeon	2.2	Protein accretion	Ng and Hung (1995)
Yellowtail	2.6 (0.3)	Growth studies	Ruchimat *et al.* (1997a)

[a] Requirements are expressed as percentage of protein.
[b] Cysteine content in parentheses.
[c] See Table 3.4, footnote *b*.

Requirement values have been reported based on maximum weight gain and the absence of lens pathology in Arctic charr and rainbow trout (Table 3.10). In both cases, the higher amount needed to prevent the lens pathology still fell within the requirement range of 2 to 3% of protein reported for other species.

3.4.6.2. Cysteine Replacement Values

As indicated above, dietary cysteine can reduce the amount of dietary methionine required for maximum growth. The cysteine replacement value for methionine on a sulfur basis has been determined to be about 60% for channel catfish (Harding *et al.,* 1977), 44% for blue tilapia (Liou, 1989), 42% for rainbow trout (Kim *et al.,* 1992a), and 40% for red drum (Moon and Gatlin, 1991) and hybrid striped bass (Griffin *et al.,* 1994b).

3.4.6.3. Utilization of Other Sulfur Sources

Robinson *et al.* (1978) evaluated the utilization of several sulfur compounds for their potential replacement value for methionine in channel catfish. Growth and feed efficiency data indicated that DL-methionine was utilized as effectively as L-methionine. Methionine hydroxy analog was only about 26% as effective in promoting growth as L-methionine. No significant growth response was observed when taurine or inorganic sulfate was added to the basal diet. Page *et al.* (1978) were also unable to detect the utilization of taurine and inorganic sulfate as sulfur sources in rainbow trout. D-Methionine has been shown to replace L-methionine on an equal basis in rainbow trout (Kim *et al.,* 1992a). L-Methionine, DL-methionine, and *N*-acetyl-DL-methionine were equally utilized by hybrid striped bass, whereas glutathione and DL-methionine hydroxy analog were only 75% as effective as L-methionine and taurine was totally ineffective (Keembiyehetty and Gatlin, 1995).

3.4.7. Aromatic Amino Acid Requirements

3.4.7.1. Phenylalanine Requirements

A relationship similar to that presented for methionine and cysteine exists for phenylalanine and tyrosine, two important aromatic amino acids. Tyrosine is considered dispensable because it can be synthesized by the fish from the indispensable amino acid phenylalanine. If tyrosine is included in the diet, it reduces the amount of phenylalanine needed in the diet. Thus, fish have a total aromatic amino acid requirement.

The phenylalanine or total aromatic amino acid requirement values for fish are presented in Table 3.11. Most requirement values fall within the

Table 3.11

Phenylalanine or Total Aromatic Amino Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	5.8	Ideal protein	Rollin (1999)
Catla	6.2	Growth studies	Ravi and Devaraj (1991)
Channel catfish	5.0	Growth studies	Robinson *et al.* (1980a)
Chinook salmon	5.1	Growth studies	Chance *et al.* (1964)
Chum salmon	6.3	Growth studies	Akiyama and Arai (1993)
Clarias hybrid	4.0	Ideal protein	Unprasert (1994)
Coho salmon	4.5	Growth studies	Arai and Ogata (1993)
Common carp	6.5	Growth studies	Nose (1979)
	4.9	Protein accretion	Ogino (1980)
European sea bass	2.6	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	2.9	A/E ratios	Kaushik (1998)
Japanese eel	5.8	Growth studies	Arai (Nose, 1979)
Japanese flounder	3.8	A/E ratios	Forster and Ogata (1998)
Milkfish	5.2	Growth studies	Borlongan (1992)
Nile tilapia	5.5	Growth studies	Santiago and Lovell (1988)
Rainbow trout	4.3	Growth studies	Kim (1993)
	5.2	Protein accretion	Ogino (1980)
Red drum	4.5	A/E ratios	Moon and Gatlin (1991)
Red sea bream	4.1	A/E ratios	Forster and Ogata (1998)
Turbot	5.3	A/E ratios	Kaushik (1998)
White sturgeon	5.3	Protein accretion	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.

range of 5 to 6% of protein, except for the lower value for rainbow trout and the higher value for common carp. The estimated requirement values are also somewhat lower than those based on growth studies.

3.4.7.2. Tyrosine Replacement Values

Since the fish has a metabolic need for both phenylalanine and tyrosine, and only a certain portion of the phenylalanine can be converted to tyrosine and still meet the animal's need for phenylalanine, it is important to determine how much of the total aromatic amino acid requirement can be provided by dietary tyrosine. Growth studies indicate that tyrosine can replace or spare about 60% of the phenylalanine requirement in common carp (Nose, 1979), 50% in channel catfish (Robinson *et al.*, 1980a), 48% in rainbow trout (Kim, 1993), and 46% in milkfish (Borlongan and Coloso, 1993).

3.4.8. Threonine Requirements

The threonine requirement values for fish are summarized in Table 3.12. The reported values range from 2 to 5% of protein. It is difficult to offer any explanation for the lack of agreement in these requirement values. Additional research is needed to determine if this wide range of values represents a true difference in the threonine requirement or just a difference in the techniques used to determine the requirement values.

DeLong *et al.* (1962) found the threonine requirement of chinook salmon to be the same when determined at rearing temperatures of 8 and 15°C. These findings were not expected, as these workers had previously reported

Table 3.12

Threonine Requirements

Fish	Requirement[a]	Based on	Reference
Atlantic salmon	3.2	Ideal protein	Rollin (1999)
Catla	5.0	Growth studies	Ravi and Devaraj (1991)
Channel catfish	2.0	Growth studies	Wilson *et al.* (1978)
Chinook salmon	2.2	Growth studies	DeLong *et al.* (1962)
Chum salmon	3.0	Growth studies	Akiyama *et al.* (1985a)
Clarias hybrid	2.8	Ideal protein	Unprasert (1994)
Coho salmon	2.0	Growth studies	Arai and Ogata (1993)
Common carp	3.9	Growth studies	Nose (1979)
	3.3	Protein accretion	Ogino (1980)
European sea bass	2.6–3.0	Growth studies	Tibaldi and Tulli (1999)
	2.7	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	2.8	A/E ratios	Kaushik (1998)
Hybrid striped bass	2.6	Growth studies	Keembiyehetty and Gatlin (1997)
Japanese eel	4.0	Growth studies	Arai (Nose, 1979)
Japanese flounder	2.3	A/E ratios	Forster and Ogata (1998)
Milkfish	4.5	Growth studies	Borlongan (1991)
Nile tilapia	3.8	Growth studies	Santiago and Lovell (1988)
Rainbow trout	3.4	Protein accretion	Ogino (1980)
	3.2–3.7	Growth studies	Rodehutscord *et al.* (1995)
Red drum	2.8	A/E ratios	Moon and Gatlin (1991)
	2.3	Growth studies	Boren and Gatlin (1995)
Red sea bream	1.8	A/E ratios	Forster and Ogata (1998)
Rohu	4.3	Growth studies	Murthy and Varghese (1996)
Turbot	2.9	A/E ratios	Kaushik (1998)
White sturgeon	3.3	Protein accretion	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.

the protein requirement of chinook salmon to increase from 40% at 8°C to 55% at 15°C (DeLong *et al.*, 1958).

3.4.9. Tryptophan Requirements

The tryptophan requirement values for fish are presented in Table 3.13. The requirement appears to be about 0.5 to 1% of protein for the various species studied. The high value of 1.4% of protein for rainbow trout may have been overestimated because no dietary levels between 0.25 and 0.50% of diet were fed (Poston and Rumsey, 1983).

Table 3.13

Tryptophan Requirements

Fish	Requirement[a]	Based on	Reference
Catla	1.0	Growth studies	Ravi and Devaraj (1991)
Channel catfish	0.5	Growth studies	Wilson *et al.* (1978)
Chinook salmon	0.5	Growth studies	Halver (1965)
Chum salmon	0.7	Growth studies	Akiyama *et al.* (1985b)
Clarias hybrid	0.6	Ideal protein	Unprasert (1994)
Coho salmon	0.5	Growth studies	Halver (1965)
	0.5	Growth studies	Arai and Ogata (1993)
Common carp	0.8	Growth studies	Nose (1979)
	0.3	Growth studies	Dabrowski (1981)
	0.6	Protein accretion	Ogino (1980)
European sea bass	0.6	A/E ratios[b]	Kaushik (1998)
Gilthead sea bream	0.6	Growth studies	Luquet and Sabaut (1974)
	0.6	A/E ratios	Kaushik (1998)
Japanese eel	1.1	Growth studies	Arai (Nose, 1979)
Japanese flounder	0.5	A/E ratios	Forster and Ogata (1998)
Milkfish	0.6	Growth studies	Coloso *et al.* (1992)
Nile tilapia	1.0	Growth studies	Santiago and Lovell (1988)
Rainbow trout	0.5	Growth studies	Walton *et al.* (1984b)
	0.6	Growth studies	Kim *et al.* (1987)
	1.4	Growth studies	Poston and Rumsey (1983)
	0.5	Protein accretion	Ogino (1980)
Red drum	0.8	A/E ratios	Moon and Gatlin (1991)
Red sea bream	0.6	A/E ratios	Forster and Ogata (1998)
Rohu	0.6	Growth studies	Khan and Jafri (1993)
Sockeye salmon	0.5	Growth studies	Halver (1965)
Turbot	0.6	A/E ratios	Kaushik (1998)
White sturgeon	0.3	Protein accretion	Ng and Hung (1995)

[a] Requirements are expressed as percentage of protein.
[b] See Table 3.4, footnote *b*.

Tryptophan deficiency results in several deficiency signs in salmonids that have not been observed in other fish species. Halver and Shanks (1960) observed scoliosis and lordosis in sockeye salmon but not in chinook salmon fed tryptophan-deficient diets. Scoliosis and lordosis have also been observed in tryptophan-deficient rainbow trout (Shanks *et al.,* 1962; Kloppel and Post, 1975; Poston and Rumsey, 1983; Walton *et al.,* 1984b) and chum salmon (Akiyama *et al.,* 1985b). These deformities were found to be reversible in rainbow trout when the fish were fed adequate dietary tryptophan (Shanks *et al.,* 1962; Kloppel and Post, 1975) and appear to be related to a depletion of 5-hydroxytryptophan in the body or brain (Akiyama *et al.,* 1986). Other tryptophan deficiency signs in rainbow trout include renal calcinosis (Kloppel and Post, 1975), caudal fin erosion, cataracts, and short gill opercula (Poston and Rumsey, 1983), and increased liver and kidney levels of calcium, magnesium, sodium, and potassium (Walton *et al.,* 1984b).

3.4.10. Comparison of Lysine Utilization in Fish and Other Animals

A comparison of the estimated lysine utilization of fish and other animals is presented in Table 3.14. These calculations were based on studies in our laboratory with channel catfish and data summarized in National Research Council (NRC) tables for poultry (NRC, 1977) and swine (NRC, 1988). It seems quite interesting that when one estimates lysine utilization (grams of growth per grams of lysine ingested) for these three species, with a wide range of relative growth rates and dietary lysine intakes, the results are very similar. These data add additional support to the findings of Bowen (1987),

Table 3.14

Estimated Lysine Utilization of Fish and Other Animals

Parameter	Channel catfish	Broiler chicken[a]	Swine[b]
Size	10–40 g	360–725 g	1–5 kg
Growth rate (g/day)	0.5	26	200
Lysine requirement (% of dietary protein)	5.0	5.7	5.8
Lysine utilization (g growth/g lysine ingested)	60.6	48.3	57.1

[a] Calculated from NRC (1977) data.
[b] Calculated from NRC (1988) data.

indicating that protein utilization is very similar among fish and other vertebrates.

3.4.11. Amino Acid Requirements of Penaeid Shrimp

Successful studies have recently been completed determining the amino acid requirements of penaeid shrimp (Table 3.15). Previous attempts to quantify the requirements had resulted in only limited success (Deshimaru and Kuroki, 1974; Akiyama, 1986). This was due primarily to the lack of a water-stable diet that would resist leaching while being slowly consumed by the shrimp. Chen *et al.* (1992) were able to determine the arginine requirement by using a microencapsulated diet. Fox *et al.*(1995) covalently bound increasing levels of supplemental lysine to wheat gluten to determine the lysine requirement in *Penaeus vannamei*. Millamena *et al.* (1996a,b, 1997, 1998, 1999) were able to utilize amino acid test diets containing casein, gelatin, and crystalline amino acids. The crystalline amino acid mixture was initially coated with gelatinized carboxymethyl cellulose and then κ-carrageenan was gelatinized to form a homogeneous gel and added to the complete diet mixture. Feeding of these diets resulted in the typical dose–response curve for each of the indispensable amino acids, however, in most cases, feeding levels above the estimated requirement level resulted in a marked reduction in growth.

Table 3.15

Amino Acid Requirements of Penaeid Shrimp Based on Growth Studies

Amino acid	Requirement[a]	Species	Reference
Arginine	5.3	*Penaeus monodon*	Millamena *et al.* (1998)
	5.5	*Penaeus monodon*	Chen *et al.* (1992)
Histidine	2.2	*Penaeus monodon*	Millamena *et al.* (1999)
Isoleucine	2.7	*Penaeus monodon*	Millamena *et al.* (1999)
Leucine	4.3	*Penaeus monodon*	Millamena *et al.* (1999)
Lysine	5.2	*Penaeus monodon*	Millamena *et al.* (1998)
	3.1	*Penaeus vannamei*	Akiyama (1986)
	4.5–5.2	*Penaeus vannamei*	Fox *et al.* (1995)
Methionine	3.5	*Penaeus monodon*	Millamena *et al.* (1996a)
Phenylalanine plus tyrosine	7.7	*Penaeus monodon*	Millamena *et al.* (1999)
Threonine	3.5	*Penaeus monodon*	Millamena*et al.* (1997)
Tryptophan	0.5	*Penaeus monodon*	Millamena *et al.* (1999)
Valine	3.4	*Penaeus monodon*	Millamena *et al.* (1996b)

[a] Requirements are expressed as percentage of protein.

3.5 Other Methods of Estimating Amino Acid Needs

Various investigators have observed improved growth and feed efficiency when experimental diets for salmonids were supplemented with indispensable amino acids to simulate levels found in isolated fish protein or the respective eggs and whole-body tissue of the species being studied (Rumsey and Ketola, 1975; Arai, 1981; Ketola, 1982; Ogata *et al.,* 1983). The indispensable amino acid requirements of certain fish have also been shown to correlate well with the indispensable amino acid pattern of the whole-body tissue of that fish (Cowey and Tacon, 1983; Wilson and Poe, 1985). Therefore, it seems reasonable to suggest that these types of information may be useful in designing test diets for fish when their amino acid requirements have not been established.

3.5.1. Amino Acid Composition of Whole-Body Fish Tissue and Fish Eggs

The amino acid composition of whole-body tissue of certain fishes is presented in Table 3.16. The amino acid compositions of these five species of fish are surprisingly similar. Similar conclusions have been found by others (Mambrini and Kaushik, 1995), who used factorial correspondence analysis to compare the indispensable amino acid composition data in the literature. They also found that while proteins of different tissues may have different indispensable amino acid patterns; the indispensable amino acid profile of a given tissue seemed to be conserved among species and was unaffected by factors such as temperature, feeding rate, and fish size. Kaushik (1998) has also reported the whole-body amino acid composition of two size classes each of European sea bass, gilthead sea bream, and turbot and found no significant difference in the amino acid composition regardless of size or species.

The amino acid composition of eggs of various fish has been summarized by Ketola (1982). In general, the amino acid composition appears to vary more than the whole-body composition data presented in Table 3.16. Ketola (1982) also points out that although the amino acid content of the fish eggs appears to differ from the reported dietary requirements of the fish, the composition of the eggs has provided useful data in formulating test diets for Atlantic salmon and rainbow trout.

3.5.2. Relationships of Composition Data to Requirement Data

On the basis of observations in other animals, Cowey and Tacon (1983) suggested that the indispensable amino acid requirements of a fish should

Table 3.16

Amino Acid Composition of Whole-Body Tissue of Certain Fish[a]

Amino acid	Rainbow trout[b]	Atlantic salmon[b]	Coho salmon[c]	Cherry salmon[d]	Channel catfish[e]
Ala	6.57	6.52	6.08	6.35	6.31
Arg	6.41	6.61	5.99	6.23	6.67
Asp	9.94	9.92	9.96	9.93	9.74
Cys	0.80	0.95	1.23	1.34	0.86
Glu	14.22	14.31	15.25	15.39	14.39
Gly	7.76	7.41	7.31	7.62	8.14
His	2.96	3.02	2.99	2.39	2.17
Ile	4.34	4.41	3.70	3.96	4.29
Leu	7.59	7.72	7.49	7.54	7.40
Lys	8.49	9.28	8.64	8.81	8.51
Met	2.88	1.83	3.53	3.14	2.92
Phe	4.38	4.36	4.14	4.63	4.14
Pro	4.89	4.64	4.76	4.33	6.02
Ser	4.66	4.61	4.67	4.48	4.89
Thr	4.76	4.95	5.11	4.63	4.41
Trp	0.93	0.93	1.40	0.83	0.78
Tyr	3.38	3.50	3.44	3.58	3.28
Val	5.09	5.09	4.32	4.85	5.15

[a] Expressed as g/100 g amino acids.
[b] Data from Wilson and Cowey (1985).
[c] Data from Arai (1981).
[d] Data from Ogata *et al.* (1983).
[e] Data from Wilson and Poe (1985).

be related to, or even governed by, the pattern of amino acids present in muscle tissue. They showed a high correlation between the requirement pattern found by feeding experiments for the 10 indispensable amino acids as determined for carp by Nose (1979) using amino acid test diets and the pattern of the same amino acids in the whole-body tissue of growing carp.

Wilson and Poe (1985) have tested this hypothesis in channel catfish. These workers obtained a regression coefficient of 0.96 when the indispensable amino acid requirement pattern for the channel catfish was regressed against the whole-body indispensable amino acid pattern found in a 30-g channel catfish. A lower regression coefficient of 0.68 was found when the requirement pattern was regressed against the channel catfish egg amino acid pattern. Nose and Murai (1990) have also compared regression coefficients of the indispensable amino acid patterns for requirement values and whole-body and egg amino acid composition data for carp and coho

salmon. These workers also found that the correlation coefficients for the whole-body data were higher than those for the egg data. Coefficients for carp were 0.75 for the egg data and 0.86 for the whole-body data; similarly, values for coho salmon were 0.90 for the egg data and 0.95 for the whole-body data. The agreement was much better for these species than indicated above for channel catfish. Thus, Nose and Murai (1990) concluded that the amino acid patterns of either whole body or eggs could be used in formulating fish feeds until reliable requirement data were available for that species.

3.5.3. Use of Composition Data to Estimate Requirements

3.5.3.1. Protein Accretion

Ogino (1980) estimated the indispensable amino acid requirements of common carp and rainbow trout based on the amounts of each amino acid retained in the carcass of the animal fed a high-quality protein diet. This method has been used to estimate the amino acid requirements of the Mozambique tilapia (Jauncey *et al.,* 1983), Siberian sturgeon (Kaushik *et al.,* 1991), and white sturgeon (Ng and Hung, 1995). Even though it is generally accepted that this technique provides an ideal pattern of indispensable amino acids for protein accretion, it is questionable whether the net amino acid retention values can actually be used to establish the amino acid requirements for maximum growth. In general, the requirement values estimate by this method are lower than those determined by growth studies. This may be due to the fact that normally only about 30 to 40% of dietary nitrogen is retained by growing fish.

3.5.3.2. A/E Ratios and the Ideal Protein Concept

Arai (1981) used A/E ratios [(indispensable amino acid content/total indispensable amino acid content including cysteine and tyrosine) × 1000] of whole-body coho salmon fry to formulate test diets for this fish. Fish fed casein diets supplemented with amino acids to simulate the A/E ratios of whole-body tissue showed much improved growth and feed efficiency. Ogata *et al.* (1983) used A/E ratios based on amino acid composition data from cherry salmon to design test diets for cherry and amago salmon fry. A casein diet supplemented with amino acids to simulate the A/E ratios of cherry salmon resulted in better growth in both species than diets containing casein alone, casein plus amino acids to simulate the A/E ratio of eyed cherry salmon eggs, or white fish meal.

A/E ratios have been used as a means of estimating the requirements of all indispensable amino acids when only one is known by relating the A/E

ratio of each indispensable amino acid to that of the A/E ratio of the known amino acid times the requirement value for the known amino acid (Moon and Gatlin, 1991). This technique has also been used by Forster and Ogata (1998) to estimate the amino acid requirements of the Japanese flounder and red sea bream. The relationship between whole-body amino acid patterns and amino acid requirement patterns discussed above is very similar to the ideal protein concept that has been advocated for use in expressing the amino acid requirements of swine (Agricultural Research Council, 1981). The ideal protein concept is based on the idea that there should be a direct correlation between the whole-body amino acid pattern of the animal and the dietary amino acid requirements of that animal. In addition, since lysine is normally the first limiting amino acid in most feedstuffs, the requirements for the other indispensable amino acids are expressed relative to the lysine requirement. Thus, if one knows the dietary lysine requirement and the whole-body amino acid composition of an animal, then one should be able to estimate the dietary requirement for the remaining indispensable amino acid requirements of the animal relative to the lysine requirement. A comparison of the amino acid requirement values as determined by conventional means in our laboratory and as estimated based on the ideal protein concept for channel catfish is presented in Table 3.17. These data show excellent agreement. This procedure is essentially the same as the one discussed above for the use of A/E ratios.

Table 3.17

Amino Acid Requirement Values as Determined by Conventional Means and as Estimated[a]

		Requirement[b]	
Amino acid	Amino acid ratio	Determined	Estimated
Lysine	100	5.1	—
Arginine	78	4.3	4.0
Histidine	25	1.5	1.3
Isoleucine	50	2.6	2.6
Leucine	87	4.4	4.4
Met + Cys	44	2.3	2.2
Phe + Tyr	87	5.0	4.4
Threonine	52	2.0	2.7
Tryptophan	9	0.5	0.5
Valine	61	3.0	3.1

[a] Based on the ideal protein concept for channel catfish.
[b] Requirements are expressed as percentage of protein.

We have used the ideal protein concept as a means of estimating the amino acid requirements of a *Clarias* hybrid from Thailand (Unprasert, 1994). Requirement values were determined for lysine, methionine, threonine, tryptophan, and leucine by growth studies. Requirement values for the remaining indispensable amino acids were then estimated relative to those of each of the amino acids determined by growth studies. The values estimated relative to lysine, methionine, and threonine were very similar to the values obtained from the growth studies. Thus, it was concluded that lysine, methionine, or threonine requirement values obtained from growth studies could be used to estimate the other amino acid requirements based on the ideal protein concept. However, less precise data were obtained when requirement values for tryptophan and leucine were used to estimate the other requirement values. We had difficulty obtaining competent amino acid analysis for the whole-body fish samples, which is of extreme importance for this procedure to work.

To use this procedure, all one needs to do to formulate diets based on the amino acid needs of a certain fish species is to determine the whole-body amino acid composition and the lysine requirement of that specific species. This new procedure should be much less time-consuming and less costly than determining the amino acid requirements of the fish by conventional means.

3.5.3.3. Species Comparisons

Akiyama *et al.* (1997) have conducted a rather extensive comparison of A/E ratio data and amino acid requirement data for a wide variety of fish species. They point out that even though there is very little variation in the amino acid composition of the whole-body tissue of the fish studied, there is considerable variation in the requirement values for certain amino acids determined in different laboratories both among and within species. These workers calculated the dissimilarity or distance index for all of the whole-body A/E ratio data and amino acid requirement data and constructed dissimilarity diagrams for each of the fish species for which such data are available. The diagram for the requirement data was about twice as large as the diagram for the A/E ratio data, indicating the higher variability in the requirement data. Species location within the diagram for the A/E ratio data appeared to be random, irrespective of the classification into families or phylogenetic categories. The diagram for the requirement data did indicate some relationship to the family classification or phylogenetic categories. Carp was adjacent to catla, both of which belong to the same family, Cyprinidae. Furthermore, chum salmon, chinook salmon, and coho salmon, in the family Salmonidae, were located close to each other. Thus, based on amino acid composition data one would suggest that the

requirements for all species would be quite similar, however, there does seem to be some evidence for differences in requirements among fish species or families.

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4

The Lipids

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4.1 Introduction

Students entering the field of fish nutrition might be forgiven for forming two impressions. First is the impression that because publications on lipids often dominate research journals and conference proceedings on fish nutrition, lipids are the most important nutrients for fish. They are not. Lipids are neither more important nor less important than any of the other groups of nutrients—proteins, carbohydrates, vitamins, or inorganic elements. Second is the impression that because we write more about lipids than other nutrients, we know more about lipids than about other nutrients. We do not. Rather, we probably know less about the nutritional requirements of fish, or any other animal for that matter, for lipids than for other nutrients, which is precisely why there is so much fish lipid nutritional research. Why should this be so? One reason is the relative complexity of lipid chemistry, that is quickly encountered by first-time students in the arcane nomenclatures and terminologies of fatty acids. Another, more fundamental reason is that our understanding of the chemistry of the cell's hydrophobic phase, i.e., of the physicochemistry of the cell membrane bilayer and the reactions that occur in it, lags well behind our understanding of the cell's aqueous phase chemistry. By the year 2000 we had a detailed biochemical understanding of amino acids and carbohydrates, their biosynthetic and catabolic pathways, their enzymology, and their molecular biology and genetics and, also, a detailed knowledge of their nutrition. In contrast, we are still defining the anabolic and catabolic pathways for particular fatty acids, especially the polyunsaturated fatty acids (PUFA), and our understanding of PUFA enzymology, much less PUFA molecular biology and genetics, is still rudimentary. It is not surprising, therefore, that we still do not really know what constitutes desirable, much less optimal, dietary requirements for particular PUFA for *Homo sapiens,* much less for farmed animals including fish.

We have noted elsewhere (Sargent *et al.,* 1993a) that enzyme–substrate interactions in carbohydrate, protein, and nucleic acid metabolism depend chiefly on strong ionic and hydrogen bond interactions between substrate and product molecules and enzymatic proteins, generally resulting in high specificities in enzyme-catalyzed reactions. However, enzyme–substrate interactions in lipid and fatty acid metabolism depend much more on weak "hydrophobic" interactions based on van der Waals and dispersion forces, which often result in lower specificities in enzyme-catalyzed reactions involving lipids and fatty acids. Thus, the exact and invariant amino acid compositions of proteins reflect the high specificity of coupling amino acids with transfer RNAs in protein synthesis, whereas the relatively variable fatty acid compositions of triacylglycerol fats and oils reflect the relatively

low specificity of incorporation of fatty acids into these lipids. Walter de la Mare wrote:

"It's a very odd thing-
As odd as can be-
That whatever Miss T. eats
Turns into Miss T."

De la Mare's verse is true for Miss T's tissue protein, for example, her muscle actomyosin, which has the same amino acid composition irrespective of what plant or animal protein she eats. However, it is not true for her body fat, whether in adipose tissue or cell membranes, whose fatty acid composition is very significantly influenced by what she eats. As we shall see later, defining the exact dietary requirements of essential fatty acids in animals requires considering not only the relative as well as the absolute amounts of individual fatty acids in the animals' diets, but also the animals' innate abilities to metabolize these fatty acids, whether anabolically or catabolically. This is because an excess of one essential fatty acid in the diet, say arachidonic acid 20:4*n*-6, alters the dietary requirement for another essential fatty acid, say eicosapentaenoic acid 20:5*n*-3. Moreover, the final level of 20:5*n*-3 in the animal is also influenced by an animal's innate, i.e., genetically determined, ability to biosynthesize that fatty acid from its shorter-chain precursor, 18:3 *n*-3, and also by its innate ability, again, genetically determined, to catabolize both the precursor 18:3*n*-3 and the end product 20:5*n*-3. In short, what determines the levels of essential fatty acids in Miss T's tissues depends on a complex interplay between Miss T's genes and her environment, in this case her diet. In contrast, the requirement for one essential amino acid, say leucine, is not changed by an excess of another essential amino acid, say lysine, such that given a sufficiency of leucine in Miss T's diet, her genetic constitution ensures that her many tissue proteins contain the required level of leucine. Considerations such as these underlie why a full understanding of lipid nutrition is so elusive and, also, why many researchers find this an attractive and challenging field of research.

Since we wrote the chapter "The Lipids" in 1986 for the 1989 edition of John Halver's *Fish Nutrition* (Sargent *et al.,* 1989), much research has been conducted on fish lipid nutrition and a plethora of research publications have appeared. This article is an effort to update and, above all, to overview fish lipid nutrition so as to define, first, "where we are now" particularly in terms of general concepts of fish lipid nutrition; second, the current problems in fish lipid nutrition; and, third, and more speculatively, what the future may hold. We start directly in Sections 4.2 and 4.3 with basics, without which this field cannot be fully understood, namely, the structural and functional chemistry of lipids, emphasizing lipids in fish. Our account rests heavily on various reviews on marine lipids and fish lipid nutrition

published by us and our colleagues since the first edition of this chapter (Henderson and Tocher, 1987; Sargent *et al.*, 1990, 1993a, b, 1995a–d, 1997, 1999a, b; Sargent, 1995a, b, 1997; Sargent and Henderson, 1995; Henderson, 1996; Bell, 1998; Sargent and Tacon, 1999). We hope that the readers of this review will understand that we cannot provide here complete coverage of the now very extensive literature on fish lipid nutrition. Our laboratory database of references is now several times longer than the length permitted for a review chapter of this type.

4.2 Structures and Biosynthesis

4.2.1. Lipid Classes: Structures

4.2.1.1. Triacylglycerols

These consist of three molecules of fatty acids esterified to the *sn*-1, *sn*-2, and *sn*-3 positions of L-glycerol (Fig. 4.1). A single fatty acid may be esterified to all three positions of the glycerol, e.g., as in trioleoylglycerol (triolein), where oleic acid is the only fatty acid esterified; or two fatty acids may be esterified, as in dioleoylmonopalmitoylglycerol; or three fatty acids may be esterified, as in oleoyllinoleoylpalmitoylglycerol. In general, saturated fatty acids and monounsaturated fatty acids are preferentially located in the *sn*-1 and *sn*-3 positions of glycerol, whereas PUFA are preferentially located in the *sn*-2 position of glycerol. However, many exceptions exist to this general rule, e.g., tridocosahexaenoyl(tri-22:6*n*-3)glycerol can be a major component of the triacylglycerols in the eye lipids of some fish (e.g., Nicol *et al.*, 1972).

4.2.1.2. Wax Esters

These molecules consist of a single molecule of a fatty acid esterified to a single molecule of a fatty alcohol (Fig. 4.2). Wax esters are very abundant in marine zooplankton, particularly, in calanoid copepods and in euphausiids (red feed and krill, respectively), which form major natural feeds for many

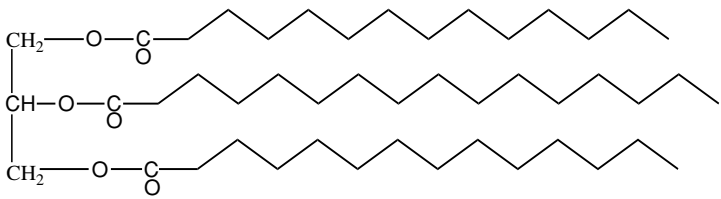

FIG. 4.1

A triacylglycerol: three fatty acids esterified to L-glycerol.

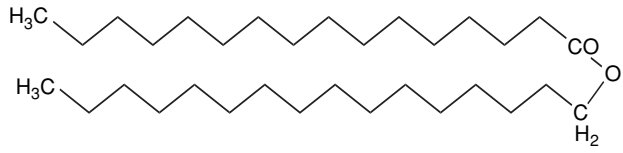

FIG. 4.2

A wax ester consisting of a fatty acid (*top*) esterified to a fatty alcohol (*bottom*).

species of zooplankton-consuming marine fish. They can also be present in considerable amounts in the body tissues and in the eggs of some fish species. The fatty alcohols in marine wax esters are generally saturated or monounsaturated and, in the case of high-latitude marine zooplankton, can be very rich in 20:1*n*-9 and 22:1*n*-11 moieties. The fatty acids of marine wax esters can be saturated, monounsaturated, or polyunsaturated. When consumed by zooplanktonivorous fish, wax esters are converted to triacylglycerols. Thus, the large amounts of 20:1*n*-9 and 22:1*n*-11 fatty acids in the triacylglycerols of many "northern hemisphere fish oils," i.e., oils from species such as herring, sand eels, and capelin, are derived directly from the oxidation of the corresponding fatty alcohols ingested from zooplankton wax esters (Sargent and Henderson, 1995). These fatty acids are present at only low percentages in "southern hemisphere fish oils," which are correspondingly richer in *n*-3 PUFA, especially 20:5*n*-3 (Sargent and Henderson, 1995).

4.2.1.3. Phosphoglycerides

The term "phospholipids" is usually taken to mean phosphoglycerides, which have a common backbone of phosphatidic acid (Fig. 4.3), i.e.,

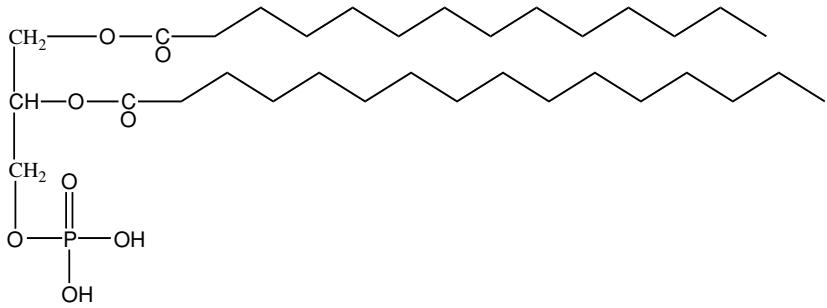

FIG. 4.3

Phosphatidic acid, the backbone of the phosphoglycerides.

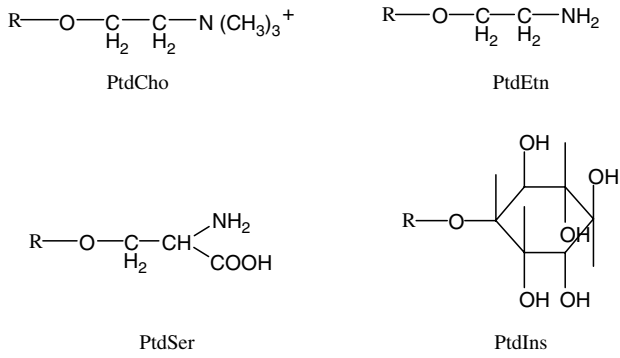

FIG. 4.4

Phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns). R, phosphatidyl group (see Fig. 4.3).

di-fatty acyl esters of L-glycerol 3-phosphate. Saturated and monounsaturated fatty acids are preferentially esterified at position *sn*-1 of the L-glycerol, with polyunsaturated fatty acids preferentially esterified at position *sn*-2. However, as with triacylglycerols, there are many exceptions to this generalization, i.e., the didocosahexaenoylphosphoglycerides, which are abundant in the eye epithelia of fish, specifically in rod outer membrane segments (Bell and Tocher, 1989; Bell and Dick, 1991). Phosphatidic acid is esterified to the "bases" choline, ethanolamine, serine, and inositol to form the major phosphoglycerides of animal, including fish, tissues, namely, phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns) (Fig. 4.4).

4.2.1.4. Sphingolipids

Sphingomyelin is strictly a phospholipid but differs fundamentally from phosphoglycerides in that it is not constituted from phosphatidic acid. It consists of the amino alcohol "base" sphingosine whose primary alcohol group is esterified to phosphocholine and whose amino group is amide-linked to a long-chain fatty acid. Sphingomyelin (Fig. 4.5) is one of the sphingolipids, as are the cerebrosides, in which the phosphocholine group of sphingomyelin is replaced by one or more sugars including glucose and galactose (Fig. 4.6).

4.2.1.5. Sterols

The major sterol by far in animal, including fish, tissues is cholesterol (Fig. 4.7). It can exist esterified to a fatty acid, e.g., as the cholesteryl esters

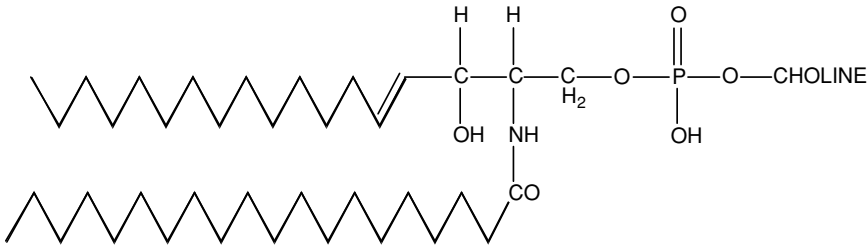

FIG. 4.5

Sphingomyelin.

of blood plasma, but also exists in the nonesterified form, as in cell plasma membranes.

4.2.2. Fatty Acid Structures

With the exception of cholesterol all of the aforementioned lipid classes contain fatty acids, esterified to alcohol groups in the case of the glycerides and to amino groups in the case of the sphingolipids. Fatty acids are designated on the basis of their chain lengths, their degree of unsaturation (number of double bonds), and the position of their double bonds. Thus, 14:0 and 16:0 designate fatty acids with 14 and 16 carbon atoms, respectively, and with no double bonds. The terms 18:1*n*-9 and 18:1*n*-7 designate fatty acids with 18 carbon atoms whose single double bonds are, respectively, 9 and 7 carbon atoms from the methyl end of the molecule. Fatty acids 18:1*n*-9 and 18:1*n*-7 are identical to $18{:}1^{\Delta 9}$ and $18{:}1^{\Delta 11}$, respectively, Δ signifying the

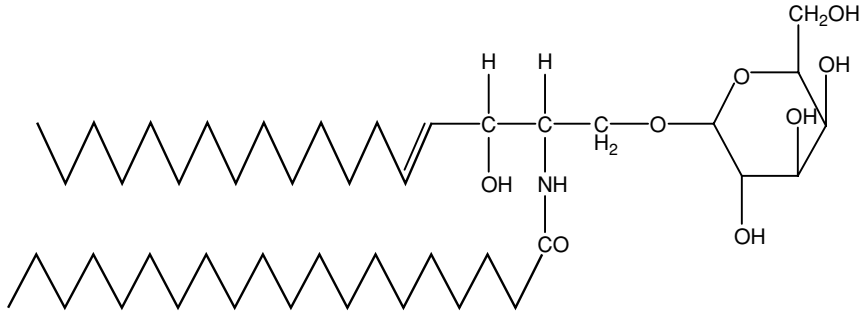

FIG. 4.6

A cerebroside.

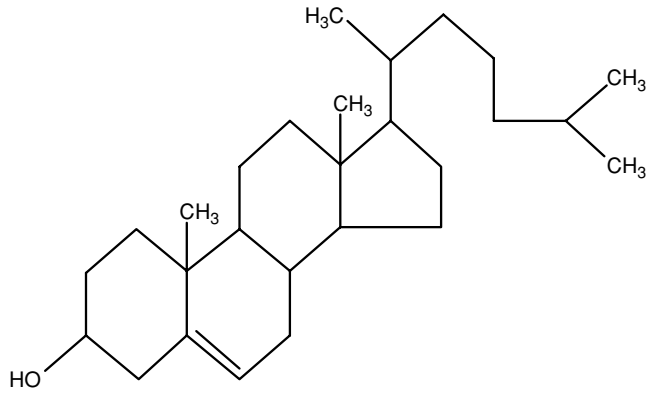

FIG. 4.7

Cholesterol.

position of the double bond from the carboxyl end of the molecule. In PUFA, i.e., fatty acids with two or more double bonds, which are nearly always in the *cis* rather than the *trans* configuration, the double bonds are interrupted by a single methylene (CH_2) group to generate "methylene-interrupted *cis*-diene" structures. Thus, by specifying the position of the first double bond, as in 18:3*n*-3, where the first double bond is three carbon atoms from the methyl terminus, the entire structure is defined. Alternatively, PUFA can be specified fully using the Δ nomenclature, as in $18{:}3^{\Delta 9,12,15} = 18{:}3n\text{-}3$, $20{:}5^{\Delta 5,8,11,14,17} = 20{:}5n\text{-}3$, or $22{:}6^{\Delta 4,7,10,13,16,19} = 22{:}6n\text{-}3$. Fatty acids with *trans* double bonds occasionally occur, e.g., elaidic acid, the *trans* isomer of 18:1*n*-9. Finally, fatty acids have a plethora of trivial English names such as palmitic acid 16:0, oleic acid 18:1*n*-9, and linolenic acid 18:3*n*-3, often reflecting their common origins as in palm, olive, and linseed oils, respectively, or more formalized Greek–Latin names such as eicosapentaenoic acid 20:5*n*-3 and docosahexaenoic acid 22:6*n*-3, reflecting the numbers of carbon atoms (20 and 22) and double bonds (5 and 6) they contain.

4.2.2.1. Saturated Fatty Acids

These occur naturally in animal, including fish, lipids, with chain lengths ranging from C_{14} to C_{24}. Phosphoglycerides seldom contain significant amounts of saturated fatty acids other than 16:0, 18:0, and, to a lesser extent, 20:0, this restriction reflecting the relatively invariant geometry (width) of the phosphoglyceride-rich bilayers that constitute animal cell membranes, especially the plasma membrane and the endoplasmic reticulum and its derivatives.

4.2.2.2. Monounsaturated Fatty Acids

These, again, occur naturally, with chain lengths from C_{14} to C_{24}, with, again, the phosphoglycerides seldom having chain lengths other than C_{16}, C_{18}, and, to a lesser extent, C_{20}. However, sphingolipids are commonly rich in nervonic acid, 24:1*n*-9. Abundant fatty acids in many fish triacylglycerols are 20:1*n*-9 (gadoleic acid) and 22:1*n*-11 (cetoleic acid), which are derived from the corresponding 20:1*n*-9 and 22:1*n*-11 fatty alcohols in zooplanktonic wax esters.

4.2.2.3. Polyunsaturated Fatty Acids

Marine organisms, especially algae, can contain a plethora of PUFA of chain length C_{16} (with two to four double bonds), C_{18} (with two to five double bonds), C_{20} (with two to five double bonds), and C_{22} (with two to six double bonds) (Sargent *et al.,* 1995c). These PUFA are generally of the *n*-3 series, although representatives of the *n*-6 and, in the case of C_{16} PUFA, the *n*-1 series also occur. However, in fish the main PUFA to be considered are 20:4*n*-6 (arachidonic acid; AA) and its metabolic precursor 18:2*n*-6 (linoleic acid; LA), together with 20:5*n*-3 (eicosapentaenoic acid; EPA) and 22:6*n*-3 (docosahexaenoic acid; DHA) and their metabolic precursor 18:3 *n*-3 (linolenic acid; LNA). Another term in common usage in the aquaculture field is HUFA, the abbreviation for highly unsaturated fatty acids. This is often used without proper definition, but, in this laboratory at least, we define it strictly as PUFA having carbon chain lengths $\geq C_{20}$ and with three or more double bonds.

4.2.3. Fatty Acid Biosynthesis

The saturated fatty acids 16:0 and 18:0 can be biosynthesized *de novo* by all known organisms, including fish, by the conventional pathway catalyzed by the cytosolic fatty acid synthetase, which occurs and has been characterized in fish (Sargent *et al.,* 1989). Likewise, all organisms including fish are capable of desaturating 16:0 and 18:0 by the microsomal fatty acid Δ^9 desaturase to yield, respectively, $16{:}1^{\Delta 9} = 16{:}1n\text{-}7$ (palmitoleic acid) and $18{:}1^{\Delta 9} = 18{:}1n\text{-}9$ (oleic acid). Fatty acid Δ^9-desaturase has been particularly well characterized, and its gene cloned in carp, where the enzyme is involved in enhancing monounsaturated fatty acid production in response to a lowered environmental temperature so as to maintain membrane fluidity (Tiku *et al.,* 1996). Not well studied in fish is the extent to which 16:1*n*-7 and, especially, 18:1*n*-9 are chain elongated to higher homologs, including 18:1*n*-7, 20:1*n*-9, 22:1*n*-9, and 24:1*n*-9, by the conventional microsomal elongation pathway that occurs in higher terrestrial mammals. It is known,

however, that the 22:1*n*-11 moiety, which can be abundant in fish triacylglycerols, is derived from the corresponding fatty alcohol in the wax esters of zooplankton, and the same holds for the 20:1*n*-9 fatty acid in fish triacylglycerols. The 22:1*n*-11 alcohol in zooplankton is considered to be derived from the corresponding fatty acid biosynthesized in zooplankton by a fatty acid Δ^9-desaturase acting on 20:0 to generate $20{:}1^{\Delta 9} = 20{:}1n\text{-}11$, which is then chain elongated to 22:1*n*-11 (see Sargent and Henderson, 1995). Irrespective of the special origin of 22:1*n*-11 and 20:1*n*-9 fatty acids in fish oils, and of particular relevance to fish nutrition, is the extent to which the pathways of saturated and monounsaturated fatty acid biosyntheses, including pathways of further elongation of these fatty acids, occur in fish. Many species of fish, including most marine species, are very well supplied with fatty acids in their natural diets. Under these conditions, endogenous *de novo* biosynthesis of fatty acids including their chain elongations is likely to be repressed. Therefore, it is not unreasonable to consider the possibility that species which have evolved with high-fat diets have a limited *de novo* fatty acid biosynthetic ability or, in the extreme, have lost this ability. In the absence of definitive information, therefore, it would be wise to ensure that fish farmed on relatively high-fat diets have suitable quantities of a suitable range of fatty acids. This may be particularly important in early developmental stages of fish. There will normally be no problems for C_{16} and C_{18} saturated and monounsaturated fatty acids but it should not be assumed that the same holds for longer chain fatty acids, including nervonic acid, 24:1*n*-9, which is a major fatty acid of the sphingolipids of neural tissues. This is an area of uncertainty in fish nutrition, which is worthy of further investigation. It is considered again in Section 4.6.

The pathway whereby C_{20} and C_{22} are biosynthesized from their C_{18} precursors is particularly important in fish. The pathway is complex but is now reasonably well understood and is the same, at least qualitatively, in fish (Buzzi *et al.,* 1996, 1997) as in rats (Voss *et al.,* 1991) (Fig. 4.8). Several features of the pathway are notable.

1. The reactions, with one exception, occur in the microsomal fraction of the liver, and the same enzymes, whether fatty acid desaturases indicated horizontally or fatty acid elongases indicated vertically in Fig.4.8, act on the *n*-3, the *n*-6, and the *n*-9 fatty acid series. However, the affinity of the enzymes, especially the desaturases, is higher for the *n*-3 than for the *n*-6 series, and both of these affinities are higher than for the *n*-9 series. Thus, 18:1*n*-9 is converted through the pathway only in the absence of both 18:3*n*-3 and 18:2*n*-6, i.e., in the absence of the "dietary essential fatty acids."
2. Particular problems exist with the insertion of the last, Δ^4, double bond in 22:6*n*-3, which does not occur through direct Δ^4 desaturation of its immediate precursor 22:5*n*-3. Rather, 22:5*n*-3 is chain elongated to 24:5*n*-3,

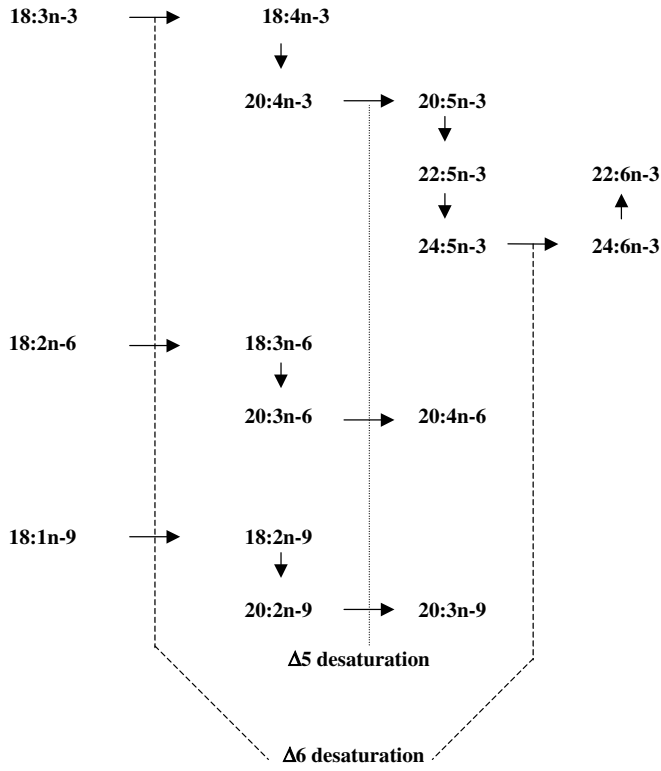

FIG. 4.8

Pathways of biosynthesis of C_{20} and C_{22} PUFA from *n*-3, *n*-6, and *n*-9 C_{18} precursors. Vertical downward arrows represent fatty acid chain elongation reactions. Horizontal arrows represent fatty acyl desaturations. The single vertical upward arrow represents peroxisomal chain shortening. Note that fatty acid Δ^6 desaturation occurs at two steps in the scheme; fatty acid Δ^5 desaturation occurs at only one step.

which is then converted by Δ^6 desaturation to 24:6*n*-3, which is then converted, by a chain-shortening reaction in the peroxisomes, to 22:6*n*-3.

3. Whereas 22:6*n*-3 is the main end product of further desaturation and elongation of 18:3*n*-3, 20:4*n*-6 is the main end product of desaturation and elongation of 18:2*n*-6. However, 20:4*n*-6 can be further desaturated and elongated to 22:5*n*-6 to some extent, with insertion of the last Δ^4 double bond in 22:5*n*-6 being achieved in exactly the same manner as for 22:6*n*-3. As far as is known, 20:3*n*-9 is not further elongated and desaturated to higher, further unsaturated *n*-9 homologs.
4. Fatty acid Δ^5 desaturation acts at only one-step in the pathway, involving 20:3*n*-6 or 20:4*n*-3, whereas fatty acid Δ^6 desaturation occurs at two steps,

the first involving 18:2*n*-6 or 18:3*n*-3 and the second involving 24:4*n*-6 or 24:5*n*-3. It is not known whether the same fatty acid Δ^6-desaturase catalyzes each of these steps or whether different Δ^6-desaturases (isoenzymes) are involved for the C_{18} and C_{24} PUFA.

5. The conversion of 18:3*n*-3 to 20:5*n*-3 and thence to 22:6*n*-3 is well established for many freshwater species of fish (see Sargent *et al.,* 1995a), with all the steps in the pathway in Fig. 4.8 having been established in rainbow trout (*Onchorynchus mykiss*) (Buzzi *et al.,* 1996, 1997). However, the overall conversion of 18:3*n*-3 to 22:6*n*-3 occurs poorly, if at all, in those species of marine fish studied so far. This difference is fundamental to elucidating the essential fatty acid requirements of fish (see Section 4.5).

Current knowledge of the extent to which marine fish can elongate and further desaturate 18:3*n*-3 is now considered. Early nutritional evidence suggested that 18:3*n*-3 and/or 18:2*n*-6 could satisfy the essential fatty acid requirements of freshwater fish, whereas the *n*-3 highly unsaturated fatty acids (HUFA), i.e., 20:5*n*-3 and 22:6*n*-3, were required to satisfy the essential fatty acid requirements of marine fish (Yone, 1978; Watanabe, 1982; Kanazawa, 1985). Dietary conversion studies performed in turbot (*Scophthalmus maximus*) using radioactive substrates *in vivo* strongly suggested that this marine species was unable to produce 20:5*n*-3 and 20:4*n*-6 from 18:3*n*-3 and 18:2*n*-6, respectively (Owen *et al.,* 1975; Cowey *et al.,* 1976). However, these *in vivo* experiments were unable to locate precisely where and how the deficiency in the elongation pathway occurred, although they were consistent with turbot having limited fatty acid Δ^5-desaturase activity. Cultured fish cell lines have been intensively studied to illuminate the problem (Tocher *et al.,* 1989, 1998). In particular, early comparative studies with cultured cell lines of turbot, Atlantic salmon (*Salmo salar*), and rainbow trout established that the limited ability of turbot to convert C_{18} to C_{20} PUFA was due either to limited activities of the C_{18} to C_{20} fatty acid elongase, or the fatty acid Δ^5-desaturase converting either 20:3*n*-6 to 20:4*n*-6 or 20:4*n*-3 to 20:5*n*-3 (Tocher *et al.,* 1989; Tocher and Sargent, 1990). More recently, the availability of specific substrates such as [U-^{14}C]18:4*n*-3, (d5)18:4*n*-3, [U-^{14}C]20:4*n*-3, and (d5)20:4*n*-3 has further illuminated the problem. Thus, there is now compelling evidence that cultured cells from turbot have a very low C_{18}-to-C_{20} fatty acid elongating activity, whereas their fatty acid Δ^5-desaturase activity is higher than that of cultured cells from Atlantic salmon (Ghioni *et al.,* 1999). However, neither cell line produced significant amounts of 22:6*n*-3 from C_{18} or C_{20} substrates. In contrast, cultured cells from gilthead sea bream (*Sparus auratus*) have active C_{18}-to-C_{20} and C_{20}-to-C_{22} fatty acid elongase activities but have a very low fatty acid Δ^5-desaturase activity (Tocher and Ghioni, 1999), as had been indicated earlier in *in vivo* studies (Mourente and Tocher, 1993a, 1994). These findings need to be confirmed

in the species in question *in vivo,* as there is no certainty that enzymes in the PUFA elongation–desaturation pathway continue to be expressed in cultured cells exactly as they are *in vivo.* Irrespective, the results establish that both of the marine species studied have at least some activity for both the C_{18}–to-C_{20} fatty acid elongase and the Δ^5-desaturase. This is consistent with the genes determining both enzymes being present in both species. Clearly, however, the expression of the genes differs in cultured cells from the different species. Thus, the fatty acid Δ^5-desaturase gene but not the C_{18}–C_{20} elongase gene is well expressed in turbot cells. In contrast, the C_{18}–C_{20} and C_{20}–C_{22} elongase gene(s) but not the Δ^5-desaturase gene is well expressed in sea bream cells. Such results have important implications for future molecular genetic research in fish lipid nutrition. Thus, it can be deduced that the apparent inability of marine fish to convert 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3 is due not to the complete absence of the required genes in a particular species, but to one or more of the required genes not being sufficiently well expressed. The problem, therefore, is how to switch on the recalcitrant genes rather than to introduce them by genetic engineering.

4.2.4. Lipid Class Biosynthesis

The pathways of *de novo* glyceride biosynthesis are the same in fish as in higher terrestrial mammals (Sargent *et al.,* 1989). However, as will be seen later (Section 4.6) there is now considerable evidence that at least some species of fish larvae, both freshwater and marine, may have a limited capacity to biosynthesize phosphoglycerides *de novo.* This reflects the fact that many fish larvae receive a luxus of phosphoglycerides in their natural diets, whether from yolk sac lipids prior to first feeding or from natural prey at and after first feeding. The same situation may well hold for *de novo* biosyntheses of cholesterol and, also, the sphingolipids, which have scarcely been studied in fish.

Apart from *de novo* biosyntheses, fatty acid retailoring by acyl exchange reactions is an important process determining the final fatty acid compositions of tissue phosphoglycerides. Administered fatty acids are readily incorporated into the phosphoglycerides of fish tissues, e.g., when incubated with hepatocytes (Buzzi *et al.,* 1997), but the extent to which this reflects *de novo* biosyntheses, i.e., net formation of phosphoglycerides, or fatty acyl exchange reactions, i.e., turnover of existing phosphoglycerides, has not been determined in fish. Fission of cell membranes, e.g., as occurs in the Golgi apparatus when forming vesicles, and fusion of cell membranes, e.g., as occurs in vesicle exocytosis at synaptic junctions, are both now known to depend on the formation of lysophosphoglycerides (Weigert *et al.,* 1999;

Schmidt *et al.,* 1999). Particular roles for specific fatty acids, especially PUFA, may exist in these processes which have not been studied in fish so far.

4.3 Functions

4.3.1. Energy Production

A major role of fatty acids in all organisms is to generate metabolic energy in the form of ATP via mitochondrial β-oxidation, a process which has been well established in fish (see Sargent *et al.,* 1989). Lipids, and specifically fatty acids, are the favoured source of metabolic energy in fish, especially marine fish, as evidenced by the very high oil levels (more than 20% of the wet weight) that can be achieved by fish such as capelin and herring. Fatty acids not only are the major source of metabolic energy in fish for growth from the egg to the adult fish (Tocher *et al.,* 1985a, b), but also are the major source of metabolic energy for reproduction (Henderson *et al.,* 1984a, b; Sargent *et al.,* 1989). Indeed, as pointed out elsewhere (Sargent *et al.,* 1995a), the very high oil levels in those industrial fish species from which fish oil is extracted exist largely for reproduction. This has recently been emphasized by the clear correlation that exists between oil levels and fecundity and, therefore, recruitment in wild populations of cod (Marshall *et al.,* 1999).

Given that fish oil is the major lipid component by far of current fish feeds, the fatty acids that are potential sources of metabolic energy in fish feeds include 16:0 and 18:1*n*-9; 20:1*n*-9 and 22:1*n*-11, which are particularly abundant in the so-called northern fish oils; and *n*-3 HUFA 20:5*n*-3 and 22:6*n*-3. It is certain that 16:0, 18:1*n*-9, 20:1*n*-9, and 22:1*n*-11 are heavily catabolized to generate metabolic energy in fish because they are all consumed in large amounts during the growth of farmed fish species, e.g., the salmonids, and, specifically, during the formation of roe by female fish (Henderson *et al.,* 1984a, b; Henderson and Almater, 1989). The evidence available for fish (Sargent *et al.,* 1989) is consistent with all of these saturated and monounsaturated fatty acids, including 22:1*n*-11, being readily catabolized by mitochondrial β-oxidation in fish. As noted earlier, 22:1*n*-11 seldom, if ever, exists in significant amounts in phosphoglycerides, so that its only role in fish is as a source of metabolic energy. Eicosapentaenoic acid, 20:5*n*-3, is readily β-oxidized by mitochondria in rats and, indeed, induces the formation of mitochondria in rats (Madsen *et al.,* 1999). However, 22:6*n*-3 is a poor substrate for mitochondrial β-oxidation in rats, where its catabolism requires peroxisomal β-oxidation (Madsen *et al.,* 1999). This is because, as insertion of the Δ^4 double bond in 22:6*n*-3 requires a special mechanism (described

above), so does it removal. Thus, the initial 2,3-(α,β)-dehydrogenation of 22:6*n*-3, which is the first step in the β-oxidation of all fatty acids, must be followed in the case of 22:6*n*-3 by an NADPH-dependent 2,4-dienoyl–CoA reductase and then by a 3-*cis*–2-t*rans*-isomerase so as to enable completion of oxidation of its β carbon. These processes, which are well evolved in peroxisomes, have yet to be studied in fish. However, peroxisomes are present in fish tissues including liver (Sargent *et al.,* 1989) and there are no reasons to believe that the mechanisms for oxidizing 22:6*n*-3 are different in fish and rats.

The specificity of fatty acid oxidation in fish is important in determining the fatty acid composition of triacylglycerols deposited in fish adipose tissue. This composition, in turn, is important not only for the well-being of the fish, particularly its successful reproduction, but also for the consumer, particularly in terms of the levels of the health-promoting 20:5*n*-3 and 22:6*n*-3 in farmed fish. In the absence of definitive biochemical information, only general observations can be made. Fish oils with levels of 20:5*n*-3 substantially higher than those of 22:6*n*-3 are readily available. These include the sardine, anchovy, and menhaden oils that form the basis of the "18:12" (EPA:DHA) formulations marketed as human health supplements. However, with one known exception, there are no fish oils with markedly higher levels of 22:6*n*-3 than 20:5*n*-3. That exception is the oil from the eye socket of the tuna (Sawada *et al.,* 1993). Indeed, tuna lipids, generally including both triacylglycerols and phosphoglycerides, consistently have much higher levels of 22:6*n*-3 than 20:5*n*-3 (Menzell and Olcott, 1964; Watanabe *et al.,* 1995; Murase and Saito, 1996). The underlying mechanisms determining such high levels of 22:6*n*-3 in tuna oils are unknown. Clearly, input of dietary fatty acids and oxidation of ingested fatty acids must both be considered. The former cannot readily account for the high levels of 22:6*n*-3 in tuna oils because the levels of 22:6*n*-3 in the natural diets of tuna do not correlate with the levels of 22:6*n*-3 accumulated by tuna and related fish (Saito and Ishihara, 1996; Saito *et al.,* 1997). Therefore, selective catabolism of 20:5*n*-3 relative to 22:6*n*-3 by tuna is the more likely possibility. Tunas, like certain scombroid fish, are warm-blooded and have active brain and eye heating systems. Tullis *et al.* (1991) have shown that the heater tissue of scombroid fish has an extremely high aerobic oxidative capacity which is likely to be fueled by fatty acid oxidation. Selective catabolism of saturated and especially monounsaturated fatty acids rather than PUFA, as has been established for Antarctic fish in general (Sidell *et al.,* 1995), could contribute very substantially to the accumulation of 22:6*n*-3 by fish with heater tissue, especially if 20:5*n*-3, but not 22:6*n*-3, is readily oxidized by mitochondria in heat generating tissues. Moreover, the maximal swimming speed of salmon has been positively correlated with levels of C_{18} fatty acids including 18:1*n*-9 and 18:2*n*-6 in

its musculature, indicating that these fatty acids rather than long-chain *n*-3 PUFA are the preferred substrates for fueling swimming muscles (McKenzie *et al.,* 1998). Thus, selective oxidation of saturated and monounsaturated fatty acids, and also 20:5*n*-3, by swimming muscles may also account for the selective retention of 22:6*n*-3 in fish, including especially scombroids and tunas. This is supported by a little known survey establishing a direct correlation between the levels of 22:6*n*-3 in fish and the fish's natural mobility for a wide range of species (Shulman and Yakovleva, 1983). Our own recent studies (Bell *et al.,* 1998) of the levels and fatty acid compositions of farmed Scottish salmon suggest that the ratio of 22:6*n*-3 to 20:5*n*-3 is consistently higher in the triacylglycerols deposited in the fish than in the fish oils currently used in salmon feeds. Likewise, Brodtkorb *et al.* (1997) have shown that the ratio of 22:6*n*-3 to 20:5*n*-3 was increased in the muscle of Atlantic salmon compared to that in the diet, consistent with selective oxidation of 20:5*n*-3, resulting in a selective retention of 22:6*n*-3 by the fish.

As well as having important roles in generating energy for heat production in warm-blooded fish and for swimming in fish in general, fatty acids also have a major role in providing energy for reproduction and particularly egg production (Henderson *et al.,* 1984a, b; Henderson and Almatar, 1989). Selective oxidation of 20:1*n*-9 and 22:1*n*-11 in triacylglycerol depots certainly occurs in capelin during the formation of roe as these fatty acids are abundant in depot triacylglycerols of the parent fish but present in roe in only very small amounts (Henderson *et al.,* 1984a, b). In addition, the higher ratio of 22:6*n*-3 to 20:5*n*-3 in the roe of capelin (Henderson *et al.,* 1984a) and in fish roe in general (Tocher and Sargent, 1984; Tocher *et al.,* 1985a, b), compared to that in body triacylglycerols, is consistent with selective catabolism of 20:5*n*-3 relative to 22:6*n*-3 in fatty acid oxidative processes producing energy for gonadogenesis. These considerations give insights into possible strategies for generating fish body oils enriched in 22:6*n*-3 relative to other fatty acids. This is an important area for future research, not least in relation to developing alternatives to fish oils in farmed fish feeds compatible with retaining high levels of 22:6*n*-3 in the flesh (see Section 4.8).

4.3.2. Membrane Structure and Functions

The phosphoglycerides that constitute cell membrane bilayers in fish generally contain 16:0, 18:1*n*-9, 20:5*n*-3, and 22:6*n*-3 as their principal fatty acids. The former two are located preferentially in the *sn*-1 position of the glycerol backbone of the phosphoglycerides, the latter two preferentially in the *sn*-2 position. Additionally, 22:6*n*-3 is generally present at about twice the level of 20:5*n*-3 in fish phospholipids. However, that generalization must be qualified with respect to phosphoglyceride classes and also with respect to

particular tissues. The following account rests mainly on extensive studies, especially of molecular species compositions of phosphoglycerides, in our own laboratory (Bell and Tocher, 1989; Bell and Dick, 1990, 1991, 1993a, b; M. V. Bell *et al.,* 1997). First, with respect to phosphoglyceride classes, the highest levels of 22:6*n*-3 are usually contained in phosphatidylethanolamine (which includes the plasmalogens, i.e., alk-1-enyl/acyl as distinct from acyl/acyl phosphoglycerides), with 16:0/22:6*n*-3, 18:0/22:6*n*-3, and 18:1*n*-9/22:6*n*-3 molecular species all being present. Phosphatidylserine is also rich in 22:6 *n*-3, with 18:0/22:6*n*-3 being an abundant molecular species. Phosphatidylcholine commonly has the lowest levels of 22:6*n*-3, in fish tissues, being rich instead in 16:0 and 18:1*n*-9, and appears to be more easily influenced by dietary fatty acids than the other phosphoglycerides. A special situation exists for phosphatidylinositol, which has the highest level of arachidonic acid, 20:4*n*-6, of all the fish phosphoglycerides and is also rich in 18:0. Indeed the *sn*-2 position of fish phosphoinositides has a distinct preference for C_{20} PUFA. Thus, high levels of 20:5*n*-3 can also be found in phosphatidylinositol, its fatty acid composition can be readily influenced by changes in dietary levels of both 20:4*n*-6 and 20:5*n*-3, and it can also accumulate 20:3*n*-6. Second, with respect to variations in the fatty acid compositions of phosphoglycerides in fish tissues, particularly high levels of 22:6*n*-3 exist in neural tissues, whether brain or eyes, to the extent that di-22:6*n*-3 species of both phosphatidylserine and phosphatidylethanolamine can account for up to 60 and 72%, respectively, of the total molecular species of these phosphoglycerides. Fish sperm can also contain high levels of dipolyunsaturated phosphoglycerides (M. V. Bell *et al.,* 1997). As discussed in Section 4.5, the abundance of 22:6*n*-3 in neural tissue has particular implications for fish larval nutrition.

The precise reason(s) for the preponderance of long-chain *n*-3 PUFA, specifically 20:5*n*-3 and, especially, 22:6*n*-3, in fish phosphoglycerides and, above all, the remarkable preponderance of 22:6*n*-3 in neural tissue, which occurs in vertebrates generally, albeit not to the same extent as in fish, remains elusive. Traditionally it was believed that the preponderance of 22:6*n*-3 in fish tissues was an adaptation to low temperatures to maintain membrane fluidity (homeoviscous regulation). However, the abundance of 22:6*n*-3 in both tropical species such as the warm-blooded tuna and polar species such as the cod belies that notion. Indeed, it is now recognized that the main determinant of fluidity in phosphoglycerides is the ratio of monounsaturated to saturated fatty acids, which is increased and decreased, respectively, in response to lowered and elevated environmental temperatures (Wodtke and Cossins, 1991). More sophisticated and credible explanations for the abundance of 22:6*n*-3 in the phosphoglycerides of fish (and indeed of marine organisms in general) start with the recognition that

22:6*n*-3 is unique among fatty acids in having the maximum number of *cis*-methylene-interrupted double bonds possible in a C_{22} fatty acid. As such it has a unique conformation dictated by a helical twist, or an "angle iron" shape, induced by its *cis*-methylene interrupted dienes that results in the molecule assuming a squat, compact form, with an overall length similar to that of 16:0 (Applegate and Glomsett, 1986). Such a structure favors the formation of the hexagonal (inverted micellar) phase rather than the conventional bilayer phase in phosphoglycerides, above all in di-22:6*n*-3-phosphoglycerides containing small head groups such as phosphatidylserine and phosphatidyl ethanolamine. Therefore, bilayers containing 22:6 *n*-3 phosphoglycerides, and above all di-22:6*n*-3-phosphatidylserine and di-22:6*n*-3-phosphatidylethanolamine, will be "energized" by the tendency of these molecules to form hexagonal phases, and this will facilitate conformational changes, especially very fast conformational changes, in membrane proteins that might otherwise be energetically unfavorable (Brown, 1994). Such changes are particularly important in visual and neuromuscular processes where di-22:6*n*-3-phosphoglycerides are particularly abundant. Moreover, the intrinsic structure of 22:6*n*-3 is inherently resistant to temperature and pressure changes, such that its effects continue to be exerted essentially independently of these environmental variables (Rabinovich and Ripatti, 1991). The advantages of such properties in the aquatic and, especially, the marine environment are self-evident and may well have been exploited evolutionary by the single-cell, motile and phototactic organisms (the flagellates) that produce 22:6*n*-3 at the base of the marine food web and from which the bulk, if not all, of the 22:6*n*-3 in the marine ecosystem is ultimately derived (Sargent *et al.,* 1995c). In this context, the sperm cell may be considered as a chemotactic rather than a phototactic flagellate. Such considerations, though speculative, are well consistent with more metabolically specific roles of 22:6*n*-3, e.g., its particular role in Ca^{2+}-ATPase activity in the sarcoplasmic reticulum of heart and skeletal muscle, which has long been established in higher mammals and more recently established in fish (Ushio *et al.,* 1997).

As described in previous reviews (Henderson and Tocher, 1987; Sargent *et al.,* 1993b, 1995b; Sargent, 1995a; Tocher, 1995), a major role for the C_{20} PUFA, especially 20:4*n*-6, is as precursors for the group of highly biologically active compounds, the eicosanoids which are C_{20} derivatives of C_{20} PUFA. These include cyclic compounds, comprised *inter alia* of prostaglandins, prostacyclins, and thromboxanes and formed initially by the action of cyclooxygenase on C_{20} PUFA, and linear compounds, including the leukotrienes and lipoxins, formed initially by the action of lipoxygenases on C_{20} PUFA. These enzymes act on free C_{20} PUFA released from plasma membrane phosphoglycerides by the action of phospholipase A_2. The eicosanoids are

autocrines, i.e., hormone-like compounds produced by cells to act in their immediate vicinity with a short half-life. Virtually every tissue in the body produces eicosanoids and they have a wide range of physiological actions, e.g., in blood clotting, the immune response, the inflammatory response, cardiovascular tone, renal function, neural function, and reproduction.

Eicosanoid production is associated very broadly with stressful situations and is a normal physiological process, with excess eicosanoid production often occurring in pathological conditions. In higher terrestrial mammals 20:4*n*-6 is the chief precursor of the eicosanoids, generating 2-series prostanoids and 4-series leukotrienes. However, 20:5*n*-3 competitively interferes with eicosanoid production from 20:4*n*-6 catalyzed by cyclooxygenase and lipoxygenases, and is itself converted to 3-series prostanoids and 5-series leukotrienes, which are generally much less biologically active than the corresponding 2-series prostanoids and 4-series leukotrienes produced from 20:4*n*-6 (Fig. 4.9). Thus, eicosanoid actions are determined by the ratio of 20:4*n*-6 to 20:5*n*-3 in cellular membranes, this in turn being determined by the dietary intake of *n*-6 and *n*-3 PUFA. There is now a large body of evidence supporting the notion that high incidences of cardiovascular and inflammatory conditions and cancers in developed societies are associated

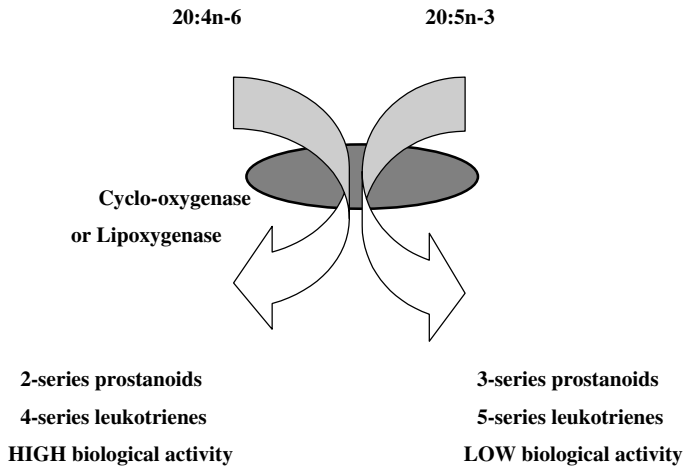

FIG. 4.9

Arachidonic acid, 20:4*n*-6, and eicosapentaenoic acid, 20:5*n*-3, compete with the same cyclooxygenase or the same lipoxygenases to produce 2-series prostanoids/4-series leukotrienes and 3-series prostanoids/5-series leukotrienes, respectively. Therefore, the ratio of 20:4*n*-6 to 20:5*n*-3 determines the ratio of high-activity to low-activity eicosanoids.

with an over high dietary intake of 18:2*n*-6 relative to 18:3*n*-3, which generates over high levels of 20:4*n*-6 in cells and thence over high levels of eicosanoids (Anonymous 1992, 1994a; Okoyuma *et al.,* 1997). Dietary supplementation with 20:5*n*-3, e.g., as fish oil or fish oil concentrates, can be beneficial under these conditions by damping down excess eicosanoid production from 20:4*n*-6 (Anonymous, 1992, 1994a, 1999).

With the exception of the phosphoinositides, the cellular phosphoglycerides of most species of fish, especially marine fish, have a large excess of 20:5*n*-3 relative to 20:4*n*-6. Despite this, 20:4*n*-6 is the chief source of biologically active eicosanoids in fish, where it is concentrated in phosphoinositides. It is tempting to conclude that the phosphoinositides are the origin of the 20:4*n*-6 used to produce eicosanoids in fish but there is no clear-cut experimental evidence for this conclusion. It has been established in fish, as in mammals, that 20:5*n*-3 and also 20:3*n*-6 (dihomo-γ-linolenic acid) competitively depress the production of eicosanoids from 20:4*n*-6 (Bell *et al.,* 1994a), as does 20:4*n*-3 (Ghioni, Tocher, and Sargent, unpublished data), which can be produced by elongation of stearidonic acid, 18:4*n*-3, which is a significant constituent of many fish oils. Therefore, in fish, as in mammals, eicosanoid production is influenced by the cellular ratio of 20:4*n*-6 to 20:5*n*-3, although, clearly, the optimal ratio of 20:4*n*-6 to 20:5*n*-3 for eicosanoid production is much lower in fish than in mammals. It remains the case, however, that an imbalanced ratio of 20:4*n*-6 to 20:5*n*-3 can be as damaging in fish as in mammals, as discussed later for marine fish larvae (Section 4.5).

The importance of 22:6*n*-3 in fish neural tissue, evidenced by the abundance of di-22:6*n*-3 species of phosphatidylethanolamine and phosphatidylserine in brain and eye, has been noted above, and, as discussed later, provision of an adequate dietary supply of 22:6*n*-3 is particularly important in marine fish larval nutrition for normal development of neural and visual functions. The fatty acid 22:6*n*-3 is no less important in this respect in human neural tissue and, therefore, in prenatal and early postnatal life when development of the neural and visual systems take place. There is now clear cut evidence that suboptimal provision of 22:6*n*-3 during prenatal and early postnatal life can generate visual and mental subnormalities in infants (Anonymous, 1994b). Indeed, there is accumulating evidence that a range of psychiatric disorders including schizophrenia may involve abnormalities in the metabolism of brain phospholipids and their associated PUFA, 22:6*n*-3, 20:5*n*-3 and 20:4*n*-6 (Horrobin *et al.,* 1999; Horrocks and Yeo, 1999; Okuyama *et al.,* 1997; Puri *et al.,* 1999; Peet *et al.,* 1999). There is also accumulating evidence that dietary supplementation with fish oils and fish oil concentrates can be beneficial in various psychiatric disorders (Horrocks and Yeo, 1999; Puri *et al.,* 1999).

These beneficial effects of long-chain *n*-3 PUFA in a range of important human disorders stem basically from a dietary excess of *n*-6 PUFA, specifically 18:2*n*-6, which is a major component of the vegetable seed oils whose production is rapidly increasing on a global scale, relative to 18:3*n*-3, whose chief dietary source for man is green leaf vegetables. The problem can be expressed alternatively as a relative deficiency of 18:3*n*-3, which can most effectively be reversed by providing the biologically active end product *n*-3 PUFA, 20:5*n*-3 and 22:6*n*-3. Fish are far and away the greatest providers of 20:5*n*-3 and 22:6*n*-3 in the human diet, so that fish have a critical role to play in human nutrition. Fish nutrition is concerned with both optimizing the health and welfare of the farmed animal and optimizing its nutritional value for the consumer. Therefore, a major concern of fish nutrition is to generate an end product with high levels of health-promoting 20:5*n*-3 and 22:6*n*-3 for the consumer. These HUFA have unique, important, and parallel roles to play in both fish and human nutrition and it is natural that they feature prominently in fish lipid nutrition.

4.4 Fatty Acids and Dietary Energy

As described above, an important role of lipids is as a source of energy-rich fatty acids. Dietary lipids are a major provider of energy in all fish, especially carnivorous species, which includes most marine fish, where carbohydrate plays little role as an energy source due to its low abundance in natural diets. In contrast, herbivorous/omnivorous species such as carp (*Cyprinus carpio*) consume plant material containing easily digestible sugars and also complex indigestible polysaccharides such as cellulose, chitin, and lignin, which the fish cannot digest easily without the assistance of specific gut flora (Watanabe, 1982; Smith, 1989). However, the extent to which fish in general, particularly marine species, can utilize dietary carbohydrate is debatable. One possible result of adaptation to an almost totally carnivorous habit is that marine fish in general may have a limited ability to digest and utilize dietary carbohydrate (Smith, 1989), in which case the energy requirements of most marine fish will be met primarily by dietary lipids and protein. Thus, the diets formulated for the aquaculture industry contain predominantly protein and lipid, with small amounts of vitamins and minerals. Easily digestible, water-soluble mono- or disaccharides such as glucose and fructose are obviously not an option in fish diets. However, for many species, polysaccharides such as starch and dextrin can be included in formulated diets at levels of up to approximately 10% (and higher in some species), although digestibility normally has to be improved by processing or cooking the ingredients prior to inclusion in the diet (Smith, 1989).

As protein is generally the most expensive of the basal components, the major issue regarding the dietary formulations for fish has been to supply the minimum protein requirements for optimal or maximal growth with an appropriate balance of other nutrients to supply the required energy. This balance is termed the ratio of digestible protein:digestible energy (DP:DE). Some protein is inevitably utilized for energy through direct oxidation of amino acids via the tricarboxylic cycle, or after conversion of amino acids to glucose via gluconeogenesis, but these processes can be minimized by including an abundance of energy-providing nutrients in the diet. Lipid as the primary energy-providing nutrient in fish diets has a major protein-sparing effect in many fish species (Wilson, 1989; Sargent *et al.,* 1989). Because of the metabolic interactions among protein, lipid, and carbohydrate described above, definition of the exact dietary lipid requirements for fish is not particularly meaningful. However, it has long been known that, in general, lipid in the range of 10–20% of the dry weight of the diet is sufficient to allow protein to be effectively utilized for fish growth without depositing excessive lipid in the fish tissues (Cowey and Sargent, 1979; Watanabe, 1982; Sargent *et al.,* 1989). Nonetheless, the precise amount of lipid required depends on the dietary protein level and, in some cases such as carp, also on the dietary carbohydrate level (Watanabe, 1982; Sargent *et al.,* 1989). More detailed accounts of the nutritional energetics of fish and the role of protein as an energy source and its interaction with other dietary components, including lipids, are provided elsewhere in this book. Therefore, this section focuses primarily on the use of so-called "high-energy" diets, which have become increasingly widespread in aquaculture. These diets are, in reality, "high-oil/fat" diets. They can have consequences for the farmed fish by altering its lipid and fatty acid metabolism, with implications for its health and welfare and, also, for the consumer in terms of the quality and taste of the end product (Bellestrazzi and Mion, 1993) and its health-promoting properties (Sargent and Tacon, 1999).

4.4.1. High-Energy (Fat) Diets

High-energy (fat) diets are formulated to increase the growth performance of the fish for a given amount of feed by maximally exploiting the protein-sparing effect of high-energy lipid and allowing as much of the dietary protein as possible to be converted into muscle protein. Although protein sparing by dietary lipid is well documented, the limits to its effectiveness have not been accurately defined for any fish species so that recent dietary formulations have tended to continue the upward trend in dietary lipid. In many cases this has successfully increased weight gains. However, the use of increasingly high-lipid diets is not without controversy, and

it is debatable whether the protein-sparing effect alone accounts for all of the weight gains observed.

A problem in considering the effects of high-energy diets in aquaculture is that what precisely constitutes a high-energy or high-fat diet is all too often undefined. Few data are available in the literature, and because commercial pressure to increase growth rates and reduce production times is the main rationale behind high-energy diets, most information concerns studies on the four main species farmed in Europe: Atlantic salmon, rainbow trout, gilthead sea bream, and sea bass (*Dicentrarchus labrax*). In rainbow trout, dietary lipid at 21% increased growth over that of fish consuming diets with 8 and 11% dietary lipid (Luzzana *et al.,* 1994). In brown trout (*Salmo trutta*) a diet containing 29% lipid increased the growth rate compared to a diet containing 21% lipid (Arzel *et al.,* 1993). In Atlantic salmon, even higher lipid levels have been investigated and diets containing 47 and 38% lipid both increased growth in comparison to a diet containing 31% lipid (Hemre and Sandnes, 1999). In a recent study with sea bass, a diet of 19% lipid showed increased growth compared with diets containing 11 and 15% dietary lipid (Lanari *et al.,* 1999). A limit to the growth-promoting effect of high dietary lipid was indicated in the study on sea bass by Peres and Oliva-Teles (1999) with sea bass, where the growth rate was higher in fish on a diet containing 24% lipid compared to fish on diets with lower (12 and 18%) and higher (30%) lipid levels. An indication that the growth-promoting effects of dietary lipid may vary with the stage of development is the observation that increasing the dietary lipid from 12 to 20% did not alter the growth of sea bass larvae (Salhi *et al.,* 1994). However in sea bass fingerlings, growth and protein sparing were both improved by increasing the dietary lipid from 9 to 15% (Vergara *et al.,* 1996). The importance of optimising ration size when feeding high-energy diets to avoid unwanted adiposity was also investigated in sea bream (Company *et al.,* 1999).

4.4.2. Dietary Lipid Levels and Tissue Lipid Levels

It has long been known that there is a strong relationship between the dietary lipid levels and the levels of lipid in the carcass of fish (Cowey, 1993). Deposition of excess lipid in the carcass will clearly be a more serious problem in those species that tend to store lipid in the flesh, although it appears that flesh lipid levels are increased in most species when fed high-fat diets. Many recent studies have shown that a potential and, perhaps, detrimental effect of high-fat diets is the deposition of excess lipid in the flesh. Thus, feeding diets with high lipid levels has been shown to increase flesh lipid levels in freshwater fish, including catfish (Stowell and Gatlin, 1992; Anwar and Jafri, 1995) and silver perch (*Leiopotherapon bidyanus*) (Anderson

and Arthington, 1989), in marine fish such as turbot (Stephan *et al.,* 1996), Atlantic mackerel (*Scomber scombrus)* (Hemre *et al.,* 1997), sea bass (Catacutan and Coloso, 1995), and red drum (*Sciaenops ocellatus*) (Williams and Robinson, 1988), and in salmonids including brown trout (Arzel *et al.,* 1993), rainbow trout (Luzzana *et al.,* 1994; Weatherup *et al.,* 1997; Dias *et al.,* 1999), chinook salmon (*Oncorhychus tshawytscha*) (Silver *et al.,* 1993), and Atlantic salmon (Bell *et al.,* 1998; Hemre and Sandnes, 1999).

High-energy feeds have recently been used widely in the salmon industry, where the upper level for dietary lipid in the 1970s was about 20%, whereas in the late 1990s this level had almost doubled, with Einan and Roem (1997) suggesting a diet with a lipid level of 35% to be optimal for growth of Atlantic salmon. However, the Atlantic salmon industry, particularly the processing and retailing sectors, now recognizes the problem of deposition of excessive dietary lipid in the flesh as a major issue relating to carcass and product quality (Hillestad *et al.,* 1998). Increased flesh lipid in salmon can lead to problems of so-called "free oil," reduced visualization of carotenoid pigment, and subsequent consumer and processor resistance to the oily texture, poor smoking performance, and potential rancidity problems (Bell *et al.,* 1998; Refsgaard *et al.,* 1998; Hillestad *et al.,* 1998). It is particularly interesting that, in salmon consuming a diet of a given oil content, the level of lipid in the flesh can vary considerably between populations and between individuals in the same population (Bell *et al.,* 1998; Refsgaard *et al.,* 1998). For example, it was observed that the lipid levels in individual salmon receiving a diet containing 28% lipid could vary from less than 5% to more than 18% of the wet weight of the flesh (Bell *et al.,* 1998). It is not clear to what extent environmental and/or genetic factors contribute to the observed biological variation (Olsen and Skjervold, 1995; Bell *et al.,* 1998).

4.4.3. Dietary Lipid Levels and Essential Fatty Acids

As well as providing energy, dietary lipid is also important as the source of essential fatty acids (EFA). The precise nature of the EFA and their absolute dietary requirements vary with species and are described in Section 4.5. However, it is also apparent that the quantitative requirement for EFA may vary with the total dietary lipid level, and this may also vary with the stage of development (Izquierdo, 1996). For instance, the requirement for *n*-3 HUFA appeared to increase as the level of lipid in the diet increased in both red sea bream (*Pagrus major*) fingerlings (Takeuchi *et al.,* 1992a) and yellowtail (*Seriola quinqueradiata*) fingerlings (Takeuchi *et al.,* 1992b). However, in contrast, there was no apparent variation in the *n*-3 HUFA requirement of larval gilthead sea bream as the dietary lipid level increased from 12 to 20% (Salhi *et al.,* 1994).

4.4.4. Dietary Lipid Levels and Antioxidant Requirements

Feeding very high levels of unsaturated lipid can increase the prooxidant stress on fish consuming the diets. Thus, as the lipid content of the diet increases, the dietary *n*-3 HUFA levels also increase and the resulting increased unsaturation index of the diet must be balanced by an increasing dietary antioxidant content, especially vitamin E (tocopherol). It was shown in Atlantic salmon that, as the dietary lipid increased, vitamin E levels in the flesh decreased (Hemre and Sandnes, 1999). It has also been shown in tilapia that the vitamin E requirement increased as the level of dietary lipid increased (Satoh *et al.,* 1987). For instance, in blue tilapia (*Oreochromis aureus*) the vitamin E requirement at 3% dietary lipid was 10 mg/kg diet, whereas at 6% dietary lipid the vitamin E requirement was 25 mg/kg, when both diets contained 120 mg/kg butylated hydroxyanisole (BHA) (Roem *et al.,* 1990). The flesh vitamin E level has been shown to influence the development of rancid taste after slaughter in both Atlantic salmon (Waagbo *et al.,* 1993) and cultured mackerel (Hemre *et al.,* 1997). Muscle homogenates from rainbow trout and sea bass fed high-fat diets were more susceptible to lipid peroxidation than fish fed low-fat diets (Dias *et al.,* 1999). The relationship between dietary lipid and fatty acid contents and dietary antioxidant requirements is discussed more fully in Section 4.7.

4.4.5. Dietary Lipid Levels, Lipogenesis, and Fatty Livers

The level of dietary lipid has other effects on lipid metabolism in fish including modulation of lipogenesis. It has long been established that increased dietary lipid levels depress *de novo* fatty acid synthesis through inhibition of several enzymes involved in hepatic lipogenesis, including acetyl coenzyme A carboxylase, fatty acid synthetase, and NADPH-generating enzymes such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Sargent *et al.,* 1989). The perceived view was that, in comparison to mammals, where dietary lipid as low as 2.5% can cause inhibition of lipogenesis, inhibition of lipogenesis in fish was obvious only with diets containing in excess of 10% lipid (Sargent *et al.,* 1989). With almost all species a diet would not be considered "high energy" with a lipid level lower than 10%, so it can safely be assumed that high-energy diets inhibit lipogenesis in fish. For instance, in hepatopancreas from carp fed a diet containing 18% lipid, there were decreased levels of lipogenic, gluconeogenic, and amino acid-degrading enzyme activities in comparison with fish fed diets containing 9% lipid (Shimeno *et al.,* 1995). The activities of various hepatic enzymes including fatty acid synthetase, glucose-6-phosphate dehydrogenase, and malic enzyme were reduced by decreasing the ratio of digestible

protein to digestible energy (DP/DE) through an increase in dietary lipid in rainbow trout and sea bass (Dias *et al.,* 1999). These enzyme activities were also decreased in yellowtail liver (Shimeno *et al.,* 1996) and carp hepatopancreas (Kheyyali *et al.,* 1989). Lipogenesis was also decreased in rainbow trout liver in response to increased dietary ratios of lipid to carbohydrate (Corraze *et al.,* 1993a; Brauge *et al.,* 1995). At an enzyme kinetic level, a high-fat diet in rainbow trout was shown to cause a significant decrease in the specific activity, catalytic efficiency, and V_{max} of hepatic glucose-6-phosphate dehydrogenase, whereas the K_m was unaffected (Sanchez-Muros *et al.,* 1996).

High-fat diets have also been shown to affect liver histology in ways consistent with the development of fatty liver pathology. Thus, increased fatty infiltration of liver has been reported in sunshine bass (*Morone chrysops* × *M. saxatilis*) fed a diet containing 16% lipid in comparison with a diet containing 13% lipid (Gallagher, 1996). Compared to fish fed diets containing 15 and 22% lipid, sea bream fed diets containing 27% lipid showed foci of swollen hepatocytes characterized by displaced nuclei and large lipid droplets in the cytoplasm (Caballero *et al.,* 1999).

4.5 Optimal Levels and Ratios of Dietary *n*-3 and *n*-6 Polyunsaturated Fatty Acids

As well as being a major energy source, dietary lipids are also a source of fatty acids required for the synthesis of new cellular lipid for growth and reproduction and for turnover of existing lipid. Therefore, lipid requirements can be broken down into three main categories, gross lipid requirement in terms of energy provision as discussed in Section 4.4, and qualitative and quantitative essential fatty acid requirements, which are the subject of this section.

All vertebrate species have absolute dietary requirements for certain PUFA. If a dietary deficiency occurs, the animal stops growing and reproducing, develops various pathologies, and eventually dies. The various deficiency symptoms and pathologies were summarized in the previous edition of this book (Sargent *et al.,* 1989). The PUFA in question are termed "essential fatty acids" (EFA) and they include members of both the *n*-6 and the *n*-3 series typified by linoleic acid, 18:2*n*-6, and α-linolenic acid, 18:3*n*-3. All vertebrate species probably require both *n*-6 and *n*-3 PUFA, i.e., both *n*-6 and *n*-3 PUFA are EFA for vertebrates, and the biologically active forms of EFA are generally the C_{20} and C_{22} metabolites of 18:2*n*-6 and 18:3*n*-3, *viz.,* 20:4*n*-6 + 22:5*n*-6 and 20:5*n*-3 + 22:6*n*-3, which are often termed HUFA (highly unsaturated fatty acids). Some, but not all, vertebrate species can convert C_{18} PUFA to the higher C_{20} and C_{22} HUFA through a

series of alternating desaturation and chain elongation reactions mediated by microsomal systems containing elongases and Δ^6 and Δ^5 fatty acid desaturases (Cook, 1996), as described in detail in Section 4.3. In species that cannot perform these conversions, the C_{20} and C_{22} HUFA themselves are dietary EFA and their C_{18} homologs do not satisfy EFA requirements. In species that can perform the conversions, C_{18}, $C_{20,}$ and C_{22} HUFA can all be termed EFA, with the C_{20} and C_{22} HUFA being more effective nutritionally than their C_{18} counterparts. Although the foregoing statements apply to all vertebrate species including fish, the extent to which they apply quantitatively to a given species varies widely. Therefore, a vital area in lipid nutrition in fish is the provision of sufficient amounts of the correct EFA to satisfy the requirements for normal growth and development, requirements that can vary quantitatively during the life of the fish. As noted in Section 4.3, competitive interactions exist between the *n*-6 and the *n*-3 PUFA, in terms of both the elongation and desaturation reactions converting C_{18} to C_{20} and C_{22} PUFA, and the direct action of PUFA as occurs in eicosanoid production. Therefore, accurate definition of EFA requirements for a given fish species involves determining not only the absolute requirements of each series of PUFA, but also the optimal balance between the two series. Reflecting its importance, this area has been the subject of several reviews in recent years (Sargent *et al.,* 1995a, 1997, 1999a, b; Izquierdo, 1996).

4.5.1. Marine Fish

Current estimates of the EFA requirements of marine fish species investigated so far indicate that the *n*-3 EFA requirements can be met only by 20:5*n*-3 together with 22:6*n*-3, often collectively called the *n*-3 HUFA (Tables 4.1 and 4.2). This situation is the logical result of adaptations to a combination of the predominant PUFA in the marine food web and the carnivorous lifestyle of virtually all the marine species investigated so far, including those listed in Tables 4.1 and 4.2. Thus, the PUFA in the marine food web are dominated by *n*-3 HUFA originating from the primary producers in the marine food web, *viz.*, the marine algae that constitute the phytoplankton and have 20:5*n*-3 and 22:6*n*-3 in abundance, with 18:3*n*-3 and 18:2*n*-6 being less prominent (Sargent *et al.,* 1995b–d). Moreover, the great majority of marine fish, including all species currently farmed or under development for farming, are carnivores, consuming a predominantly piscine natural diet for most of their life history. Carnivorous marine fish consume smaller fish that, *a priori,* are rich in 20:5*n*-3 and 22:6*n*-3 derived from phytoplankton via zooplankton and, consequently, have no need to convert their very limited dietary intake of 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3. It appears that the capacity for this conversion has effectively been lost during evolution.

Table 4.1

Essential Fatty Acid (EFA) Requirements of Larval and Early Juvenile Stages of Some Marine and Freshwater Fish[a]

Species	EFA	% of dry diet	Ref.[b]
Freshwater			
Carp (*Cyprinus carpio*)	*n*-6 PUFA	1(0.25% 18:2*n*-6)	1
	n-3 PUFA	~0.05	
Rainbow trout (*Oncorhynchus mykiss*)	DHA essential	?	2
Striped bass (*Morone saxatilis*)	18:3*n*-3	?	
	n-3 HUFA	>0.5	3
Marine			
Cod (*Gadus morhua*)	EPA	?	4
	DHA	~1	5
Gilthead sea bream (*Sparus aurata*)	*n*-3 HUFA	5.5 (DHA:EPA = 0.3)	6
	n-3 HUFA	1.5 (DHA:EPA = 2)	7
	n-3 HUFA	1.5 (in phospholipid)	8
	DHA:EPA	~2	9
Red sea bream (*Pagrus major*)	*n*-3 HUFA	2.1 (with 1.0% DHA)	10
	DHA	1.0–1.6	10
	EPA	2.3	10
Striped jack (*Pseudocaranx dentex*)	DHA	1.6–2.2	11
	EPA	<3.1	11
Yellowtail (*Seriola quinqueradiata*)	*n*-3 HUFA	3.9 (DHA:EPA = 0.5)	12
	DHA	1.4–2.6	12
	EPA	3.7	12
Mahimahi (*Coryphaena hippurus*)	*n*-3 HUFA	0.6–1.0	13
Turbot (*Scophthalmus maximus*)	DHA required	?	14

[a] DHA, docosahexaenoic acid (22:6*n*-3); EPA, eicosapentaenoic acid (20:5*n*-3); HUFA, highly unsaturated fatty acids.

[b] *Key to references:* (1) Radunzneto *et al.* (1996); (2) Wirth *et al.* (1997); (3) Webster and Lovell (1990); (4) Zheng *et al.* (1996); (5) Takeuchi *et al.* (1994); (6) Rodriguez *et al.* (1994a); (7) Rodriguez *et al.* (1998); (8) Salhi *et al.* (1999); (9) Rodriguez *et al.* (1994b); (10) Furuita *et al.* (1996a); (11) Takeuchi *et al.* (1996); (12) Furuita *et al.* (1996b); (13) Ostrowski and Kim (1993); (14) Reitan *et al.* (1994).

A similar situation exists in extreme terrestrial carnivores such as the cat (Rivers *et al.,* 1975). It is likely that no marine fish species can convert any substantial amount of 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3. Therefore, the *n*-3 and *n*-6 EFA in marine fish species, including all the major cultured species such as sea bream, sea bass, turbot, and halibut (*Hippoglossus hippoglossus*), are the HUFA 20:4*n*-6, 20:5*n*-3, and 22:6*n*-3.

Juveniles of the golden gray mullet, *Liza aurata,* are well established as predominantly herbivorous in the wild. Mourente and Tocher (1993b) established that juvenile *Liza aurata* have a very limited ability to convert C_{18}

Table 4.2

Essential Fatty Acid (EFA) Requirements of Older Juvenile and Preadult Marine and Freshwater Fish[a]

Species	EFA	% of dry diet	Ref.[b]
Freshwater			
Rainbow trout (*Oncorhyncus mykiss*)	18:3*n*-3	0.7–1.0	1
	n-3 HUFA	0.4–0.5	2
Chum salmon (*Oncorhyncus keta*)	18:2*n*-6 & 18:3*n*-3	1.0 of each	3
Coho salmon (*Oncorhyncus kisutch*)	18:2*n*-6 & 18:3*n*-3	1.0 of each	4
Cherry salmon (*Oncorhyncus masou*)	18:3*n*-3 or *n*-3 HUFA	1.0	5
Arctic charr (*Salvelinus alpinus*)	18:3*n*-3	1.0–2.0	6
Carp (*Cyprinus carpio*)	18:2*n*-6	1.0	7
	18:3*n*-3	0.5–1.0	7
Grass carp (*Ctenopharyngodon idella*)	18:2*n*-6 & 18:3*n*-3	1.0 & 0.5	8
Tilapia			
Oreochromis zilli	18:2*n*-6	1.0	9
O. nilotica	18:2*n*-6	0.5	10
Eel (*Anguilla japonica*)	18:2*n*-6 & 18:3*n*-3	0.5 of each	11
Ayu (*Plecoglossus altivelis*)	18:3*n*-3 or 20:5*n*-3	1.0	12
Milkfish (*Chanos chanos*)	18:2*n*-6 and 18:3*n*-3	0.5 of each	13
Channel catfish (*Ictalurus punctatus*)	18:3*n*-3	1.0–2.0	14
	n-3 HUFA	0.5–0.75	14
Whitefish (*Coregonus laveratus*)	*n*-3 HUFA	0.5–1.0	15
	18:3*n*-3	>1.0	16
Sheatfish (*Silurus glanis*)	18:3*n*-3	1.0	17
Striped bass (hybrid)	*n*-3 PUFA	1.0	18
Marine			
Turbot (*Scophthalmus maximus*)	*n*-3 HUFA	0.8	19
	AA	~0.3	20
Red sea bream (*Pagrus major*)	20:5*n*-3 or *n*-3 HUFA	0.5	21
	20:5*n*-3	1.0	22
	22:6*n*-3	0.5	22
Gilthead sea bream (*Sparus aurata*)	*n*-3 HUFA	0.9 (DHA:EPA = 1)	23
	n-3 HUFA	1.9 (DHA:EPA = 0.5)	24
	DHA:EPA	0.5	25
Striped jack (*Pseudocaranx dentex*)	22:6*n*-3	1.7	26
Sea bass (*Dicentrarchus labrax*)	*n*-3 HUFA	1.0	27
Yellowtail flounder (*Pleuronectes ferrugineus*)	*n*-3 HUFA	2.5	28
Silver bream (*Rhabdosargus sarba*)	*n*-3 HUFA	1.3	29
Korean rockfish (*Sebastes schlegeli*)	*n*-3 HUFA	0.9	30
	EPA or DHA	1.0	31
Red drum (*Sciaenops ocellatus*)	*n*-3 HUFA	0.5–1.0 (0.3-0.6 EPA + DHA)	32

[a] DHA, docosahexaenoic acid (22:6*n*-3); EPA, eicosapentaenoic acid (20:5*n*-3); HUFA, highly unsaturated fatty acids.

[b] *Key to references:* (1) Castell *et al.* (1972); (2) Takeuchi and Watanabe (1976); (3) Takeuchi *et al.* (1979); (4) Yu and Sinnhuber (1979); (5) Thongrod *et al.* (1990); (6) Yang *et al.* (1994); (7) Takeuchi and Watanabe (1977); (8) Takeuchi *et al.* (1991); (9) Kanazawa *et al.* (1980); (10) Takeuchi *et al.* (1983); (11) Takeuchi *et al.* (1980); (12) Kanazawa *et al.* (1982); (13) Bautista and de la Cruz (1988); (14) Satoh *et al.* (1989); (15) Thongrod *et al.* (1989); (16) Watanabe *et al.,* (1989); (17) Borgut *et al.* (1998); (18) Gatlin *et al.* (1994); (19) Gatesoupe *et al.* (1977); (20) Castell *et al.* (1994); (21) Yone (1978); (22) Takeuchi *et al.* (1990); (23) Kalegeropulos *et al.* (1992); (24) Ibeas *et al.* (1994b); (25) Ibeas *et al.* 1997; (26) Takeuchi *et al.* (1992c); (27) Coutteau *et al.* (1996); (28) Whalen *et al.* (1999); (29) Leu *et al.* (1994); (30) Lee *et al.* (1993); (31) Lee *et al.* (1994); (32) Lochmann and Gatlin (1993).

PUFA to C_{20} and C_{22} HUFA *in vivo,* being entirely similar to the carnivorous turbot in this respect. The abundance of 20:5*n*-3 and 22:6*n*-3 in marine algae (see Sargent *et al.,* 1995b–d) ensures that even a herbivorous marine fish can receive a sufficiency of these HUFA in its natural diet and has little or no need to convert C_{18} PUFA to C_{20} and C_{22} HUFA, so that these HUFA are its EFA.

4.5.1.1. Embryos and Yolk Sac Larvae

The lipid and fatty acid compositions of fish eggs and lipid metabolism during embryonic and early yolk sac larval stages were described in the previous edition of this book (Sargent *et al.,* 1989). Therefore, this section only briefly summarizes these areas, focusing on new data related mainly to similar studies on additional species which have furthered our understanding of the role of lipids and fatty acids in the earliest life stages of marine fish.

Although the lipid content and lipid class composition of fish eggs vary considerably with species, in many marine fish the eggs have relatively low levels of lipid (usually <5% of the wet weight) which is predominantly polar lipids (60–90% of the total lipid on average) (Sargent *et al.,* 1989). This situation is found in eggs from herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), whiting (*Merlagus merlangus*), saithe (*Pollachius virens*) (Tocher and Sargent, 1984), cod (*Gadus morhua*) (Fraser *et al.,* 1988), and halibut (Falk-Petersen *et al.,* 1989). Gilthead sea bream (Mourente and Odriozola, 1990; Ronnestad *et al.,* 1994), Senegal sole (*Solea senagalensis*) (Vazquez *et al.,* 1994), common dentex (*Dentex dentex*) (Mourente *et al.,* 1999), sea bass (Ronnestad *et al.,* 1998), and turbot (Silversand *et al.,* 1996) have higher levels (>50%) of neutral lipids, with the eggs from all these species having oil globules similar to those found in the relatively lipid–rich eggs of sand eel (*Ammodytes lancea*) and capelin (*Mallotus villosus*) (Tocher and Sargent, 1984). The polar lipids of marine fish eggs are dominated by phospholipids, particularly phosphatidylcholine (PtdCho), followed by phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), and phosphatidylinositol (PtdIns). The neutral lipids are mainly triacylglycerols and smaller amounts of cholesterol, although some lipid–rich eggs have globules that are predominantly steryl and/or wax esters (Sargent *et al.,* 1989).

The total lipid of eggs from most marine fish studied is rich in *n*-3 HUFA, usually present at higher percentages than in the total lipid of other tissues, probably reflecting in part the preponderance of phospholipids in eggs, which, like phospholipids in other tissues, are generally higher in HUFA than neutral lipids (Sargent *et al.,* 1989; Rainuzzo, 1993). Therefore, the eggs of herring, haddock, cod, whiting, saithe, plaice, halibut, turbot,

Senegal sole, and common dentex all contain high levels of 20:5*n*-3 and 22:6*n*-3 (Tocher and Sargent, 1984; Falk-Petersen *et al.,* 1989; Rainuzzo, 1993; Parrish *et al.,* 1993; Vazquez *et al.,* 1994; Evans *et al.,* 1996; Silversand *et al.,* 1996; Mourente *et al.,* 1999). The fatty acid compositions of egg total lipid vary with species, again reflecting partly differences in lipid class compositions, but are relatively more resistant to dietary changes than the fatty compositions of other tissues (Sargent *et al.,* 1989). For instance, the levels of 22:6*n*-3 and total *n*-3 HUFA were almost identical between wild and captive turbot eggs, whereas the levels of 18:2*n*-6 and, to a lesser extent, 20:1*n*-9 were much higher in eggs from captive fish (Silversand *et al.,* 1996). These data support the previously appreciated resistance of egg composition to dietary changes but, at the same time, show that egg fatty acid composition can be affected by diet as evinced by elevated 18:2*n*-6 (Silversand *et al.,* 1996). The effects of broodstock diet on the fatty acid composition of marine fish eggs and its relationship to egg quality criteria are described in Section 4.5.1.4. In addition, the fatty acid composition of halibut eggs has been shown to vary throughout the spawning season and between first-time spawners and repeat spawners (Evans *et al.,* 1996; Parrish *et al.,* 1993).

Previously, lipid metabolism during embryogenesis and early larval development had been studied in relatively few species (see Sargent *et al.,* 1989). However, in recent years the number of species studied has expanded greatly, to include dolphin fish (*Coryphaena hippurus*) (Ostrowski and Divakaran, 1991), plaice (*Pleuronectes platessa*) turbot (Rainuzzo *et al.,* 1992), gilthead sea bream (Ronnestad *et al.,* 1994), Senegal sole (Vazquez *et al.,* 1994; Mourente and Vazquez, 1996), cod (Fraser *et al.,* 1988; Finn *et al.* 1995), halibut (Ronnestad *et al.,* 1995), sea bass (Ronnestad *et al.,* 1998), and common dentex (Mourente *et al.,* 1999). These studies establish that the utilization of lipids and fatty acids during embryonic and early larval development varies considerably between species. It had been reported previously that lipids were utilized as an energy source mainly after hatching in red sea bream and flounder (*Pseudopleuronectes americanus*) (Sargent *et al.,* 1989), whereas, in Atlantic herring and cod, phospholipid was utilized during both embryogenesis and to a greater extent during early larval development (Tocher *et al.,* 1985a; Fraser *et al.,* 1988). In dolphin fish, lipids were catabolized throughout the development period but to a greater extent during embryogenesis than larval development (Ostrowski and Divakaran, 1991). Furthermore, in both red sea bream and red drum, neutral lipids were the main lipids utilized, whereas polar lipids and specifically PtdCho were catabolized primarily in herring and cod, although neutral lipid utilization increased after hatching (Sargent *et al.,* 1989). It was postulated that catabolism of phospholipids for energy may be a common characteristic of fish eggs that were rich in phospholipids (Sargent *et al.,* 1989). This postulate continues to hold

since PtdCho was also catabolized primarily in the phospholipid-rich eggs of halibut and plaice, but not in turbot eggs where neutral lipids account for more than 50% of total lipid (Rainuzzo *et al.,* 1992; Finn *et al.,* 1995; Ronnestad *et al.,* 1995). In contrast, in marine pelagic eggs that contain higher lipid levels, reflecting high levels of neutral lipid in oil globules or otherwise, such as from sea bream, sea bass, Senegal sole, and dentex, lipids are utilized primarily after hatching and mainly as neutral lipid, whether from the oil globule or otherwise (Ronnestad *et al.,* 1994, 1998; Mourente and Vazquez, 1996; Mourente *et al.,* 1999). Therefore, in marine fish eggs it appears that, in general, lipid utilization occurs to a greater extent after hatching, particularly in neutral lipid-rich eggs, possibly reflecting the greater energy demands of the mobile, free-swimming yolk sac larvae compared to the embryonic egg phase. In relation to specific lipid classes, two main patterns of utilization are apparent in marine fish, obviously directly related to egg lipid compositions. Phospholipid-rich eggs tend to utilize phospholipids, particularly PtdCho, whereas neutral lipid-rich eggs utilize primarily triacylglycerols and also steryl and wax esters where present. Another relatively common feature observed during development is the conservation and/or synthesis of PtdEtn, as reported in both the phospholipid-rich eggs of cod (Fraser *et al.,* 1988), plaice, and halibut (Rainuzzo *et al.,* 1992; Ronnestad *et al.,* 1995) and the neutral lipid-rich eggs of turbot (Rainuzzo *et al.,* 1992), Senegal sole (Mourente and Vazquez, 1996), and dentex (Mourente *et al.,* 1999). This results in a decrease in and normalization of the PtdCho:PtdEtn ratio as development proceeds, from the high values seen in most marine fish eggs to the values normally observed in fish tissues. This is particularly the case in phospholipid-rich eggs, dominated by PtdCho, where PtdCho is catabolized during embryogenesis.

Catabolism of lipids, whichever class, results in the release of free fatty acids, which can either be utilized for energy or reacylated back into lipid pools for other uses, which, during embryogenesis and early larval development, can be for the formation of rapidly developing larval tissues. In *n*-3 HUFA-rich marine eggs, it is perhaps obvious that PUFA and HUFA will be catabolized for energy, particularly in phospholipid-rich eggs. Catabolism of HUFA, both 22:6*n*-3 and 20:5*n*-3, for energy, has been reported in cod (Finn *et al.,* 1995), halibut (Ronnestad *et al.,* 1995), Senegal sole (Vazquez *et al.,* 1994; Mourente and Vazquez, 1996), and dentex (Mourente *et al.,* 1999). In dentex, PUFA in both PtdCho and triacylglycerol were utilized, along with other fatty acids, generally in line with their order of abundance (Mourente *et al.,* 1999). Similarly, in cod the fatty acids in PtdCho were catabolized nonselectively (Finn *et al.,* 1995). However, in an early study in Atlantic herring, many of the PUFA liberated by the catabolism of PtdCho were selectively retained in the neutral lipid pool (Tocher *et al.,* 1985b). Although

also utilized for energy, PUFA were relatively conserved in comparison with saturated and monounsaturated fatty acids during development of Senegal sole (Mourente and Vazquez, 1996). In cod, monounsaturated fatty acids in triacylglycerol were selectively catabolized in comparison with PUFA (Finn *et al.,* 1995). In halibut, 22:6*n*-3 was a quantitatively important fuel, with almost 40% of the 22:6*n*-3 from PtdCho hydrolysis being catabolized, but with over 60% of the 22:6*n*-3 being selectively retained at the same time in PtdEtn (38%) and neutral lipids (23%) (Ronnestad *et al.,* 1995). Fraser *et al.*(1988) had reported earlier that about 33% of the 22:6*n*-3 released during PtdCho catabolism in cod eggs was incorporated into TAG and steryl esters. Similar retention of 22:6*n*-3 in PtdEtn was observed in Senegal sole and dentex (Mourente and Vazquez, 1996; Mourente *et al.,* 1999). Some selective retention of 20:5*n*-3 was also reported in halibut (Ronnestad *et al.,* 1995) and 20:4*n*-6 was selectively retained during development of Senegal sole (Mourente and Vazquez 1996). Based on studies mainly with plaice, Rainuzzo (1993) suggested that utilization of HUFA, including 22:6*n*-3, occurred mainly in earlier stages of development when yolk was still present, whereas, later, in nonfeeding larvae, 22:6*n*-3 and 20:4*n*-6 were selectively retained in PtdEtn, at the expense of other fatty acids, including 20:5*n*-3, which were mainly catabolized. Thus, HUFA, as well as having well-established roles in membrane structure and function, can also serve as important energy sources during embryonic and early larval development of marine fish.

4.5.1.2. Larvae and Early Postlarvae

Marine fish larvae pose the aquaculturist great problems, due primarily to their small size and their often poorly developed digestive system, which has hampered the development of appropriate fabricated first feeds. Ten years ago, the technical problems associated with presenting defined micro diets to marine fish larvae had prevented the accurate determination of lipid and EFA requirements in these crucial early life stages. Even now, recent studies on the development of fabricated, defined microdiets for first-feeding marine fish larvae are relatively few (Salhi *et al.,* 1994, 1999; Bessonart *et al.,* 1999; Halfyard *et al.,* 1999). Poor acceptance of fabricated first feeding diets may be related to the particles presented having an unsuitable size spectrum or to their aggregation or settling properties prior to consumption. It is also likely that the larvae prefer to capture and ingest moving prey or require specific gustatory stimuli to ingest captured prey. As a result, live feeds are currently still the option of choice in most situations until the larvae are large enough to be maintained on a fabricated diet. However, the use of live feeds has presented significant problems in accurately defining lipid and EFA requirements in marine fish larvae, although this is an area that has received considerable attention in recent years (Brown *et al.,* 1989,

1997; Brown and Jeffrey, 1992; Izquierdo, 1996; Rainuzzo *et al.,* 1997; Reitan *et al.,* 1997; Shansudin *et al.,* 1997; Sargent *et al.,* 1997, 1999a, b; McEvoy and Sargent, 1998; Dhert *et al.,* 1998).

Several types of live feeds of variable sizes have been used, with their specific utilization depending on the size of the larvae at emergence and during the subsequent growth period. The principal live feeds used have been rotifers (*Brachionus* sp.) and brine shrimp (*Artemia* sp.) nauplii. Rotifers, being smaller, are fed to small larvae including very early larval stages. *Artemia* nauplii, being larger, are fed to larger larvae including later developmental stages. The major factor in the choice of these organisms has been their ready availability and ease of culture, with *Artemia* being particularly useful in that they can be packaged, stored, and transported easily in an arrested form of development as dry cysts. However, *Artemia* is a nutritionally poor diet for marine fish larvae in that it generally lacks the *n*-3 HUFA essential for marine larvae, being rich instead in 18:3*n*-3. Because many different strains of *Artemia* exist, one option has been to seek strains that are nutritionally more suitable. Strains with significant levels of 20:5*n*-3 have been reported, but none so far with significant levels of 22:6*n*-3 (Navarro *et al.,* 1991, 1992a, b, 1993a). This limitation is related, at least partly, to the propensity of *Artemia* to retroconvert 22:6*n*-3 to 20:5*n*-3 (Evjemo *et al.,* 1997; Navarro *et al.,* 1999).

Therefore before use, *Artemia* must be "enriched" with *n*-3 HUFA prior to feeding to marine fish larvae and several different strategies can be employed (Dhert *et al.,* 1998; McEvoy and Sargent, 1998). Phytoplankton are the natural food of rotifers and *Artemia,* and provide one method of enriching the animals. Judicious choice of algal species, including a marine *Chlorella* or diatoms such as *Tetraselmis,* which are rich in 20:5*n*-3, or prymnesiophytes such as *Nannochloropsis* sp. or *Isochrysis galbana,* which are rich in 22:6*n*-3, can readily increase the levels of both 20:5*n*-3 and 22:6*n*-3 in rotifers fed algae (Brown *et al.,* 1989, 1992, 1997; Reitan *et al.,* 1993, 1997; Tamura *et al.,* 1993). Such procedures, however, are much less effective in increasing the 22:6*n*-3 levels of *Artemia* nauplii. Nonetheless, the use of "green water" in larval production systems, i.e., marine fish larvae cultured in the presence of one or more specific algal species together with rotifers initially and *Artemia* nauplii later, remains common practice (Navarro and Amat, 1992; Brown and Jeffrey, 1992; Reitan *et al.,* 1993; Mourente *et al.,* 1993; Kashiwakura *et al.,* 1994; Olsen *et al.,* 1997). The procedure can be effective and may involve nutritional and environmental benefits other than EFA nutrition. However, it remains to some extent empirical in that it is difficult to control the levels of *n*-3 HUFA in both the algae and the live feed organisms, which can vary both with time and developmental stage, during culture of the larvae.

Early work supplemented rotifers and *Artemia* nauplii with *n*-3 HUFA used baker's yeast, which had been cultured in media containing fish oil, so-called ω-yeast (Sargent *et al.,* 1989). In recent years, methods for directly enriching

Artemia nauplii and rotifers using *n*-3 HUFA-rich oils have been extensively developed (Dhont *et al.,* 1991; Olsen *et al.,* 1993; Coutteau and Sorgeloos, 1997; Coutteau and Mourente, 1997; Sargent *et al.,* 1997; Dhert *et al.,* 1998; McEvoy and Sargent, 1998; Gara *et al.,* 1998). The general enrichment process involves incubating the *Artemia* nauplii or rotifers with a water-miscible preparation of an *n*-3 HUFA-rich oil for a relatively short period of time, so that the enriching preparation is ingested into the gut of the live animals, which are then immediately fed to the larvae. The procedure is, in effect, a "bioencapsulation" of the enriching oil (Coutteau and Sorgeloos, 1997) (see also Section 4.6). With rotifers it is also possible to use a long-term enrichment period which is combined with growth of the rotifer (Planas and Cunha, 1999). The most commonly used enriching preparations are micellar emulsions of marine fish oils and, as such, are basically triacylglycerol micelles (Izquierdo *et al.,* 1992; Perez *et al.,* 1994; Sargent *et al.,* 1997; Ando *et al.,* 1997). Increased understanding of the lipid and EFA requirements of larval marine fish in recent years has resulted in a trend toward the use of a speciality oil, tuna orbital oil, which has, respectively, high and low levels of 22:6*n*-3 and 20:5*n*-3 (Bell *et al.,* 1996a; McEvoy *et al.,* 1997; Ando *et al.,* 1997; Gara *et al.,* 1998), and ethyl and methyl ester concentrates of 20:5*n*-3 and 22:6*n*-3 (Takeuchi *et al.,* 1992; Rainuzzo *et al.,* 1994). Phospholipid rich preparations have also been used, including triacylglycerol oils emulsified with marine and soya oil lecithins (Rainuzzo *et al.,* 1994; McEvoy *et al.,* 1996, 1997; Tocher *et al.,* 1997; Salhi *et al.,* 1999). Commercial products designed to enrich live feeds for larval culture have included oil emulsions (e.g., Selco, SuperSelco INVE Aquaculture, Lochnsri, Belgium), microcapsules (e.g., Frippak Booster), and dried marine fungi (AlgaMac 2000 and 3000 Aquafauna Biomarine, Hawthorne, CA). The use of live feed enrichment procedures is now widespread both in research on nutritional requirements and in commercial marine larval fish production systems (Ostrowski and Kim, 1993; Mourente *et al.,* 1993; Naess *et al.,* 1995; Nery *et al.,* 1995; Fernandez-Reirez *et al.,* 1995; McEvoy *et al.,* 1996; Blair *et al.,* 1998a, b; Gara *et al.,* 1998).

Using a combination of fabricated microdiets and, predominantly, enriched live feeds, the EFA requirements of the larval and very early juvenile stages of a number of marine fish species have been determined (Table 4.1). The exact levels of EFA reported can vary between studies, dependent upon the precise parameter measured, such as survival, growth, and vitality (Furita *et al.,* 1996), as well as the overall dietary lipid level (Salhi *et al.,* 1994). Even so, one overall impression of the data to date is that larval requirements for *n*-3 HUFA are generally greater than those of juveniles and preadult fish (cf. Table 4.2), with the caveat that there are relatively few species where the requirements at larval and later juvenile stages can be compared directly (Takeuchi *et al.,* 1990, 1992c, 1996; Ibeas *et al.,* 1994a, b; Rodriguez *et al.,*

1994, 1998a; Furuita *et al.,* 1996a; Salhi *et al.,* 1999). A second impression is that the requirement of marine fish larvae for 22:6*n*-3 is usually higher than that for 20:5*n*-3, which means that the EFA requirements are usually satisfied by a lower level of 22:6*n*-3 than can be achieved with 20:5*n*-3 alone (Watanabe, 1993). This may also be the case with later juveniles, but again, direct comparative data are few, although with gilthead sea bream it appears that the optimal dietary ratio of 22:6*n*-3 to 20:5*n*-3 in the larval stage is about 2 (Rodriguez *et al.,* 1994, 1997, 1998a), whereas it is less than 1 in older juveniles (Ibeas *et al.,* 1997). Red drum larvae fed a diet with a ratio of 22:6*n*-3 to 20:5*n*-3 of almost 4 exhibited significantly superior performance in a salinity challenge test (Brinkmeyer and Holt, 1998). Moreover, stress resistance was correlated with levels of 22:6*n*-3 but not with levels of 20:5*n*-3 or total *n*-3 HUFA in mahimahi larvae (dolphin fish, *Coryphaena hippuus*) (Kraul *et al.,* 1993). One reason for the higher dietary requirement for 22:6*n*-3 is for rapidly developing visual and neural tissues, which account for a relatively greater proportion of total body mass in larval stages. The importance of 22:6*n*-3 for the proper development of these tissues has been demonstrated in larval herring (Navarro *et al.,* 1993b, c; Bell *et al.,* 1995a) and in sea bass (Navarro *et al.,* 1997; Bell *et al.,* 1996a). Thus, deficiency of dietary 22:6*n*-3 resulted in larval herring having an impaired ability to capture prey at natural light intensities, presumably due to impaired rod function in their eyes (Bell *et al.,* 1995). The avidity of neural tissues for 22:6*n*-3 was shown by the dramatic increase in this fatty acid in the brains of both turbot and sea bream when larvae of both species were weaned from a diet deficient in 22:6*n*-3 to one rich in 22:6*n*-3 (Mourente *et al.,* 1991; Mourente and Tocher, 1992, 1993c). Therefore, the delivery of sufficient 22:6*n*-3 to developing marine fish larvae is of major importance and is not without problems. Specific issues such as the peroxidation of 22:6*n*-3 in highly aerated live feed cultures (McEvoy *et al.,* 1995) and its retroconversion to 20:5*n*-3 in *Artemia* (Evjemo *et al.,* 1997; Navarro *et al.,* 1999), both of which limit the ultimate level of 22:6*n*-3 that can be delivered to larvae by these techniques, remain to be solved.

In turbot, the early supply of 22:6*n*-3 was found to be essential for correct pigmentation (Reitan *et al.,* 1994) and in Japanese flounder (*Paralichthys olivaceus*) pigmentation success was related to dietary 20:4*n*-6 and also to HUFA levels, including 20:4*n*-6, in neural tissues (Estevez and Kanazawa, 1996; Estevez *et al.,* 1997). Subsequent work on turbot confirmed the essentiality of 22:6*n*-3 for normal pigmentation but also showed that 20:4*n*-6 levels in neural tissue lipids were negatively correlated with pigmentation and that the optimum dietary 20:5*n*-3 level was more dependent on the dietary 20:4*n*-6 than the dietary 22:6*n*-3 levels, indicating the importance of feeding the correct dietary ratio of 22:6*n*-3:20:5*n*-3:20:4*n*-6 (Estevez *et al.,* 1999).

Arachidonic acid, 20:4*n*-6, had previously been shown to influence growth in larval gilthead sea bream (Rodriguez *et al.,* 1994) and excess 20:4*n*-6 (4% of the dry weight of *Artemia* nauplii) was shown to inhibit growth and increase mortality in larval yellowtail (Ishizaki *et al.,* 1998). In contrast, Zheng *et al.* (1996) found that dietary 20:4*n*-6 had no effect on the growth or vitality of larval cod. However, at a fixed dietary *n*-3 HUFA level and fixed ratio of 22:6*n*-3 to 20:5*n*-3, a level of 20:4*n*-6 of up to 1.5 and 1% of the dry weight of the diet was found to improve growth, respectively, in larval sea bream (Bessonart *et al.,* 1999) and larval Japanese flounder (Estevez *et al.,* 1997). Therefore, there is increasing qualitative evidence pointing to the importance and probable essentiality of dietary 20:4*n*-6 for optimal growth and development of marine fish larvae (Rodriguez *et al.,* 1994; Ishizaki *et al.,* 1998; Estevez *et al.,* 1999; Bessonart *et al.,* 1999). However, there are as yet no hard data for the optimal quantitative requirements of 20:4*n*-6 for marine larval fish.

A possible alternative to enrichment of nutritionally deficient live prey such as rotifers and *Artemia* nauplii is to use zooplankton species that naturally have more favorable PUFA and HUFA compositions (Virtue *et al.,* 1995; Shansudin *et al.,* 1997; Evjemo and Olsen, 1997). In recent years this has become an increasingly important area of endeavor. Zooplankton can either be harvested directly from the sea by filtration or be extensively cultured in ponds or tanks, or fish larvae can be introduced into seawater mesocosms enriched with nutrients to stimulate phytoplankton growth and thereby contain natural zooplankton in abundance. In all cases, there are problems associated with the use of essentially wild zooplankton in that their naupliar and early copepodite stages (which are the size required for marine fish larvae culture) generally contain relatively low levels of total lipid (and therefore have a low energy content) whose fatty acid composition can vary seasonally, due largely to changes in the dominant phytoplankton prey species present. In addition, the use of wild zooplankton can introduce potentially pathological organisms. The problem of energy supply can be overcome by cofeeding with energy-rich *Artemia* nauplii and by feeding zooplankton at specific critical periods of development (Naess *et al.,* 1995; McEvoy *et al.,* 1998). Cultured marine copepods have been successfully used to raise larval mahimahi (Kraul *et al.,* 1993) and also in rearing turbot and halibut larvae, where they have been shown to improve pigmentation success significantly (Naess *et al.,* 1995; McEvoy *et al.,* 1998). The increased pigmentation was associated with higher levels of *n*-3 HUFA in the zooplankton-fed larvae, suggesting that pigmentation in flatfish appears to be influenced by dietary fatty acids as well as overall nutritional status (Gara *et al.,* 1998; Olsen *et al.,* 1999). Recent pilot-scale intensive culture of the harpacticoid copepod, *Tisbe* sp., was shown to be more successful than conventional rotifer

feeding for growth and development of larvae of the American plaice (*Hippoglossoides platessoides*) (Nanton and Castell, 1998). However, *Tisbe* was less successful as a first feeding organism for larvae of haddock than rotifers (Nanton and Castell, 1998). This may have been due to the benthic *Tisbe* being unavailable to the pelagic haddock larvae.

4.5.1.3. Juveniles and Adults

Once weaned, an adequate supply of *n*-3 HUFA and, particularly, 22:6*n*-3 is no longer a major problem so that determining the quantitative dietary EFA requirements becomes easier. A summary of the data currently available for the requirements of juvenile and preadult marine fish are listed in Table 4.2. For several species including turbot, red sea bream, sea bass, red drum, and rockfish (*Sebastes schlegeli*), the EFA requirements can be met by levels of *n*-3 HUFA of less than or up to 1% of the dry weight of the diet (Gatesoupe *et al.,* 1977; Yone, 1978; Takeuchi *et al.,* 1990; Lochman and Gatlin, 1993; Lee *et al.,* 1994; Coutteau *et al.,* 1996). Other species appear to require higher levels, such as 1.3, 1.7, and 2.5% for silver bream (*Rhabdosargus sarba*), striped jack (*Pseudocaranx dentex*), and yellowtail flounder (*Pleuronectes ferrugineus*), respectively (Takeuchi *et al.,* 1992c; Leu *et al.,* 1994; Whalen *et al.,* 1999). Several other marine species that are becoming important in mariculture including halibut, cod, and various soles have yet to have their EFA requirements determined quantitatively.

As mentioned previously with larval fish, the quantitative EFA requirements of juvenile marine fish vary with dietary lipid levels (Takeuchi *et al.,* 1992a, b). However, various studies with gilthead sea bream have established that the precise PUFA composition of the diet can also affect quantitative EFA requirements. Thus, the requirements can vary with differing dietary 22:6*n*-3:20:5*n*-3 ratios (Kalogeropulos *et al.,* 1992; Ibeas *et al.,* 1994b, 1996). This is perhaps obvious considering that these acids do not usually have the same EFA value for fish, with 22:6*n*-3 generally having a higher EFA value (Watanabe, 1993). Therefore, at a dietary ratio of 22:6*n*-3 to 20:5*n*-3 of 0.5 (11% lipid in the diet) the requirement for total *n*-3 HUFA was reported to be *circa* 1.9% of the diet (Ibeas *et al.,* 1994b), whereas at a dietary 22:6*n*-3:20:5*n*-3 ratio of 1 (12% lipid in the diet) the requirement was only 0.9% (Kalogeropoulos *et al.,* 1992). However, the requirement may also vary with the size of the fish, in that the fish studied by Ibeas *et al.* (1994) had an initial weight of 42 g, whereas in a later study using diets with the same lipid level and 22:6*n*-3:20:5*n*-3 ratio but with fish of only 11-g initial weight, the requirement was reported as "at least 1%" (Ibeas *et al.,* 1996). Although a 22:6*n*-3:20:5*n*-3 ratio of 2 appears optimal for sea bream larvae and a ratio of 1 gave the lower *n*-3 HUFA requirement in juveniles, a ratio of

22:6*n*-3:20:5*n*-3 of 0.5 gave best growth in sea bream juveniles fed diets containing the same dietary levels of lipid and total *n*-3 HUFA (Ibeas *et al.,* 1997).

As with larvae, the requirement of juvenile marine fish for *n*-6 HUFA, specifically 20:4*n*-6, has not been determined quantitatively. Early studies suggested that 20:4*n*-6 was possibly required for optimal growth, development, and health of turbot juveniles (Bell *et al.,* 1985a). Subsequently, dietary trials with newly weaned fish produced results consistent with the hypothesis that 20:4*n*-6 was essential for turbot and a value of about 0.3% of the dry weight of the diet (between 0.25 and 0.5%) could be estimated under the conditions of the experiment (Castell *et al.,* 1994; Bell *et al.,* 1995b). There have been no more recent studies to further our knowledge of the dietary requirements of juvenile and subadult marine fish for 20:4*n*-6.

4.5.1.4. Broodstock

Broodstock nutrition is vital to producing high-quality eggs and larvae with fatty acid contents optimized to give the developing embryos and larvae the best chance of success (Tandler *et al.,* 1995). The fatty acid and, especially, the lipid class compositions of fish eggs are generally more conserved and relatively less influenced by diet than other tissue lipid and fatty acid compositions, reflecting the importance of specific compositions in the gametes. However, it has become clear in recent years that, despite this tendency toward conservation, it is possible to alter the fatty acid composition of marine fish eggs in relatively small but potentially very important ways.

One possible way of developing ideal broodstock diets is to determine the compositions of eggs from wild broodstock and to try to reproduce these compositions in eggs from captive fish (Silversand *et al.,* 1996; Pickova *et al.,* 1997). In this respect, lipid content, class composition, and fatty acid composition can all be potential determinants of egg quality (Devauchelle and Coves, 1988; Rainuzzo, 1993). An early study with Atlantic halibut had shown no significant differences in lipid and fatty acid compositions between "viable" and "nonviable" eggs (Bruce *et al.,* 1993). However, there have been many studies showing that egg fatty acid compositions can be affected by broodstock diets in various species, including sea bass (Thrush *et al.,* 1993; Bell *et al.,* 1997b) sea bream (Mourente and Odriozola, 1990a; Fernandez-Palacios *et al.,* 1995; Almanso *et al.,* 1999), striped jack (Vassallo-Agius *et al.,* 1998), cod (Silversand *et al.,* 1995), and yellowtail (Verakunpiriya *et al.,* 1996). Several studies have also shown that various egg quality criteria, including hatching and fertilization rates and early survival, were positively correlated with increased levels of *n*-3 HUFA, and interestingly, levels of

20:4*n*-6 in sea bream (Harel *et al.,* 1992; Fernandez-Palacios *et al.,* 1995; Rodriguez *et al.,* 1998b), sea bass (Bruce *et al.,* 1999), and cod (Pickova *et al.,* 1997). Increasingly, work has focused not simply on the overall levels of *n*-3 HUFA and 20:4*n*-6, but also on the 22:6*n*-3 : 20:5*n*-3 ratio in eggs, which has been shown to be positively correlated with egg quality criteria (Pickova *et al.,* 1997). What is required is to establish the optimum ratio of 22:6*n*-3 : 20:5*n*-3 : 20:4*n*-6 in eggs (J. G. Bell *et al.,* 1997; Bruce *et al.,* 1999).

The dynamic mechanisms underlying the ultimate effects of broodstock diet in swim-up larvae are beginning to be investigated. Thus, the relationships between broodstock diet composition and broodstock tissue compositions and subsequent egg compositions and egg quality have been studied in sea bream (Harel *et al.,* 1992), as have the effects of diet on lipid metabolism during embryonic (Mourente and Odriozola, 1990a) and subsequent larval development (Mourente and Odriozola, 1990b). Similarly, the relationships among diet, vitellogenin composition, and final egg compositions were studied in cod (Silversand *et al.,* 1995). The timing of dietary intervention in relation to season has also been studied in sea bass (Navas *et al.,* 1997; Carnevali *et al.,* 1998).

Much less work has been done on male broodstock, despite the fact that all animal sperm are normally rich in 22:6*n*-3, indicating a possible role for this fatty acid in sperm function (Tinoco, 1982). Work with females suggests that male broodstock diet could influence the level of 22:6*n*-3 in sperm and, therefore, have potential effects on sperm quality possibly leading to effects on fertilization success. In humans, 22:6*n*-3 levels were significantly lower in sperm from infertile patients compared with normal individuals (Zalata *et al.,* 1998). In a study on fish, sperm from wild sea bass was compared with sperm from captive sea bass fed either a commercial pellet diet or trash fish (Bell *et al.,* 1996b). Whereas the sperm from wild males and captive males fed the trash fish diet were similar, the sperm from fish fed the pelleted diet had an elevated ratio of 20:5*n*-3 to 20:4*n*-6, which was argued may have consequences for reproductive performance (Bell *et al.,* 1996b).

4.5.2. Freshwater Fish

Current estimates of EFA requirements for those freshwater fish species studied so far indicate that the *n*-3 EFA requirements can generally be met by 18:3*n*-3 (Tables 4.1 and 4.2). Since 20:5*n*-3 and 22:6*n*-3 are the biologically active forms of *n*-3 EFA in vertebrates, we can deduce that 18:3*n*-3 is converted to 20:5*n*-3 and 22:6*n*-3 in most freshwater fish cited in Tables 4.1 and 4.2. However, the situation is less straightforward for freshwater than marine fish (reviewed by Sargent *et al.,* 1989). The differences between marine and freshwater fish in Tables 4.1 and 4.2 can generally be accounted

for by considering the natural diets of the species and also whether the species is herbivorous, omnivorous, or carnivorous. Freshwater microalgae unlike marine microalgae generally have 18:3*n*-3 rather than 20:5*n*-3 and 22:6*n*-3 as their principal PUFA (Ahlgren *et al.,* 1992). In addition, 18:2*n*-6 is not prominent in marine microalgae but it can be abundant in freshwater microalgae (Ahlgren *et al.,* 1992). The major PUFA in the green leaves of terrestrial and freshwater plants is 18:3*n*-3 and 18:2*n*-6 is abundant in the seed oils of plants. Freshwater insects can have substantial amounts of both 20:5*n*-3 and 20:4*n*-6 in their lipids, but 22:6*n*-3 is usually very low or absent and 18:2*n*-6 and 18:3*n*-3 are frequently the major PUFA in this invertebrate group (Stanley–Samuelson *et al.,* 1988; Ogg *et al.,* 1993). Bell *et al.* (1994b) showed that other freshwater invertebrates contained mainly 18:3*n*-3, 18:2 *n*-6, and 20:5*n*-3 as their principal PUFA and Henderson *et al.* (1996) showed that insect larvae (bloodworms) used commercially as fish feed have 18:2*n*-6 as their major PUFA, with 18:3*n*-3, 20:4*n*-6, and 20:5*n*-3 being present only in low amounts. Therefore, although lipids in freshwater organisms are generally less well defined than their marine counterparts, it is clear that C_{18} PUFA are at least as well represented as C_{20} PUFA at the base of the freshwater food webs and that both 18:3*n*-3 and 18:2*n*-6 are present. This correlates with the widespread ability of freshwater fish to convert C_{18} PUFA to the biologically active C_{20} and C_{22} HUFA and with freshwater fish having substantial dietary requirements for both *n*-6 and *n*-3 PUFA. Therefore, most freshwater fish, including many anadromous species such as salmon, possess both the fatty acid Δ^6- and Δ^5-desaturases required for the production of 20:5*n*-3 and 22:6*n*-3 from 18:3*n*-3, and, of 20:4*n*-6 from 18:2*n*-6 (Sargent *et al.,* 1993b, 1995a).

Within the above generalization, it should be noted that at least one freshwater species is now known to be incapable of converting 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3. Henderson *et al.* (1995) have shown that mature pike, *Esox lucius,* an extreme carnivore consuming largely smaller fish does not convert either 18:2*n*-6 or 18:3*n*-3 to their corresponding HUFA to any significant extent. Moreover, studies with caracchoid fish have established that juvenile stages of the herbivorous silver dollar pirhana, *Mylassoma aureum,* reared on oat flakes readily convert 18:2*n*-6 to 20:4*n*-6 and 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3 (Henderson *et al.,* 1996). Juvenile stages of the carnivorous red pirhana, *Serassalmus natteri,* reared on mosquito larvae also readily convert 18:2*n*-6 to 20:4*n*-6 and 18:3*n*-3 to 20:5*n*-3 and probably also 22:6*n*-3. The mosquito larvae have 18:2*n*-6 as their major PUFA and are relatively deficient in C_{20} and C_{22} HUFA. Therefore, a strict carnivore such as the mature pike that consumes fish has little or no ability to convert C_{18} PUFA to the C_{20} and C_{22} HUFA, but a strict carnivore such as the juvenile pirhana that consumes insects (as well as presumably other larval and juvenile fish) has this ability.

The above studies and similar studies with marine species (see Section 4.5.1) emphasize the importance of considering the PUFA content of the natural diets of different species in assessing their EFA requirements. However, the natural diet of a given species can change substantially during development, so that the extent to which a given species can convert C_{18} PUFA to HUFA may also change with development. In particular, it is possible that early developing stages that consume relatively small food items such as insects and zooplankton may readily convert C_{18} PUFA to HUFA, whereas more mature stages that become piscivorous may have lost this ability. Little knowledge is available in this area. However, Atlantic salmon postsmolts readily convert 18:2*n*-6 and 18:3*n*-3 to their higher homologs, even though the fish in question have been farmed on diets rich in C_{20} and C_{22} PUFA for many generations (J. G. Bell *et al.,* 1993, 1997).

4.5.2.1. Embryos and YolkSac Larvae

As with marine fish eggs, the lipid content and lipid class composition of freshwater fish eggs varies between species, but in general freshwater fish eggs have lipid contents in the range 2.5–10% of the wet weight (Henderson and Tocher, 1987). Lower lipid levels (<5%) were found in eggs from roach (*Leucisus rutilis*), perch (*Percia fluviatilis*), northern pike, and tilapia, whereas the eggs of salmon, rainbow trout, striped bass (*Morone saxatilis*), and whitefish (*Coregonus albula*) had lipid contents of >5% (Henderson and Tocher, 1987). As with marine fish eggs, the eggs with higher lipid contents have higher levels of neutral lipids, stored almost invariably in the form of oil globules or droplets, in addition to the phospholipid-rich yolk lipid (Henderson and Tocher, 1987; Wiegand, 1996a). The phospholipids of freshwater fish eggs are generally dominated by PtdCho (Henderson and Tocher, 1987) as in marine fish eggs, but there are exceptions including striped bass eggs, whose phospholipids are reported to contain predominantly PtdIns (Gallagher *et al.,* 1998) and sturgeon (*Acipenseridae* sp.) eggs, which have relatively high PtdEtn (Gershanovich, 1991). Neutral lipids are often triacylglycerols with smaller amounts of cholesterol, as in the eggs from trout, salmon, and ayu (*Plecoglossus altivelis*) (Katoh *et al.,* 1989). However, some lipid-rich eggs can have globules that are predominantly wax esters, as in gourami (*Trichogaster cosby*), or wax and/or steryl esters as in some freshwater Perciformes and striped bass (Henderson and Tocher, 1987; Anderson *et al.,* 1990; Wiegand, 1996a).

The total lipid of eggs from a range of freshwater fish, including trout, perch, roach, whitefish striped bass, walleye (*Stizostedion vitreum*), pike, and Australian freshwater species, contained high levels of total PUFA (Henderson and Tocher, 1987; Anderson *et al.,* 1990; Gunesekera *et al.,* 1999). They were generally rich in *n*-3 HUFA (Henderson and Tocher, 1987),

although not to such high levels as found in some marine fish (Wiegand, 1996a). In contrast, the eggs of most freshwater fish contained higher levels of *n*-6 PUFA, particularly 20:4*n*-6 and 18:2*n*-6, than found in marine fish eggs (Anderson *et al.,* 1990; Wiegand, 1996a). As with marine fish eggs, the specific fatty acid compositions of freshwater fish eggs varies with species and are affected to some extent by diet (Henderson and Tocher, 1987; Katoh *et al.,* 1989). The eggs of wild striped bass and chinook salmon both contained higher levels of *n*-3 HUFA and higher *n*-3:*n*-6, ratios than eggs from equivalent cultured populations (Ashton *et al.,* 1993; Harrell and Woods, 1995). Eggs from cultured Atlantic salmon had an increased level of 20:5*n*-3 and a decreased level of 20:4*n*-6, which greatly increased the ratio of 20:5*n*-3:20:4*n*-6 in the cultured eggs compared to wild eggs (Pickova *et al.,* 1999). Decreased 20:4*n*-6 in eggs from cultured fish in comparison with wild fish was also observed in striped bass (Gallagher *et al.,* 1998). In contrast, the above study with Atlantic salmon recorded similar levels of 22:6*n*-3 in the eggs of wild and cultured fish (Pickova *et al.,* 1999) and levels of 22:6*n*-3 in walleye (*Stizostedion vitreum*) eggs from different geographical populations, including a domesticated population, were more conserved than levels of other HUFA (Czesny and Dabrowski, 1998). There appears to be selection pressure to maintain levels of 22:6*n*-3 in eggs within a species-specific range (Wiegand, 1996a).

There is less information available on lipid and fatty acid utilization during embryonic and early larval development in freshwater fish. However, based on the available data it appears that the patterns of utilization are similar to those in marine fish (see Henderson and Tocher, 1987). Therefore, in general, lipid utilization in freshwater fish eggs can occur during the whole developmental period including embryogenesis, as in pike (Desvilettes *et al.,* 1996) and striped bass (Chu and Ozkizilcik, 1995), although it occurs to a greater extent after hatching in goldfish (*Carassius auratus*) (Wiegand, 1996b) and sturgeon (Gershanovich, 1991). The specific classes utilized again depend largely on the type of eggs with neutral lipid–rich eggs primarily utilizing neutral lipids as in striped bass (Chu and Ozkizilcik, 1995) and triacylglycerol in sturgeon (Gershanovich, 1991), whereas phospholipid-rich eggs such as those from African catfish (*Clarias gariepinus*) utilized primarily PtdCho (Verreth *et al.,* 1994a). Phospholipid was also the predominant lipid catabolized in goldfish (Wiegand, 1996b) but in pike, PtdCho, triacylglycerol, and steryl esters were all catabolized, whereas PtdEtn appeared to be selectively retained or biosynthesised, as was also observed in some marine fish and African catfish (Verreth *et al.,* 1994a; Desvilettes *et al.,* 1997). In rainbow trout, phospholipid was slowly and continuously metabolized, but triacylglycerol was not utilized until after hatching, whereas in Atlantic salmon, triacylglycerol was catabolized throughout

development, with PtdCho also continuously catabolized so that, by swim-up fry stage, the PtdCho:PtdEtn ratio, initially very high, approached that of muscle (Henderson and Tocher, 1987).

As with marine fish and irrespective of lipid class utilized, all types of fatty acids, saturated, monounsaturated, and PUFA, can be metabolized for provision of energy during development of freshwater fish eggs. However, selective retention of HUFA has been quite consistently observed in the relatively few studies of lipid metabolism during development of freshwater fish eggs. In goldfish eggs catabolizing PtdCho, there was a selective incorporation and retention of the released 22:6*n*-3, 20:5*n*-3, and 20:4*n*-6 in neutral lipids, similar to that previously observed with herring (Wiegand, 1996b). During yolk sac absorption in pike, PUFA released by the hydrolysis of PtdCho were incorporated into the larval body (Desvilettes *et al.,* 1997). In the neutral lipid-rich eggs from both trout cod, *Maccullochella macquariensis,* and Murray cod, *Maccullochella peelii peelii, n*-3 HUFA and, especially, 20:4*n*-6 tended to be conserved during development (Gunasekera *et al.,* 1999), whereas saturated and monounsaturated fatty acids were utilized primarily during catabolism of triacylglycerols in sturgeon (Gershanovich, 1991). Therefore, although data are few, utilization of HUFA for energy provision during embryonic and early larval development may not be as important in freshwater fish eggs as it appears to be in marine fish eggs.

4.5.2.2. Larvae and Fry

In many cultured freshwater species, including trout and salmon, the newly hatched larvae or fry are large enough to accept finely ground fabricated pelleted diets whose composition can be strictly defined and controlled to ensure maximal growth and survival of the developing larvae. Therefore, the problem of defining precise EFA requirements for freshwater larvae and fry would appear not to be as great a problem as it is with marine fish. However, because practical aspects of feeding newly hatched freshwater fish are not major problems and, consequently, the salmon, trout, carp, and catfish aquaculture industries have been very successful in rearing high-quality fry, relatively few detailed studies have been conducted. So, as with marine fish larvae, there are few data on the precise EFA requirements of first-feeding freshwater fish larvae and fry (see Table 4.1). Larvae of the anadromous striped bass display significantly higher growth when raised on 20:5*n*-3-rich *Artemia* strains, in comparison with 18:3*n*-3-rich strains (Webster and Lovell, 1990). Salmonids including trout and anadromous salmon also show a higher growth performance when fed the end product HUFA directly than when fed 18:3*n*-3 (Sargent *et al.,* 1989). Indeed, rainbow trout larvae fed *Artemia* nauplii enriched with 18:3*n*-3 and 18:4*n*-3 had poorer growth compared to larvae fed a commercial feed containing *n*-3 HUFA and showed

no sign of conversion of 18:3*n*-3 or 18:4*n*-3 to 22:6*n*-3, suggesting that 22:6*n*-3 may be essential for larval rainbow trout (Wirth *et al.,* 1997). These data suggest that in striped bass and salmonids, the activity of the fatty acid desaturation/elongation pathway in some freshwater fish can be limiting to growth. In contrast, there was no difference in growth of African catfish larvae fed *Artemia* with low or high *n*-3 HUFA levels (Verreth *et al.,* 1994b). Similarly, tilapia (*T. zilli*) larvae fed the freshwater rotifer, *B. calyciflorus,* in conjunction with different microalgae that contained 18:2*n*-6 and 18:3*n*-3 as their only PUFA, displayed only low levels of the C_{18} PUFA but relatively high levels of 22:6*n*-3, indicating that these tilapia larvae readily converted C_{18} PUFA to HUFA (Isik *et al.,* 1999).

4.5.2.3. Juveniles and Adults

A summary of the data currently available for the requirements of juvenile and preadult freshwater fish is given in Table 4.2. As alluded to earlier, freshwater species can be one of three main types, those that require mainly *n*-3 PUFA such as salmonids and whitefish, species that require mainly *n*-6 PUFA such as tilapia, and species that require significant amounts of both such as channel catfish (*Ictalurus punctatus*) and carps (common and grass). Silver perch (*Leiopotherapon bidyanus*) also require both *n*-6 and *n*-3 PUFA, but their quantitative requirements are not known and so are not included in Table 4.2 (Anderson and Arthington, 1989). Again, as described earlier, the C_{18} PUFA, 18:3*n*-3 and 18:2*n*-6, are usually effective in satisfying the EFA requirements of freshwater fish based on various criteria, but with some species, including salmonids and whitefish, *n*-3 HUFA can satisfy the EFA requirements at lower levels than 18:3*n*-3 and can increase growth over that obtained on 18:3*n*-3 alone (Sargent *et al.,* 1989; Thongrod *et al.,* 1989; Watanabe *et al.,* 1989). Similarly, growth in channel catfish is significantly improved by the inclusion of dietary *n*-3 HUFA (Satoh *et al.,* 1989b; Santha and Gatlin, 1991). Requirements of freshwater fish for *n*-6 HUFA, specifically 20:4*n*-6, are unknown, as there are no studies directed at 20:4*n*-6 in comparison with 18:2*n*-6.

It is surprising that the absolute requirement for EFA of Atlantic salmon has not been precisely defined, although comparison with other salmon and salmonid data would suggest a requirement of about 1.0% of 18:3 *n*-3 or perhaps 0–5–1.0% of *n*-3 HUFA (Table 4.2). The requirements of salmon parr may be different because, at the parr stage, Atlantic salmon naturally consume a diet consisting largely of freshwater invertebrates rich in C_{18} PUFA, both 18:3*n*-3 and 18:2*n*-6, and containing some 20:5*n*-3 but little 22:6*n*-3 (Bell *et al.,* 1994b). Atlantic salmon parr fed a diet containing a blend of vegetable oils to provide a fatty acid composition resembling their natural diet showed improved smolting performance in terms of increased

osmoregulatory ability, in comparison with fish fed the fish oil-containing diets normally fed to parr (J. G. Bell *et al.,* 1997). In addition, the hepatic fatty acid desaturation and elongation activities increased during parr–smolt transformation and this increase was significantly reduced by feeding fish oils rich in *n*-3 HUFA (J. G. Bell *et al.,* 1997). Therefore, Atlantic salmon parr may perform better on diets containing both 18:3*n*-3 and 18:2*n*-6 than on diets with high levels of *n*-3 HUFA. Other work with chinook salmon showed that a balance of *n*-6 and *n*-3 PUFA was required for optimal smolt survival (Higgs *et al.,* 1992). Another salmonid studied recently is Arctic charr (*Salvelinus alpinus*), where *n*-3 PUFA are reported as the primary EFA (Olsen *et al.,* 1991), with a requirement of about 1–2% of 18:3*n*-3, but this species also appears to require *n*-6 PUFA, although the requirement is not strictly defined but estimated at about 0.7% of 18:2*n*-6 (Yang and Dick 1993; Yang *et al.,* 1994; Ringoe and Olsen, 1994).

A potentially interesting area in freshwater fish lipid nutrition is the production of hybrids. Tilapia species are one of the few fish reported as having a strict requirement for only 18:2*n*-6 (Kanazawa *et al.,* 1980; Takeuchi *et al.,* 1983). Indeed, it has been shown that dietary 18:3*n*-3 can actually inhibit growth in blue tilapia (Stickney and Wurts, 1986). In contrast, hybrid tilapia, female Nile (*O. niloticus*) crossed with male blue (*O. aureus*), required both 18:2*n*-6 and some *n*-3 HUFA for maximal growth (Chou and Shiau, 1999). Hybrid sea bass, female striped (*M. saxatilis*) crossed with male white (*M. chrysops*), or *vice versa,* commonly known as "sunshine bass," are also interesting as they apparently reflect a mix of a freshwater and a marine species (Greenberg and Harrell, 1992). An earlier study investigating diets with varying ratios of 22:6*n*-3 to 20:5*n*-3, in comparison to an *n*-6 PUFA diet, had suggested than juvenile sunshine bass did not require *n*-3 HUFA for optimal growth since the fish consuming the HUFA-containing diets grew no better than control fish (Greenberg and Harrell, 1992). However, more recent studies have suggested that *n*-3 HUFA are essential for maximal growth, feed efficiency, and survival of sunshine bass and that elongation and desaturation of dietary 18:2*n*-6 and 18:3*n*-3 were not observed (Nematipour and Gatlin, 1993). The precise EFA requirements for striped bass, white bass, and sunshine bass, are not known, but various studies suggest that *n*-3 HUFA at about 1% of the dry weight of the diet improves growth performance in hybrids (Randall Robinette *et al.,* 1997; Gatlin *et al.,* 1994). Together, the above data suggest that hybrid striped bass may have a requirement pattern more like a marine fish.

4.5.2.4. Broodstock

Considering the importance of broodstock nutrition for reproductive performance and egg and larval quality, it is perhaps surprising that there

are relatively few studies in this area on freshwater fish. This is perhaps a reflection of the relative success of the major freshwater fin fish aquaculture industries, including salmon, trout, carp, and catfish, where production of large numbers of high quality eggs has not been a major problem, in contrast to the situation with marine fish. The little research that has been done has tended to indicate a similar scenario to that observed with marine fish, except that the relative importance of different fatty acids may be different. Thus, the fatty acid composition of diets for freshwater fish broodstock can affect the reproductive performance of the females (Santiago and Reyes, 1993; Corraze *et al.,* 1993b) and alter the fatty acid composition of the resultant eggs (Hardy *et al.,* 1990; Yuneva *et al.,* 1990), with consequences for egg quality (Pickova *et al.,* 1999; Czesny and Dabrowski, 1998). The findings are as follows.

Eggs from rainbow trout broodstock fed a corn oil diet contained increased 18:2*n*-6 and lower *n*-3 HUFA than eggs from fish fed a cod liver oil diet, but there was no difference in fecundity or egg viability (Corraze *et al.,* 1993b). In contrast, in Nile tilapia broodstock fed various oils the best reproductive performance was obtained with fish fed a soybean oil diet giving eggs with a high *n*-6:*n*-3 PUFA ratio, whereas fish fed cod liver oil had the best weight gain but the poorest reproductive performance and the lowest *n*-6:*n*-3 PUFA ratio in the eggs (Santiago and Reyes, 1993). Eggs from wild Atlantic salmon had an increased level of 20:4*n*-6, a decreased level of 20:5*n*-3 and a low 20:5*n*-3:20:4*n*-6 ratio compared to eggs from cultured fish, this being associated with increased hatching rate (Pickova *et al.,* 1999). Wild walleye eggs also contained higher levels of 20:4*n*-6 than eggs from a domesticated population and the wild eggs also had increased survival rates (Czesny and Dabrowski, 1998). These data suggest an important role for egg *n*-6 PUFA and, in particular, 20:4*n*-6 in relation to egg quality in at least some freshwater fish. However, there are also data to indicate that *n*-3 HUFA are important in broodstock diets of freshwater fish, as in another study with perch (*Perca fluviatilis*) broodstock fed diets with different levels of *n*-3 HUFA (Abi-ayad *et al.,* 1997). The eggs with the highest level of *n*-3 HUFA showed significantly higher fertilization rates, larval weights at hatch, and larval resistance to osmotic shock stress, in comparison with eggs from fish fed the lowest level of *n*-3 HUFA.

4.6 Dietary Phosphoglycerides: Inositol and Choline

The previous section considered the EFA requirements of fish largely independently of the chemical form in which they are presented to the fish, i.e., the lipid classes into which the fatty acids are esterified. However,

it is now clear that not all lipid classes are equally effective in delivering EFA to fish, especially to fish larvae. This is linked to issues of whether or not fish have a specific requirement for particular lipid classes, especially phosphoglycerides, and this is linked in turn to the fishes' requirements for phosphoglyceride bases such as inositol, choline, and sphingosine. We have recently reviewed these issues (Sargent *et al.,* 1999b) and base the following mainly on that account.

As described in Section 4.5, brine shrimp nauplii are enriched with *n*-3 HUFA by exposing the animals in seawater to micelles of triacylglycerols stabilized with emulsifiers such as lecithin (phosphatidylcholine), these micelles being filtered by the nauplii from the seawater and retained largely as triacylglycerols. The ingested micelles are metabolized by the nauplii as evidenced by the marked retroconversion of 22:6*n*-3 to 20:5*n*-3, which takes place immediately after supplementation (Navarro *et al.,* 1999). Moreover, when methyl and ethyl esters of *n*-3 HUFA (22:6*n*-3 concentrates) are used to supplement the nauplii, these are rapidly converted to triacylglycerols (Takeuchi *et al.,* 1992d; McEvoy *et al.,* 1996). Thus, supplementing oils ingested by the nauplii are at least partially assimilated by their intestinal mucosal cells and metabolized, rather than remaining entirely unassimilated and unchanged in their gut cavities. To our knowledge there has been no formal study of the rates or extents of these processes or how they are determined. Rather, it is normal practice to expose the nauplii to emulsions for fixed periods of between 1 and 24 hr so as to maximize the levels of oil ingested by the nauplii. In our experience (McEvoy *et al.,* 1996) non-supplemented, enrichment-grade brine shrimp nauplii contain circa 12% of their dry weight as total lipid, which is 23% phospholipid and 58% triacylglycerols. Enriching these nauplii for 24 hr with micelles composed of 90% triacylglycerols emulsified with 10% lecithin increases naupliar total lipid to 23% of the dry weight, with 16.5% of the total lipid now being present as phospholipids and 63.6% as triacylglycerols. Such findings are similar to those reported originally by Takeuchi *et al.* (1992d). Thus, supplementation simultaneously alters not only the fatty acid composition of the nauplii, depending on which triacylglycerol oil is used, but also their total lipid contents and composition.

Is there an optimal total lipid content in live larval feeds? and What are its optimal levels of phospholipids and triacylglycerols? The growth of fish larvae and post larvae, including ayu, striped jack, red drum, sea bass, and turbot, is known to be stimulated by including intact phospholipids in the diet (Kanazawa *et al.,* 1981; Kanazawa, 1993; Takeuchi *et al.,* 1992c; Geurden *et al.,* 1997a; Craig and Gatlin, 1997). Such growth stimulating effects are not due to the phospholipids providing EFA since soya lecithin, which lacks *n*-3 HUFA but is rich in 18:2*n*-6, is as effective as lecithin from fish eggs, which is rich in both 22:6*n*-3 and 20:5*n*-3. Moreover, Geurden *et al.* (1995, 1998)

established that the growth stimulating effects of phospholipids on carp larvae could not be mimicked by adding emulsifiers to the diet, or choline or inositol. Carp larvae fed diets deficient in phospholipids accumulate oil droplets, presumably triacylglycerols, in their intestinal mucosae (Fontagne *et al.,* 1998). Based on these studies it was proposed (Geurden *et al.,* 1995, 1997b; Fontagne *et al.,* 1998; Coutteau *et al.,* 1997) that the stimulating effects of phospholipids in larval fish growth were due not to the provision of EFA, choline, or inositol, or to emulsifying effects of phospholipids in the intestine, but to the fish larvae having a limited ability to biosynthesise phospholipids *de novo*. Therefore, the larvae have a partial dietary requirement for intact phospholipids, not least for transporting triacylglycerols from the intestinal mucosal cells into the lymph and thence into the serum as chylomicra and very low-density lipoproteins.

It can be deduced from the foregoing that fish larvae readily reacylate dietary glycerides. This is because the phosphoglycerides of nonsupplemented brine shrimp nauplii are deficient in 22:6*n*-3, whereas the phosphoglycerides of fish larvae fed nauplii supplemented with triacylglycerols rich in 22:6*n*-3 are enriched in 22:6*n*-3, i.e., the *n*-3 HUFA composition of larval body phospholipids reflects its dietary triacylglycerols. Thus, the limitation in phospholipid biosynthesis in the larvae is in forming the glycerophospho-base backbone (i.e., glycerophosphocholine, -ethanolamine, -serine, or -inositol) of the phosphoglyceride molecule (Figs. 4.3 and 4.4). Although not studied so far, the same may well apply to the sphingolipids in terms of linking sphingosine with phosphocholine or sugars (Figs. 4.5 and 4.6). Such apparent limitations in the ability of marine fish larvae to synthesize phospholipids *de novo* may not be surprising since, in their natural environment, larvae ingest live feed whose lipid is predominantly phospholipid. Thus, the larvae will seldom, if ever, be required to biosynthesise phospholipids extensively *de novo*. The same argument, of course, applies to cholesterol and, indeed, to fatty acids in general, including, especially, 20:5*n*-3 and 22:6*n*-3 and, perhaps also, special long-chain moieties such as nervonic acid, 24:1*n*-9. It is possible that early developing fish larvae have limited lipid biosynthetic capabilities in general, whether for fatty acids or lipid classes, but are instead specialized toward modifying the fatty acid composition of dietary lipids by fatty acyl exchange reactions and by fatty acyl chain shortening reactions inherent in the β-oxidation of fatty acids, which is a major source of energy in larvae. This is an important area for future research in fish lipid nutrition.

We have noted in the past (e.g., Sargent *et al.,* 1989, 1993b) and recently emphasized (Sargent *et al.,* 1999b) that the ideal diet for fish larvae is, of course, the yolk of eggs or yolk sac larvae. This is because the lipid content and composition of marine fish eggs are essentially the same as those of the resulting larvae and also of the larvae's natural copepod diet (Table 4.3).

Table 4.3

Fatty acid compositions of Total Lipid from Fish Eggs, Larvae, and Live Larval Feeds[a]

Source	Fatty acid composition (wt%)							
	22:6*n*-3	20:5*n*-3	20:4*n*-6	18:3*n*-3	18:2*n*-6	18:1*n*-9	16:0	Ref.[b]
Cod eggs	29.3	14.8	1.7	0.3	1.1	11.5	18.5	1
Cod larvae	30.2	15.0	1.8	0.6	1.7	7.6	17.4	2
Calanoid copepods	32.2	12.1	1.0	1.7	2.0	7.0	18.1	3
Artemia nauplii	0.0	3.9	1.1	22.1	5.9	17.4	11.6	4
Artemia nauplii + TOO[c]	19.4	10.8	3.0	14.2	5.7	24.4	16.7	4

[a] From Sargent *et al.* (199b).

[b] Data from (1) Tocher and Sargent (1984); (2) Klungsoyr *et al.* (1989); (3) McEvoy *et al.* (1998) for mixed zooplankton, predominantly *Eurytemora affinis, Acartia teclae* and *Centropages hamatus;* (4) McEvoy *et al.* (1996).

[c] Eighty-eight percent tuna orbital oil + 12% herring roe phospholipid.

Moreover, when a diet containing 10% marine egg phospholipid, which is essentially the phospholipid content of many marine fish eggs, is analyzed in terms of the published lipid nutrient requirements for fish, all requirements are comfortably fulfilled (Table 4.4). This is despite the published requirements being derived from various species and at various stages of

Table 4.4

Amounts of Essential Fatty Acids, Inositol, and Choline in a Diet Containing 10% Dry Weight as Marine Fish Phospholipid[a]

100 g dry wt of diet contains 10.0 g total phospholipid
10.0 g total phospholipid contains 0.42 g phosphatidylinositol
0.42 g phosphatidylinositol contains 72 mg (0.40 mmol) inositol[b]
10.0 g total phospholipid contains 6.34 g phosphatidylcholine
6.34 g phosphatidylcholine contains 624 mg choline[c]
10.0 g total phospholipid contains 1740 mg 22:6*n*-3 and 949 mg 20:5*n*-3, i.e., 2699 mg *n*-3 HUFA[d] and 118 mg (0.39 mmol) 20:4*n*-6
Ratio of 22:6*n*-3 to 20:5*n*-3 to 20:4*n*-6 in diet is 183:100:12[e]
Ratio of *n*-3 HUFA to *n*-6 HUFA is 24:1

[a] From Sargent *et al.* (1999b). The marine phospholipid is total phospholipid from cod roe (Tocher and Sargent, 1984).

[b] The published requirement for inositol for salmon is 30–40 mg per 100 g diet (Halver, 1989).

[c] The published requirement for choline for salmon is 60–80 mg per 100 g diet (Halver, 1989).

[d] The published requirement for *n*-3 HUFA for turbot is 800–1300 mg per 100 g diet (Gatesoupe *et al.*, 1977; Le Milnaire *et al.*, 1983).

[e] 20:4*n*-6 can be increased twofold in turbot larvae, without deleterious effects (Estevez *et al.*, 1999).

development. Particularly notable in Table 4.4 is the equivalence of the molar dietary requirements for inositol and 20:4*n*-6, which is precisely predicted by the marked concentration of 20:4*n*-6 in fish phosphatidylinositol and by 18:0/20:4*n*-6 being the dominant molecular species of fish phosphatidylinositol (see Section 4.3.2). Clearly, phosphatidylinositol can be assimilated from yolk sac lipids, whether in the developing egg or in yolk sac larvae, directly into embryonic or larval tissue lipids without modification, i.e., without a requirement for *de novo* biosynthesis. Equally, however, developing fish eggs and larvae have a propensity for fatty acyl exchange reactions because the di-22:6*n*-3 molecular species of phosphatidylethanolamine and phosphatidylserine that are abundant in larval eyes are absent from the nonfertilized eggs (Bell, 1989; J. G. Bell *et al.,* 1995), i.e., they must be elaborated by acyl exchange reactions of phosphatidylethanolamine and -serine during embryogenesis and early larval development. Also notable in Table 4.4 is that the levels of 22:6*n*-3 and 20:5*n*-3 provided in the 10% phospholipid diet by phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine amply meet the published *n*-3 HUFA requirements for marine fish larvae and also generate the required ratio (Reitan *et al.,* 1994) of 22:6 *n*-3 to 20:5*n*-3 of 2:1. As important, the ratio of 20:5*n*-3 to 20:4*n*-6, albeit apparently high in Table 4.4, has been shown by Estevez *et al.* (1999) to be entirely satisfactory for turbot larvae culture. These authors also showed that the levels of 20:4*n*-6 in the diet could be doubled without deleterious effects on the fish.

The foregoing strongly suggests that the EFA requirements of early developing larvae in nature are very largely met by the phospholipid content of their natural diets. This, of course, immediately offers at least a partial explanation of the superiority of live diets, especially copepods, to most fabricated diets so far studied in larval culture, since the former have the bulk of their EFA in phospholipids, in contrast to triacylglycerols in fabricated diets. It also sets a premium on developing fabricated diets with phospholipids as the major dietary lipid supplying EFA, together with triacylglycerols, as the major dietary lipid generating energy (see Section 4.8). One limitation in achieving this objective is the very limited knowledge of lipid digestion and absorption in fish larvae. In general, lipid digestion, absorption, and transport in fish are broadly very similar to those in mammals (see Sargent *et al.,* 1989) and are an area that has received only limited attention in recent years (Sheridan, 1988; Iijima *et al.,* 1990; Koven *et al.,* 1994, 1997; Olsen and Ringoe, 1998; Olsen *et al.,* 1998, 1999a, b). However, the very small size of early fish larvae, in which the digestive system is often incomplete, presents formidable practical difficulties for experimental study. Precisely how the optimal levels of phospholipids and triacylglycerols in larval diets vary with development stage and with species is a major and challenging area for future research in fish nutrition.

4.7 Fatty Acid Peroxidation

Although the absolute levels of PUFA may vary widely between species, fish are generally regarded as PUFA-rich organisms providing a virtually unique source of the *n*-3 HUFA, 22:6*n*-3 and 20:5*n*-3 (Henderson and Tocher, 1987; Sargent and Tacon, 1999). These HUFA, which are vital constituents for cell membrane structure and function, are very susceptible to attack by oxygen and other organic radicals. Resultant damage to PUFA in membrane phospholipids can have damaging consequences for cell membrane structure and fluidity, with potential pathological effects on cells and tissues (Sies, 1991).

4.7.1. Mechanisms of Peroxidation

The mechanisms by which PUFA in fish tissues undergo oxidative deterioration have been described in detail by Hsieh and Kinsella (1989). Before oxygen can damage the structural integrity of a PUFA, it has to be activated to a high-energy state. Molecular oxygen can be activated to a number of high-energy states including singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). The last three "active oxygen species" can be generated by normal cellular metabolic processes such as the mitochondrial electron transport chain, and all four can be generated in biological systems by a variety of nonenzymatic and enzymatic mechanisms (Fridovich, 1976; Buettner, 1993). Singlet oxygen can be generated nonenzymatically following photosensitization in the presence of O_2 and visible light or by enzymes such as microsomal oxidases, lipoxygenase, and prostaglandin synthetase (Korycka-Dhal and Richardson, 1978). The hydroxyl radical is a particularly powerful species formed by the addition of a third electron to H_2O_2. This occurs in biological systems following reductive cleavage of H_2O_2 by a metal ion in the reduced state, such as Fe(II) or Cu(I), by the Fenton–Haber–Weiss reaction (Buettner, 1993):

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

Hydroxyl radical may also be generated by microsomal electron transfer processes, activated polymorphonuclear leukocytes, and lipoxygenase and cyclooxygenase enzymes. The superoxide radical is produced by all aerobic cells and is abundant in neutrophils, monocytes, macrophages, and eosinophils, as well as being produced by many enzymatic oxidations including xanthine oxidase, cytochrome P450 oxidases, and aldehyde oxidase

(Hseih and Kinsella, 1989). Although not active enough to initiate autoxidation itself, superoxide radical can be converted to the hydroxyl radical as described above.

The oxidation of unsaturated fatty acids begins when a powerful species, such as the hydroxyl radical, abstracts a hydrogen atom from a methylene group of a PUFA to leave a carbon radical ($R^{\cdot}$). The carbon radical rearranges to form a conjugated diene, which easily reacts with oxygen to form a peroxy radical ($ROO^{\cdot}$), which in turn can abstract a further hydrogen from another PUFA to produce a hydroperoxide (ROOH) and another lipid radical. Hence, a propagation reaction occurs. Such reactions will then proceed rapidly unless termination occurs by reaction of two radicals to form a nonradical product or by intervention of antioxidant species which "trap" the damaging radicals in an unreactive form after donating a hydrogen atom to the peroxy radical (see Section 4.7.3). Lipid hydroperoxides can decompose to alkoxy and hydroxyl radicals, and the alkoxy radical can be converted to secondary products including aldehydes, ketones and alcohols which may themselves be cytotoxic. The mechanisms of cellular autoxidative processes are shown in Fig. 4.10.

The rate-limiting step in the autoxidation process is the propagation step whereby a peroxy radical abstracts a hydrogen atom from an unsaturated fatty acid. Thus, the ease of peroxidation is proportional to the number of double bonds present. The rates of autoxidation of 18:1*n*-9, 18:2*n*-6, and 18:3*n*-3, based on their rate of oxygen uptake, were found to be in the order 1:50:100, respectively (Gunstone and Hilditch, 1945). More recently, the rate of oxygen uptake for 20:4*n*-6 was found to be 2.9 times faster than that for 18:2*n*-6 (Porter *et al.,* 1981), whereas oxygen uptake by esters of 20:5*n*-3 and 22:6*n*-3 was about 7 and 10 times faster, respectively, than that for 18:3*n*-3 (Cho *et al.,* 1987). A diagram of the oxidation of PUFA is shown in Fig. 4.11.

4.7.2. Consequences of Peroxidation

Oxidation of PUFA in biomembranes can result in functional aberrations and pathological changes which have been reviewed extensively (Freeman and Crapo, 1982; Cross, 1987; Halliwell and Gutteridge, 1989). Animal cells have evolved a highly efficient, multilevel, antioxidant protection mechanism that is a safeguard against the adverse effects of life in an oxygen-rich environment (Fig. 4.10). In fish, which contain particularly high levels of *n*-3 HUFA that are highly susceptible to oxidative damage (Fig. 4.11), a highly efficient antioxidant protection system is essential for the physiological well-being of the animals. However, when parts of the antioxidant protective mechanism are placed under stress, perhaps due to dietary deficiencies

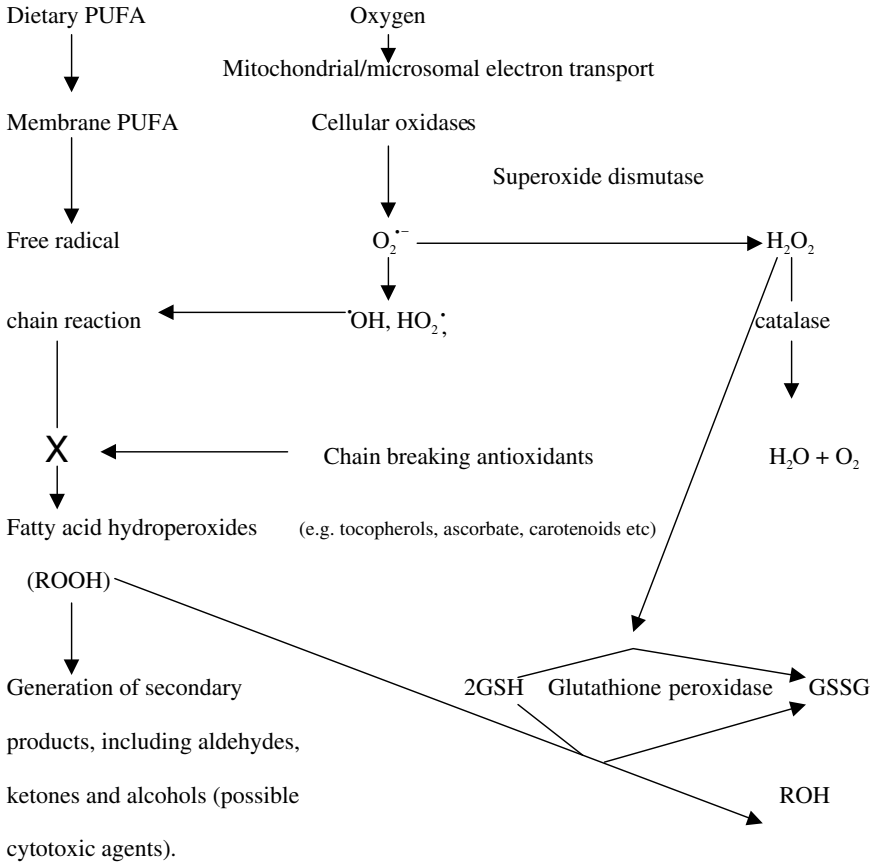

FIG. 4.10

Schematic representation of generation of reactive oxygen species, interaction with cellular PUFA, and antioxidant protective mechanisms.

of essential antioxidant nutrients or intake of oxidized (rancid) foodstuffs, then pathological consequences can result.

Reduced growth, loss of appetite, decreased feed efficiency, and increased mortality have all been reported in many species of fish fed oxidized lipid, including carp (Hata and Kaneda, 1980), channel catfish) (Murai and Andrews, 1974), yellowtail (Park, 1978), rainbow trout (Cowey *et al.,* 1984), Atlantic salmon (Ketola *et al.,* 1989), coho salmon (*Oncoryhchus kisutch)* (Ketola *et al.,* 1989), and African catfish (Baker and Davies, 1997). Additional gross pathologies include exudative diathesis (increased permeability of capillaries) in channel catfish (Murai and Andrews, 1974) and reduced

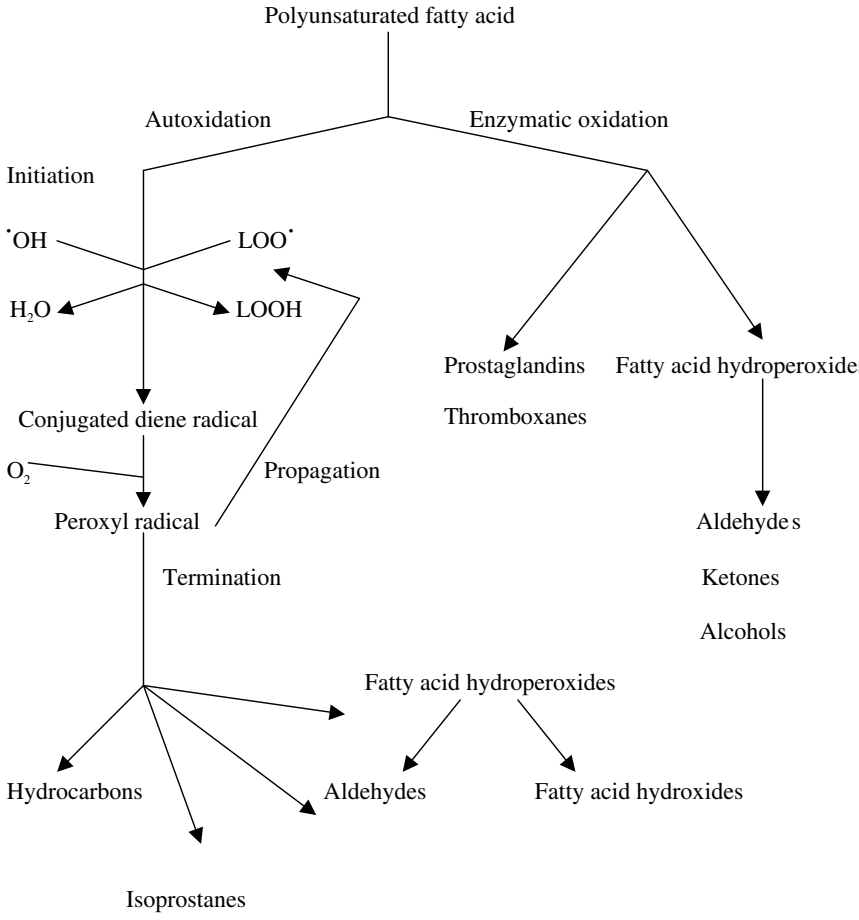

FIG. 4.11

Oxidation reactions affecting polyunsaturated fatty acids.

hematocrit, increased erythrocyte fragility, and reduced hemoglobin content in European sea bass (Messager *et al.,* 1992), rainbow trout (Moccia *et al.,* 1984), and striped jack (*Longgirostris delicatissimus)* (Wada *et al.,* 1991). A number of histological lesions due to lipid peroxidation have been identified, including myopathy of skeletal muscle in channel catfish (Murai and Andrews, 1974), European sea bass (Messager *et al.,* 1992), yellowtail (Sakaguchi and Hamaguchi, 1969), rainbow trout (Cowey *et al.,* 1984), and striped jack (Wada *et al.,* 1991) and, also, lipoid liver degeneration and accumulation of ceroid pigments (lipofuschin).

Clearly, the presence of oxidized lipid can have toxic consequences for fish, whether they arise from direct dietary input or via deficiencies in essential antioxidant nutrients. However, evidence suggests that pathological symptoms can be eliminated, or at least controlled, by supplying sufficient dietary antioxidants, particularly vitamin E, to prevent the production of excessive levels of free radical-generated toxic compounds (Cowey *et al.,* 1984; Baudin-Laurencin *et al.,* 1989; Baker and Davies, 1997).

4.7.3. Protection against Peroxidation

4.7.3.1. Tocopherols

The tocopherols and tocotrienols, the vitamin E-type compounds, are not synthesized by animals and must be obtained in the diet, ultimately from higher plant and algal sources (Hess, 1993). Vitamin E is widely regarded as the primary lipid-soluble antioxidant (Buettner, 1993; Kamal-Eldin and Appelqvist, 1996) and the relative antioxidant efficacies of the tocopherols *in vivo* have been established as $\alpha > \beta > \gamma > \delta$ (Burton and Traber, 1990). The antioxidant activities of tocopherols are imparted by their ability to donate their phenolic hydrogen atoms to lipid free radicals, resulting in the stabilization of the latter and the termination of the lipid peroxidation chain reaction (Burton and Ingold, 1989). However, tocopherols can also prevent lipid peroxidation by acting as quenchers of singlet oxygen (Gorman *et al.,* 1984). While α-tocopherol has been identified as the major naturally occurring tocopherol in the lipids of marine fish (Ackman and Cormier, 1978), β- and γ-tocopherols are absorbed and deposited in fish tissues (Hamre and Lie, 1997). However, recent studies in Atlantic salmon suggest that the uptake and deposition of γ-tocopherol may be less efficient than those of α-tocopherol, and given that the former is deposited primarily in the adipose tissue, it is likely that the latter is more important in preventing oxidative damage to PUFA components of membrane phospholipids (Parazo *et al.,* 1998).

The vitamin E requirements of many fish species of importance in aquaculture have been established over the last 20 years and generally fall in the range of 20–50 mg/kg dry feed (NRC, 1993). The deficiency symptoms are similar to those described in Section 4.7.2, above, and include muscular dystrophy, exudative diathesis, anemia, impaired erythropoiesis, erythrocyte fragility, skin discoloration, and ceroid pigment deposition (NRC, 1993). These issues are dealt with more fully in Chapter 2, "The Vitamins." More recent research has focused on the relationship of vitamin E with increased dietary PUFA, temperature, and interaction with other antioxidants.

Several nutritional studies in mammals have shown a close correlation between an increased PUFA intake, resulting in increased incorporation of

PUFA into biomembranes, and, an increased incidence of lipid peroxidation (Cho and Choi, 1994; Sugihara *et al.,* 1994). Similarly, in fish increased levels of dietary and tissue PUFA require increased dietary supplementation with vitamin E to prevent the occurrence of oxidative damage. A correlation between increased dietary PUFA and vitamin E requirement has been found in blue tilapia (Roem *et al.,* 1990), turbot (Stephan *et al.,* 1995), carp (Runge *et al.,* 1992) and Atlantic salmon (Waagboe *et al.,* 1991). When fish are subject to a state of vitamin E deficiency, there is a rapid loss of vitamin E from liver and muscle but a selective retention in the neural tissues of the brain and eye. In a study with Atlantic salmon, feeding a vitamin E-deficient diet for a period of 22 weeks resulted in liver vitamin E levels falling to 3% of their original value, whereas levels in brain and eye were reduced only to 35 and 40% of their original values (Bell *et al.,* 2000). These results suggest a selective conservation of vitamin E in tissues with a high *n*-3 HUFA content and probably reflect the functionality of *n*-3 HUFA-rich biomembranes in neural tissues. In general, levels of vitamin E are higher in fish tissues than in mammals, and this probably reflects the higher degree of antioxidant protection required in *n*-3 PUFA-rich organisms (Hamre and Lie, 1995).

In rats deficient in both vitamin E and selenium, Buttriss and Diplock (1988) observed an increase in the long-chain PUFA, 22:6*n*-3 and 20:4 *n*-6, in mitochondrial and microsomal membranes. They theorized that this increase was due to an overproduction of these PUFA arising from increased activity of the desaturation and elongation mechanisms responsible for the synthesis of PUFA. A similar effect has also been found in African catfish fed oxidized oil (Baker and Davies, 1996). In a very recent study, the ability of isolated salmon hepatocytes to desaturate and elongate 18:3*n*-3 further was increased in fish fed diets deficient in vitamin E, although no increase in membrane *n*-3 PUFA was seen (Bell *et al.,* 2000). While the mechanism of the above effects remains unclear, it appears that an elevation in oxidation potential or "peroxide tone" may increase cellular synthesis of long-chain *n*-3 PUFA. An increase in peroxide tone, whether achieved by restricted dietary intake of one or more antioxidants and/or by inclusion of dietary prooxidants in the form of oxidized triacylglycerol oils or other lipid classes, appears to result in activation of fatty acyl desaturation and elongation.

In addition to being a potent antioxidant *in vivo,* there is increasing evidence that vitamin E is an important factor in preventing oxidative deterioration of fish products *in vitro* (Hseih and Kinsella, 1989). A study with rainbow trout established that there was a dose related inhibition of the appearance of the lipid peroxidation product malondialdehyde following cold storage of the fish at −18°C for up to 8 months (Gessl *et al.,* 1995). A similar effect was observed in turbot following cold storage at 20°C for 6 months (Stephan *et al.,* 1995), while fillets of cultured Atlantic mackerel showed losses of vitamin E proportional to time of storage at −30°C, indicating

the effective antioxidant activity of vitamin E in preventing deterioration of flesh quality (Hemre *et al.,* 1997). Such evidence suggests that fish feeds should contain a level of vitamin E supplementation about four to five times the requirement for normal growth and development to protect against degradation during storage (Tacon, 1996).

4.7.3.2. Carotenoids

Several recent studies have suggested that carotenoids, including β-carotene, astaxanthin, and canthaxanthin, are potent antioxidants in *in vitro* membrane models and that they operate synergistically with vitamin E (Krinsky, 1993; Nishigaki *et al.,* 1994). Carotenoid pigments are deposited in the eggs of numerous fish species, with pigment being mobilized from the flesh of salmonids and deposited in the ovarian tissues during sexual maturation (Torrisen *et al.,* 1989; Watanabe and Miki, 1993; Hatlen, 1997). The presence of carotenoids seems to enhance egg quality, perhaps by protecting against the damaging effects of UV radiation or other environmental prooxidants (Krinsky, 1993; Chew, 1995, 1996). It seems likely that the beneficial effects of carotenoid deposition on egg quality are related to their function as antioxidants, in terms of their ability either to sequester oxidative initiators, like singlet oxygen, or to act as chain terminators by trapping hydroperoxide radicals. Studies on the etiology of the so-called M74 syndrome, affecting yolk sac larvae of Baltic stocks of Atlantic salmon, have identified a correlation between low astaxanthin levels in eggs and development of the syndrome (Pickova *et al.,* 1998). Previous studies have identified a strong correlation between high incidences of M74 and a low astaxanthin content in broodstock muscle, with significant reductions in vitamin E and ubiquinone in affected swim-up fry compared to unaffected fry (Lignell, 1993; Pettersson and Lignell, 1995). In a recent study with Atlantic salmon fed diets unsupplemented or supplemented with astaxanthin, *in vitro* stimulation of autoxidation was suppressed in muscle microsomal membranes from fish fed astaxanthin compared to those from fish lacking astaxanthin (Bell *et al.,* 2000). In addition, feeding diets containing astaxanthin appeared to reduce the plasma levels of 8-isoprostane, a product of nonenzymatic lipid peroxidation (see Fig. 4.11) (Bell *et al.,* 2000).

4.7.3.3. Ascorbic Acid and Other Synergistic Antioxidants

Antioxidant systems generally comprise more than one component and the net effect of total antioxidant activity is usually greater than the sum of the individual activities, indicating a synergistic phenomenon. Over 50 years ago Golumbic and Mattill (1941) observed that, although ascorbic acid (vitamin C) was a poor antioxidant for oils and fats, it enhanced the antioxidant efficacy of vitamin E. While ascorbic acid possesses antioxidant activity in the aqueous phase, its ability to interact with the lipid phase at

the membrane–cytosolic interface enables it to regenerate α-tocopherol from its tocopheroxyl radical, *in vivo* and *in vitro,* thus restoring the antioxidant activity of tocopherol (Freisleben and Packer, 1993). In a study with juvenile rainbow trout, Frischknecht *et al.* (1994) fed diets deficient in either vitamin E, vitamin C, or both vitamins for a period of 31 weeks. The fish fed the diet deficient in both vitamins had a high mortality and anemia after 8–12 weeks, and histopathological examination showed severe muscular dystrophy and splenic hemosiderosis. However, fish fed the diet deficient in vitamin C alone did not develop classical symptoms of deficiency (low growth, hemorrhages, gill damage, and vertebral deformations) until 16–20 weeks. Those fish fed diets deficient in vitamin E alone showed splenic hemosiderosis after 20 weeks. These results suggest a synergistic relationship between vitamin C and vitamin E in rainbow trout. In another study with rainbow trout, lipid peroxidation was increased in spermatozoa of fish fed a diet deficient in vitamin C, and the 22:6n-3 content of the spermatozoa was significantly lower in the deficient group compared to the group supplemented with 870 mg/kg of ascorbyl monophosphate (Liu *et al.,* 1997).

Antioxidant synergism has also been observed between vitamin E and selenium in trout and salmon (J. G. Bell *et al.,* 1985; Poston *et al.,* 1976) and between vitamin E and astaxanthin in salmon (Bell *et al.,* 2000). Other potential synergistic effects include regeneration of α-tocopherol from its radical by glutathione (Wefers and Sies, 1988) or dihydrolipoic acid (Freiselben and Packer, 1993). In addition, phospholipids having a primary amine group, e.g., phosphatidylethanolamine or phosphatidylserine, can function as peroxyl radical scavengers and thereby have a sparing effect on tocopherol (Lambelet *et al.,* 1984), whereas phosphatidylinositol and other acidic phosphatides can act synergistically with tocopherols due to their metal chelating activity (Pokorny, 1987; Ishihara, 1996). Recently, phospholipids were shown to enhance the antioxidant efficacy of tocopherols in oils by forming reverse micelles or microemulsions, such that tocopherols were positioned in the micelles with their active phenolic group adjacent to the polar region where peroxy radicals are concentrated (Kago and Terao, 1995). These activities of phospholipids can be important in preventing autoxidation in oil emulsions used to enrich live prey such as rotifers and *Artemia* nauplii (Sargent *et al.,* 1997).

4.8 Sources of Lipids for Farmed Fish Feeds

4.8.1. Alternatives to Fish Oils in Bulk Feeds

Fish meal and fish oil derived from industrial fisheries, e.g., capelin, herring, sand eel, mackerel, anchovy, and sardine fisheries, have been the

standard ingredients of bulk feeds for intensively farmed fish, above all salmonids and marine fish, for many years. The requirements of marine fish for 20:5*n*-3 and 22:6*n*-3 make fish oil the only commercially available source of these fatty acids, essential in marine fish feeds. As noted earlier, many freshwater fish can convert 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3 and can, therefore, be grown on diets containing 18:3*n*-3, at least in principle. Such fish include salmonids, most notablly, rainbow trout. These fish also thrive on fish oils, and this, together with the relative paucity of commercial oils rich in 18:3*n*-3 and the ready available and relative cheapness of fish oils, has resulted in the widespread use of fish oils in farmed fish feeds. However, global fisheries are now stagnating and the current yield of fish oil from industrial fisheries, circa 1.4 million tons in 1996 (Sargent and Tacon, 1999), is unlikely to be significantly exceeded in future. Fish farming consumed a total of 560,000 tons of fish oil in 1996, with farmed salmon and farmed trout consuming, respectively, 36 and 22% of that total (Sargent and Tacon, 1999). Global aquaculture has grown at 11.6% per annum compound growth since 1984 (Tacon, 1996) and is continuing to grow at a similar rate. It is clear that demand for fish oil from aquaculture must, before long, exceed supply. This problem will be exacerbated by climatic events such as El Niño, by growing environmental pressure to decrease exploitation pressure on finite marine resources and by increasing consumer perception that levels of pollutants such as dioxin in fish oils have now reached unacceptable levels. For these reasons, finding alternatives to fish oils in farmed fish feeds is becoming an increasingly urgent issue.

Finding replacement oils and fats which permit economically efficient growth of the fish is not by itself a complete solution to the problem. Such replacements already exist because it has long been known that, providing that their EFA requirements are met, catfish, carp, and trout can be successfully reared on diets rich in either beef tallow or hydrogenated fish oils (Stickney and Andrews, 1972; Takeuchi *et al.*, 1978; Henderson and Sargent, 1984). Moreover, catfish have been routinely grown commercially in the past on diets rich in corn oil. Rather, the solution to replacing fish oils requires retaining as far as possible the health-promoting properties of the end product for the consumer, which means retaining as far as possible the current high levels of 20:5*n*-3 and 22:6*n*-3 in farmed fish (Sargent and Tacon, 1999). Indeed, the beneficial effects of fish and specifically fish oils in developed societies stems fundamentally from a marked global imbalance of *n*-6:*n*-3 PUFA, caused mainly by rapid increases in recent decades in the production of vegetable oils rich in 18:2*n*-6. Thus, of the total global production of oils and fats in 1996/1997 of 93,082,000 tons, 20,799,000 tons was derived from soya, 17,077,000 tons from palm, and 11,410,000 tons from rape (O'Mara, 1998) (Table 4.5). These oils are all rich in 18:2*n*-6 and relatively lacking

Table 4.5

Fatty Acid Composition of Commercially Available Fats and Oils (Triacylglycerols)

	Lard[a]	Palm[a]	Rape[a]	Soya[a]	Olive[a]	Linseed[a]	Herring[b]	Anchovy[b]
Global Production (tons × 10^{-6}) in 1996[c]	6.1	17.1	11.4	20.8	2.0	0.7	1.4[d]	1.4[d]
Fatty acid								
16:0	26	61	5	11	14	7	13	17
16:1*n*-7	3	tr[e]	tr	tr	2	tr	7	9
18:0	15	5	2	4	3	5	1	4
18:1*n*-9	49	26	60	22	69	18	10	12
18:2*n*-6	9	7	21	54	12	17	1	1
18:3*n*-3	tr	tr	10	8	1	54	1	1
20:1*n*-9	tr	0	2	tr	tr	0	13	2
20:5*n*-3	0	0	0	0	0	0	6	17
22:1*n*-9	0	0	1	tr	0	0	0	0
22:1[f]	0	0	0	0	0	0	23	2
22:6*n*-3	0	0	0	0	0	0	6	9

[a] Data are mean values for the ranges quoted by Gunstone *et al.* (1994).
[b] Data from Sargent and Henderson (1995).
[c] Data from O'Mara (1998).
[d] Value for total global fish oil production.
[e] Trace.
[f] The *n*-9 isomer in the vegetable oils; the *n*-11 isomer in the fish oils.

in 18:3*n*-3 (Table 4.5). Linseed oil, which is one of the very few commercially available oils rich in 18:3*n*-3 and with a high ratio of 18:3*n*-3 to 18:2*n*-6 (Table 4.5), accounted for only some 661,000 tons (O'Mara, 1998). Fish oils, the only source of 20:5*n*-3 and 22:6*n*-3 (Table 4.5), accounted for 1,387,000 tons (O'Mara, 1998). Lard, derived from pork, which is rich in saturated fatty acids and deficient in *n*-3 PUFA (Table 4.5), accounted for 6,101,000 tons (O'Mara, 1998). These global tonnages, together with the compositional data in Table 4.5, establish how far the ratio of *n*-6 to *n*-3 in human diets is escalating from the desired value of circa 5:1 (Anonymous, 1992, 1994a) and emphasize how valuable fish–derived 20:5*n*-3 and 22:6*n*-3 are as nutrients for man. Simply utilizing vegetable oils rich in 18:2*n*-6 and animal fats rich in saturated fatty acids as replacements for fish oils in farmed fish feeds is tantamount to using fish to imbalance further an already imbalanced human diet.

In considering replacements for fish oils in aquaculture feeds, the following may be considered. First, levels of 20:5*n*-3 and 22:6*n*-3 in current farmed

fish feeds are well in excess of the minimum *n*-3 essential fatty acids requirements of the fish. This is palpably the case in salmon farming, where the fish oil content of the feeds now commonly exceeds 30% of the dry weight. Clearly, more judicious use of available fish oil can allow a greater tonnage of farmed fish to be produced than is currently the case. However, distributing the available fish oil over greater quantities of fish does not increase the total input of 20:5*n*-3 and 22:6*n*-3 in the human diet. Second, efforts should be made to minimize the catabolism by fish of those fatty acids that are particularly valuable in human nutrition, i.e., 20:5*n*-3 and 22:6*n*-3. As noted earlier (Section 4.3.1), 22:6*n*-3 can be selectively retained by fish, probably due to the inherent difficulties in oxidizing this fatty acid, which requires the peroxisomal rather than the mitochondrial pathway of β-oxidation. However, 20:5*n*-3 appears to be relatively easily oxidized by mitochondria and, in this respect, is similar to the saturated and monounsaturated fatty acids including 20:1*n*-9 and 22:1*n*-11, which are abundant in northern fish oils. It was also noted earlier that 18:1*n*-9 and also 18:2*n*-6 appear to be easily oxidized by fish. Therefore, it should be possible, at least in principle, to provide sufficient 18:1*n*-9 and, to some extent, 18:2*n*-6 in dietary feeds to offset partially the oxidation of 20:5*n*-3 and, if need be, the oxidation of 20:1 and 22:1 by the fish. Fatty acid 18:1*n*-9–rich vegetable oils relatively deficient in 18:2*n*-6 are readily available, e.g., olive oil and high-oleic acid sunflower oil. The majority of commonly available vegetable oils (Table 4.5) are rich in both 18:1*n*-9 and 18:2*n*-6. Third, care should be exercised in substituting fish oils with vegetable oils rich in 18:2*n*-6 for reasons of consumer health and possibly also fish health. Growth of salmon on diets containing fish meal and sunflower oil as the sole added dietary oil can cause cardiovascular disorders in the fish, especially under stress (Bell *et al.*, 1991, 1993). This is worryingly reminiscent of the deleterious effects of excessive dietary ratios of *n*-6 to *n*-3 PUFA in man. Precisely how much 18:2*n*-6 can be included in farmed fish feeds, and for how long, without deleterious effects to the fish, especially in terms of their response to stress and disease, remains to be evaluated. Fourth, much more effort is needed to evaluate the extent to which 18:3*n*-3-rich oils, specifically linseed oil, can successfully substitute for fish oils, especially in the salmonids and freshwater fish in general, which are capable of converting this fatty acid to 20:5*n*-3 and 22:6*n*-3. The early study by Castell *et al.* (1972) established that rainbow trout could be successfully reared on a diet containing 18:3*n*-3 as the sole fatty acid. This is an area that urgently needs revisiting since an end product in which 20:5*n*-3 and 22:6*n*-3 are partially replaced by 18:3*n*-3 is much more acceptable for consumer health than one where the replacement fatty acid is 18:2*n*-6. Additionally, it may be possible to select strains of fish with high activities in converting 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3, even in the presence of significant amounts

of fish oil in the fish's diet. Perhaps further in the future is the possibility of maximally activating the genes determining conversion of 18:3*n*-3 to 22:6*n*-3 in marine fish (see Section 4.3.2). Finally, it should be realized that the oil stored in large amounts in the adipocytes of fish such as salmon and other "oily" fish fed natural diets based on fish oil has specific fluidity characteristics stemming not only from its content of 20:5*n*-3 and 22:6*n*-3 but also from its content of 20:1*n*-9 and 22:1*n*-11. Replacement of this oil with "lighter" oils so as to replace C_{22} and C_{20} with C_{18} fatty acids, whether monounsaturated or polyunsaturated, may not always result in good retention of the oil within adipocytes under all conditions, not least processing conditions involving low-temperature storage and/or smoking. The successful development of alternatives to fish oil in aquafeeds requires much research if projected targets for aquaculture expansion are to be met.

4.8.2. Marine Fish Larval Feeds

Particular problems exist in providing dietary lipids for marine fish larvae, whose production has too long remained a bottleneck in marine fish farming. The problem stems fundamentally from the fact that marine fish larvae are generally very small and naturally consume very small live prey, making it difficult to recreate natural feeding conditions in marine larval production systems, especially at the high densities required for economic production. *Artemia* nauplii enriched with fish oils to provide the dietary *n*-3 HUFA essential for the larvae continue to figure prominently, perhaps too prominently, in marine larvae production and alternative strategies to larval feeding are urgently needed. Continuing development of fabricated microdiets is essential, as is the development of technology for the efficient mass production of more natural live feeds, particularly copepods. However, as noted in Section 4.6, marine fish larvae have exacting dietary lipid requirements not only for the correct balance of 22:6*n*-3, 20:5*n*-3, and 20:4*n*-6, but also probably for phospholipids. Specialty triacylglycerols enriched in or with particular blends of these PUFA are already available, e.g., tuna orbital oil, fractions of fish oils developed as human nutritional supplements highly enriched in 22:6*n*-3 and 20:5*n*-3 and, more recently, triacylglycerols containing either 22:6*n*-3, 20:5*n*-3 or 20:4*n*-6 as the major fatty acid from single-cell sources such as *Crypthecodinium cohnii* and *Mortiella* (see, e.g., Sargent *et al.,* 1999b; Estevez *et al.,* 1999). Such oils, though expensive, have ready applications in supplementing live feeds and in microdiet formulations to provide optimal HUFA requirements for fish larvae. However, the feeds so generated fall far short of natural marine larval diets in that what is required for the larvae are dietary phospholipids containing the correct blend of HUFA, especially *n*-3 HUFA-rich phospholipids.

No ready source of such phospholipids exists at present other than marine products such as roe and milt, which already have efficient, direct outlets as human foods. New sources of *n*-3 HUFA-rich phospholipids are required, possibly from single-cell culture or from chemical and/or enzymatic retailoring of *n*-3 HUFA-rich triacylglycerols with abundant plant phospholipids. What is required above all, perhaps, is the development of efficient primary production systems to underpin production of natural live feeds for marine larvae, i.e., those single-cell algae that produce the required lipid nutrients *denovo*. Such organisms are, of course, well known, e.g., *Isochrysis galbana* and *Pavlova lutheri* (see also Reitan *et al.,* 1997; Brown *et al.,* 1997), and are already finding applications in marine fish larval production, albeit on a relatively small scale. The problems to be solved here appear to be technological and economic rather than nutritional.

4.9 Prospects

The dominant ongoing issue in fish lipid nutrition is the special roles of *n*-3 HUFA, 20:5*n*-3 and, especially, 22:6*n*-3, in fish and man alike. Scientifically, 22:6*n*-3 is a unique and challenging molecule in terms of its chemical structure and properties, its biosynthesis and catabolism, and its cellular functions. Its fundamental role in neural development affords it a unique role in the nutritional biochemistry of both *Homo sapiens* (Crawford *et al.,* 1999) and fish (this chapter). Should there be any fundamental justification for aquaculture compared to other food production systems, it may well be that of continuing to provide a ready and convenient source of this nutrient for *Homo sapiens,* at a time when traditional supplies from capture fisheries are becoming, or are about to become, seriously constrained. In terms of fish nutrition, therefore, the overriding requirement for the future is to find new sources of 20:5*n*-3 and, especially, 22:6*n*-3 for fish feeds. The issue of finding replacements for fish oil was considered in the previous section. We note here simply that, without enhanced and new sources of *n*-3 HUFA, the only current practical option for continuing aquaculture expansion is to develop farmed fish as an aquatic alternative to chicken and pork, i.e., as a product whose lipids are derived primarily from terrestrial feed stocks including vegetable oils with an overhigh ratio of *n*-6:*n*-3 PUFA. We strongly caution against accepting this option over lightly. How, then, do we proceed?

We have noted elsewhere (Sargent and Tacon, 1999) that freshwater fish currently comprise the overwhelming bulk of fish farmed globally and that the species in question, mainly carp, have an innate ability to convert 18:3*n*-3

to 20:5*n*-3 and 22:6*n*-3. This property applies to salmonids also, at least in the freshwater phase of their life history. Therefore, a high priority in future research in fish lipid nutrition is to maximize the innate ability of these fish to produce *n*-3 HUFA from 18:3*n*-3. This requires, *a priori,* ready access to dietary oils rich in 18:3*n*-3 which are already available, e.g., as linseed-type oils. It is ironic that major efforts have been invested in recent decades to eliminate the already very low levels of 18:3*n*-3 in 18:2*n*-6-rich vegetable oils such as corn oil. Indeed, current research on genetically modified organisms includes efforts to engineer rape and, indeed, linseed to produce oils with very low 18:3*n*-3! We question the current drive to increase global production of vegetable oils rich in 18:2*n*-6 and deficient in 18:3*n*-3 and plead that more emphasis be given to developing those 18:3*n*-3-rich oils already available. We also plead for much more research on the molecular biology and genetics of the production system in freshwater fish that generates 22:6*n*-3 and 20:5*n*-3 from dietary 18:3*n*-3. Only in this way can factors controlling the pathway be elucidated so as to optimize its performance in those species known to possess it and, also, to select genetically using modern molecular genetic technology for those individuals in which it may be highly expressed. More challenging may be to extend such research to marine fish. The conversion of 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3 is generally very poorly expressed in marine fish, but no species so far studied does not have at least some propensity to convert 18:3*n*-3 to 22:6*n*-3. This indicates, very importantly, that marine fish have the relevant genes and the problem is, then, how to maximally activate these genes. Generating new hybrids from different species, where rate limitations in converting 18:3*n*-3 to 22:6*n*-3 occur at different steps in the conversion pathway, also holds promise. Transgenetic approaches to the problem are also eminently possible. Such approaches, of course, will bring many of the currently perceived problems and, indeed, ills of biotechnology, at least in the short term. This should not, however, deflect us from developing the necessary science, which will undoubtedly be essential in the longer term for stock improvement.

The problem of developing efficient diets for marine fish larvae, addressed in Sections 4.5 and 4.6, is also one of high priority for future research. Perhaps new and more fundamental approaches are needed here. Current efforts are basically directed, largely for economic reasons, toward generating high stocking densities of very small animals and associated high stocking densities of tiny live feeds that have naturally evolved at low stocking densities. Indeed, a fundamental adaptation of herbivorous zooplankton and larval fish consuming these zooplankton and their nauplii is an ability to filter efficiently large volumes of seawater in a medium that is intrinsically nutrient-dilute. Should we not, therefore, accept this and give much more emphasis to culturing marine fish larvae in more dilute systems, i.e.,

in larger-volume systems such as mesocosms? If so, how large can such mesocosms be in practice? and When are they no longer distinguishable from Scottish sea lochs or Norwegian fjords? In short, should not mariculture become more associated with managing and restocking limited-scale, partly enclosed marine ecosystems? Recent and ongoing developments in ecosystem modeling and management will make this option increasingly attractive in the future.

Overall, we see the future of aquaculture, particularly from its speciality standpoint of *n*-3 HUFA production, as a successful blend of advanced biotechnology and ecologically sound sustainable development. Our optimism in writing this now may well be generated by the very recent arrival of the new millennium and its new challenges, but we believe that our optimism is justified.

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5

The Minerals

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Acknowledgment

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5.1 Introduction

All forms of aquatic animals require inorganic elements or minerals for their normal life processes. Unlike most terrestrial animals, fish have the ability to absorb some inorganic elements not only from their diets but also from their external environment in both freshwater and seawater. Many essential elements are required in such small quantities that it is difficult to formulate diets and maintain an environment that is low in minerals to demonstrate a mineral deficiency. Despite new developments in the mineral nutrition of fish, most research on inorganic elements has been confined to osmoregulation, toxicity, and related physiological functions. This chapter examines the mineral requirements of finfish and compares the mineral metabolism of fish with that of other animals.

It is well recognized that all living organisms contain most naturally occurring elements in the periodic table. At present, 29 of the 90 naturally occurring elements are known to be essential for animal life. The greater proportion of living matter consists of six basic structural elements—carbon, hydrogen, nitrogen, oxygen, phosphorus, and sulfur. These elements, found at high concentrations, are required in gram amounts. In addition, five macro elements, calcium, magnesium, sodium, potassium, and chlorine (as chloride), are also required in gram quantities. The remaining elements occur in the body at much lower concentrations (milligrams or micrograms per kilogram). Initial difficulties in the accurate determination of low levels of many of these elements inevitably led to their description as "trace elements." Today modern analytical techniques now permit the accurate analysis of these elements, with consequent demonstration of their unique functions in many metabolic processes.

By the simplest definition, an essential element is one required for the maintenance of life; its absence results in death of the organism. The criterion for an essential element has been well described by Frieden (1984): "An element is considered essential when a deficient intake produces an impairment of function when restoration of physiological levels of the element prevents or relieves the deficiency. The organism can neither grow nor complete its life cycle without the element in question. The element should have a direct influence on the organism and be involved in the metabolism. The effect of an essential element can not be wholly replaced by any other element." Essentiality is generally established when it has been demonstrated by more than one independent investigator and in more than one animal species.

Fifteen trace elements are considered to be essential in animals. Among these the physiological role of a deficiency of chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, selenium, and zinc is well

recognized. Although deficiencies of nickel, vanadium, silicon, and arsenic have been demonstrated in an ultraclean environment, with the exception of silicon, the physiological function of these trace elements has not been clearly demonstrated. Other elements, including cadmium, lead, bromine, and tin, have also been claimed to be essential, but their essentiality remains to be confirmed. Most of these trace elements have been detected in fish tissues, however, the essentiality of only a few of these elements has been demonstrated.

The main functions of essential elements in the body include the formation of skeletal structure, maintenance of colloidal systems (osmotic pressure, viscosity, diffusion), and regulation of acid–base equilibrium. They are important components of hormones, enzymes, and activators of enzymes (Table 5.1). Calcium and phosphorus are required for the formation of the skeletal structures of the body. Sodium, potassium, and chloride, along with phosphates and bicarbonates, maintain homeostasis and the acid–base balance. A fixed number of specific trace metals (Fe, Mn, Cu, Co, Zn, Mo,

Table 5.1

Essential Metalloenzymes in Aquatic Animals

Trace elements	Enzyme	Function
Iron	Succinate dehydrogenase	Aerobic oxidation of carbohydrates
	Cytochromes (*a*, *b*, *c*)	Electron transfer
	Catalase	Protection against H_2O_2
Copper	Cytochrome oxidase	Terminal oxidase
	Lysyl oxidase	Lysine oxidation
	Ceruloplasmin (ferroxidase)	Iron utilization, copper transport
	Superoxide dismutase	Dismutation of the superoxide free radical ($O_2^{\cdot -}$)
Zinc	Carbonic anhydrase	CO_2 formation
	Alcohol dehydrogenase	Alcohol metabolism
	Carboxypeptidases	Protein digestion
	Alkaline phosphatase	Hydrolysis of phosphate esters
	Polymerases	Synthesis of RNA and DNA chains
	Collegenase	Wound healing
Manganese	Pyruvate carboxylase	Pyruvate metabolism
	Superoxide dismutase	Dismutation of the superoxide free radical ($O_2^{\cdot -}$)
	Glycosylaminotransferases	Proteoglycan synthesis
Molybdenum	Xanthine dehydrogenase	Purine metabolism
	Sulfite oxidase	Sulfite oxidation
	Aldehyde oxidase	Purine metabolism
Selenium	Glutathione peroxidase	Removal of H_2O_2
	Type I and III deiodinases	Conversion of thyroxide to the active form

Se, etc.) are firmly associated with a specific protein in metalloenzymes, which produce a unique catalytic function. Certain minerals, such as calcium, magnesium, and manganese, are of particular significance as enzyme activators. A nonmetal, iodine, is necessary for the biosynthesis of thyroid hormones, which in turn greatly affect development and metabolism in all vertebrates. Some biologically important compounds contain mineral as an inherent part of their structure, e.g., hemoglobin and vitamin B_{12}.

5.1.1. Difficulties in Studying Mineral Requirements of Fish

Inorganic elements, such as nutrients, are difficult to study, particularly trace elements. The exchange of ions from the aquatic environment across gills and skin of fish complicates the determination of the quantitative dietary requirements. Many trace elements are required in such small amounts that it is difficult to formulate purified diets low in mineral and maintain water sufficiently free of the test element. Despite advances in instrumental analysis of trace elements making lower detection limits possible, there are still many problems associated with their accurate measurement in fish tissue to be overcome. A critical factor in the determination of ultratrace elements, such as manganese, vanadium, and chromium, is the need for meticulous sample preparation. Techniques that involve the use of high-purity reagents, acid-cleaned glassware, and clean-room facilities should be employed to avoid contamination. Often normal values of trace elements in fish tissue vary widely in reports from laboratory to laboratory. The use of certified reference materials (CRM) is also essential to assure differences arise from the fish tissues being examined, and not the analytical techniques employed.

Although a wide range of functions has been established for the essential elements for domestic animals and humans, such information available on fish is fragmentary and incomplete. Relatively little is known about the uptake, function, and biological availability of many trace elements. Environmental concerns about air and water pollution have prompted more research on the metabolism and functional role of toxic elements. To measure the nutritional status of trace elements, information is also required on the age, sex, health, and physiological conditions (e.g., smoltification and sexual maturation) of the animal.

The most commonly used measure of nutritional status is the level of trace element in the blood, muscle, liver, and bone. For many essential elements, there is a range of tissue levels compatible with optimum growth and function. A reduced level of mineral intake influences the tissue concentration, which causes a gradual decline in the function of an organ until clinical toxicity and deficiency occur (Fig. 5.1). However, it is the intermediate stage that is difficult to establish. More desirable measures of mineral nutritional status

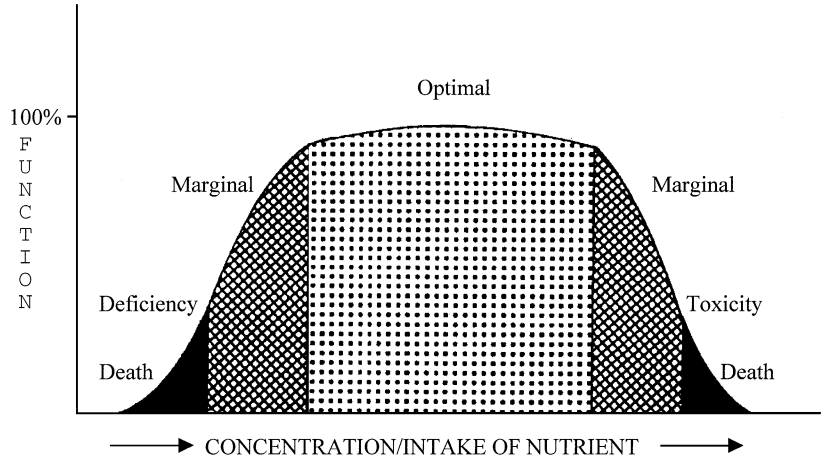

FIG. 5.1

Biological dose–response curve. Dependence of animal function on intake of an essential nutrient according to Mertz (1986).

are those which test some metabolic function of the element rather than a tissue analysis. For iodine, there is a well-defined functional test (thyroxine binding) to assess its nutritional status. However, for other elements such functional tests are not clearly established. Elucidation of techniques that will allow for the identification of subclinical, pathological change in the assessment of nutritional status remains a challenging area for mineral nutrition research.

5.1.2. Nature and Distribution of Elements in Fish

The concentration of minerals in the body of an aquatic organism depends on the food source, environment, species, stage of development, and physiological status of the animal. Most organisms accumulate and retain minerals from the environment, however, their incorporation is highly selective. In marine food chains, a unique transport of trace metals has been reported (Bernhard and Andreae, 1984). Most trace metals show the highest concentration increase at the first trophic level (seawater–phytoplankton). In zooplankton, only Cd, Cu, and Zn concentrations increase, whereas plankton-feeding fish have higher Cu and Zn levels than fish preying on invertebrates and fish feeding on invertebrates and fish. The concentration of Cd, Cu, and Zn increases along the food chain at the lower levels, reaching a maximum with crustaceans and then decreasing in fish. Information on the transport of other elements in the marine food chain is limited. An

interesting Russian study on fish from different lakes showed that the quantities of manganese, iron, copper, and zinc in the muscle and liver reflect the geochemical characteristics of the earth surrounding the lake (Love, 1980). Unlike, some soft tissues such as liver, muscle, and kidney, otolith and lenses retain metals for a longer duration and their concentration has been used to detect environmental pollution (Dove and Kingsford, 1998).

Although the distribution of many essential and toxic elements in various aquatic organisms has been reported, the complete elemental composition is reported for only a few fish species. The mineral composition of rainbow trout varies with fish size, stage of life cycle, and reproductive status (Shearer, 1984). The concentration of Ca, Cu, Fe, K, Mg, Mn, Na, P, Sr, and Zn was higher in juvenile fish than in adults. A decrease in somatic Mn, Fe, and Zn was found during gonadal maturation in female but not male fish. Disease state, water quality, and other environmental factors may also modify tissue mineral distribution (Lall and Olivier, 1993). With some exceptions, mineral element concentrations in structural organelles of tissue are less susceptible to dietary and environmental changes than are those of soft tissue and body fluids.

Generally Ca, Mg, Na, K, Fe, Zn, Cu, and Se are derived from the water to satisfy some of the nutritional requirements of fish, however, chlorides, phosphates, and sulfates are more effectively obtained from food sources (Lall, 1979; 1989). Fish eggs also absorb certain minerals from water. Zeitoun *et al.* (1976) demonstrated absorption of Na, Fe, and Zn at hatching in eggs of rainbow trout and uptake of Ca, Na, K, Fe, and Zn from the water after yolk absorption. They concluded that Cu and P found in the larvae probably originated from an unfertilized oocyte. A remarkable increase in the Mn content of *Salmo salar* and *Oryzias latipes* eggs after fertilization has been reported (Lall and Hines, 1985; Hori and Iwasaki, 1976). However, the uptake of heavy metals by eggs and by developing embryos shows some differences. The uptake of Cd by trout eggs is restricted by the chorion (Beattie and Pascoe, 1978). Waterborne Se accumulation in embryos was found to increase with the development of gills (Hodson *et al.,* 1986).

5.1.3. Skeletal Tissue Metabolism

In most vertebrates, the skeleton represents a reservoir of Ca, P, and other ions that are in a state of continual exchange with electrolytes found in blood and extracellular fluids. These functions require continuous remodeling, a process that involves three types of bony cells: osteoblasts (bone forming cells), osteocytes (entrapped inside the bone matrix), and osteoclasts (multinucleated bone resorbing cells). Osteocytes are thought to be involved in the maintenance of bone substances and the exchange of ions from body fluids. Thus, the skeleton of most vertebrae exerts a massive buffering

effect on changes in plasma electrolyte values. Among fish a variety of skeletal systems performs these functions. The physiology of skeletal tissue has been discussed in detail by Simmons (1971) and Simkiss (1974).

Morphologically, fish bones consist of the dermal bones of the head, the internal skeleton, and the scales. The histology of fish bones is basically similar to that of higher vertebrates. However, fish do not have any hematopoietic elements within the bones. Bone and scales of fish constitutes calcium hydroxyapatite salts embedded in a matrix of type I collagen fibers. They contain the bone-specific protein, octeocalcin (Nishimoto *et al.,* 1992). Scales do not contain enclosed cells, but they are covered by sclroblasts, the cells thought to be responsible for matrix production and mineralization. There are two types of fish bones, cellular and acellular. Cellular bones are confined to only a few groups of fish, e.g., Salmonidae, Cyprinadae, and Clupeidae. The skeletal tissue of higher orders of teleost fish such as Perciformes is unique among vertebrates in that it lacks osteocyte cells. Acellular bones are formed from osteoblast cells which move away from the site of mineralization as bone deposition occurs and therefore are incapable of extensive modeling (Moss, 1963). The tissues of acellular bone may not be directly involved in calcium metabolism under normal conditions (Taylor, 1985). However, Witten (1997) has demonstrated that mononucleated cells perform bone resorption in juvenile cichlids.

Scales are formed by the replacement of dermal connective tissues during intramembranous ossification. Generally these scales consist of two layers, a superficial mineralized "bony" or hydrodentine layer, which is deposited over a deeper fibrous sheet described as the "lamellar" or "fibrillar plate" (Simkiss, 1974). Undoubtedly, there are many variations in fish scales and their structures. Resorption of scales occurs during spawning migration of salmon and also during food deprivation, but the biochemical mechanism is not clear.

The elemental concentration of skeletal tissues varies with age in both cellular and acellular bones. The mineral phase of fish bone is poorly crystallized apatite (Neuman and Mulyran, 1968). The crystals in acellular bone are smaller and/or more strained than in cellular fish bone (Moss and Posner, 1960). The main constituents of fish vertebrae include calcium, phosphate, and carbonate, with small amounts of magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide, and sulfate. Calcification and changes in bone composition resulting from changes in calcium and phosphorus nutrition are considered in more detail later.

5.1.4. Mineral Interactions

Several nutrient interactions and interrelationships are well known (e.g., niacin–tryptophan and Ca–P–vitamin D) in terrestrial animal and human

nutrition (Mills, 1985; McDowell, 1992). A wide range of potential mineral–mineral and mineral–vitamin interactions has also been reported (Hilton, 1989). Interrelationships may manifest themselves as competition for binding sites on transport or storage molecules or substitution at an active site of an enzyme or as a requirement for one element for the proper metabolism of another. Antagonistic relationships occur when elements with a similar electronic configuration and ionic radius compete for binding sites, e.g., zinc and cadmium in metallothionein and magnesium/manganese substitutions at enzyme active sites. Synergistic relationships in which one element enhances the role of another, e.g., iron and copper, are difficult to explain. The complex interrelationship among copper, zinc, iron, and calcium, as well as that of copper, molybdenum, and sulfur, is of practical concern (Mertz, 1986; Davis, 1980) but beyond the scope of this chapter. Another type of interrelationship involves the interactions between the elements themselves. Selenium has a high affinity for certain toxic elements such as mercury and silver; hence selenium exerts a protective effect against the toxicity of these metals by forming complexes *in vitro,* resulting in a decrease in the biological availability of both selenium and the heavy metal.

Minerals also interact with other nutrients. A synergism between dietary selenium and vitamin E is known. Zinc is required for the metabolism of vitamin A. The strong redox potential of vitamin C may alter the valence of copper and iron and thus reduce or enhance absorption. Some mineral interactions determined with experimental animals may not be directly applicable to fish because of the differences in mineral uptake from food and water in fish (Hilton, 1989). However, the application of techniques to assess mineral interactions and bioavailability is extremely useful.

5.1.5. Environmentally Induced Toxic Elements

It has become apparent in recent years that fish and aquatic organisms are able to accumulate and retain trace elements drawn from their environment. Metals enter the hydrosphere from either natural processes or pollution through activities such as mining operations, burning of fossil fuels, agriculture, and urbanization. The solubility of trace metals in natural waters is controlled principally by the pH, type and concentration of ligands and chelating agents, and oxidation state of the mineral components and the redox environment of the system. The soluble forms are usually ions (simple or complex) or nonionized organometallic chelates or complexes. These metals are absorbed by aquatic organisms through gills and body surfaces and from ingestion of food or water.

Although aquatic organisms readily absorb metals from the environment, their ability to regulate abnormal concentrations varies with the species.

Certain fish and crustaceans are able to excrete high proportions of excessive metal intake and consequently regulate the concentration in the body at relatively normal levels (Bryan, 1976). This occurs for essential elements such as Cu, Zn, and Fe. Fingerlings and newly hatched fry are poorer regulators of these metals than nonessential minerals such as Hg, Cd, and Pb. Generally the gills, digestive tract, feces, and urine are involved in regulatory and excretory processes.

Sublethal effects of several metals on aquatic organisms have been demonstrated experimentally (Bryan, 1979). Most sublethal toxicity appears to be of a biochemical origin and causes morphological, physiological (growth, swimming performance, respiration, and reproduction), and behavioral changes (Bryan, 1976; Alabaster and Lloyd, 1980). The toxicity mechanisms of metal ions include blocking of essential biological functional groups of enzymes, displacing the essential metal ion in the biomolecule (enzyme or protein) and modifying the active conformation of the biomolecule. The concept and modes of intake and absorption of heavy metals have been discussed in detail by Simkiss and Taylor (1989).

5.1.6. Osmoregulation and Saltwater Adaptation

The problem of osmotic and ionic regulation in fish has been the subject of several reviews (Maetz, 1971; Kirschner, 1979; Evans, 1979, 1980; Eddy, 1982; Boeuf, 1987). The predominant osmotically active solutes are sodium, potassium, and chloride. Proteins constitute a small part of the osmotic concentration; however, they play an important role in the regulation of fluid across cell membranes. In many animals, trimethylamine oxide and urea also contribute to the osmotic composition of solutes. Even though calcium, magnesium, bicarbonate, and phosphate are not directly involved in osmoregulatory processes, they influence the functioning of the kidney, an important osmoregulatory organ. In various regulatory processes, respiration supplies oxygen and removes carbon dioxide, digestion maintains the level of nutrients, and osmoregulation controls the volume and composition of fluids.

Fish maintain the ionic composition and osmolarity of their body fluids at levels significantly different from those of the surrounding water. Although large areas of body surface, such as the gills, which serve a respiratory function, remain exposed to the external environment, the blood of freshwater fish is hypertonic and that of marine fish is hypotonic compared to their external medium (Fig. 5.2). Hagfish blood closely resembles seawater in its salt composition. They do not osmoregulate but instead regulate ions so that the total osmotic pressure of their plasma is almost identical to that of seawater.

In freshwater, the active uptake of salts through the gills, along with the low body surface permeability, enables them to maintain blood ion levels

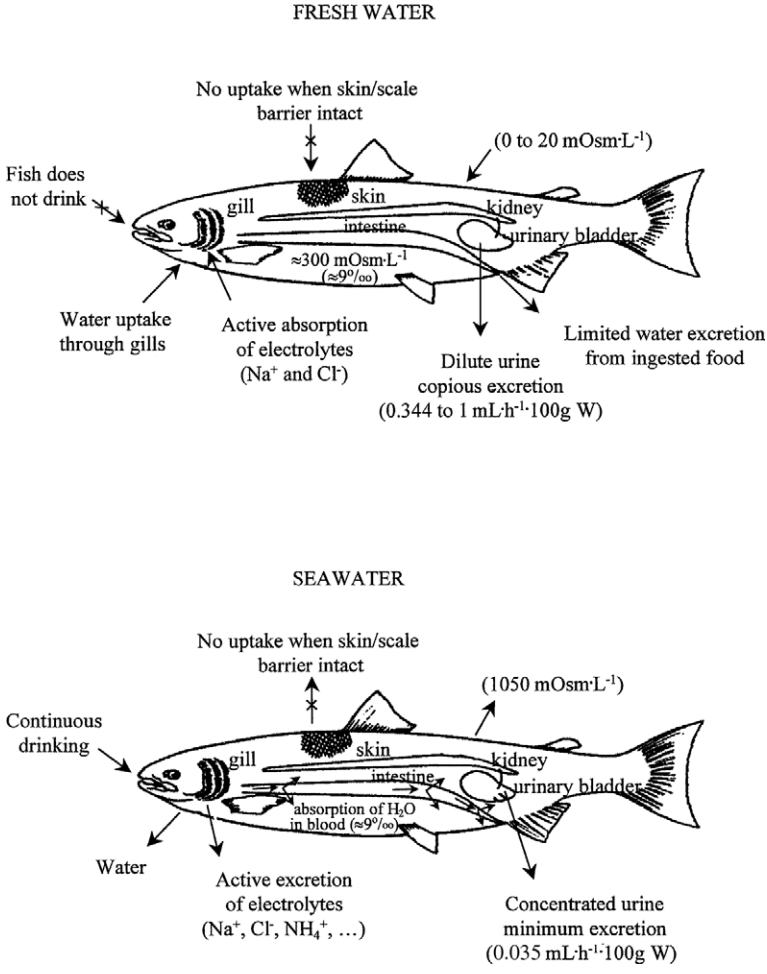

FIG. 5.2

Osmoregulation by rainbow trout in freshwater (0‰) and seawater (35‰). From Boeuf (1987) with permission.

that are more concentrated than those of the dilute milieux. Kidneys aid in osmoregulation by maintaining a high glomerular filtration rate along with tubular and bladder reabsorption of filtered ions, producing copious dilute urine. Marine fish lose water through any permeable surface and thereby increase their level of salt. They replace water loss osmotically by drinking seawater. The gut actively absorbs monovalent ions and water into the blood,

accumulating most of the divalent ions in the intestine with the same osmolarity as blood. Water conservation is further enhanced by reduced glomerular filtration, the kidney serving principally as a divalent-ion secretory organ. Excess monovalent ions derived from swallowed seawater and the passive uptake across the body surface are excreted, mainly through the gills.

Saltwater adaptation and osmotic and ionic regulation in euryhaline fish, particularly salmon, trout, and eels, are well documented (Folmer and Dickhoff, 1980; Eddy, 1982; Boeuf, 1995). Euryhaline fish withstand abrupt changes in environment salinity with several physiological adjustments. These include modification of branchial chloride cell number (Thomson and Sargent, 1977), drinking rate (Krogh, 1939), urine production rate (Hickman and Trump, 1969), gill epithlium permeability, and ion efflux (Maetz, 1971; Simkiss and Taylor, 1989). Smoltification of migratory salmonids is associated with marked changes in morphology, chemical composition, physiological and biochemical function, and behavior. The underlying mechanisms for these changes remain to be fully elucidated. It is widely accepted that the enzyme sodium-, potassium-activated adenosine triphosphatase (Na^+,K^+-ATPase) in the gill plays an important role in seawater adaptation. The activity of this enzyme increased in smolts prior to seawater entry (Zaugg and McLain, 1972). The exact mechanism by which environmental changes stimulate Na^+,K^+-ATPase activity is not clear, but there is evidence of hormonal involvement. Cortisol and prolactin influence the gill Na^+,K^+-ATPase response to salinity (Epstein *et al.,* 1971; Pickford *et al.,* 1970). Thyroxine plays an important role in parr–smolt transformation (LaRoche and Leblond, 1952), however, there is no evidence of thyroid stimulation of gill ATPase activity in fish.

Evidence indicates that nutritional status influences the adaptation of euryhaline fish to seawater. Feeding a diet high in salt content enables trout and salmon to adapt to seawater more easily (Zaugg and McLain, 1969; Basulto, 1976). Furthermore, starved rainbow trout are unable to maintain their gill Na^+,K^+-ATPase activity at the same level as fed animals (Jürss *et al.,* 1983). Free amino acids, apart from their metabolic function, play an important role in osmotic regulation mechanism during salinity changes in rainbow trout (Jürss, 1980; Jürss *et al.,* 1983). The concentration of several amino acids in the muscle of rainbow trout held in seawater was higher than in animals starved in freshwater (Jürss, 1980).

5.1.7. Bioavailability of Minerals

To determine how efficiently the body utilizes dietary mineral elements, one must know the availability of that element from a feed ingredient or complete diet. Generally, chemical analysis does not indicate the level of

biologically effectiveness of a nutrient. Many mineral bioavailability problems are being increasingly recognized in human and animal nutrition (Forbes and Erdman, 1983). It has been shown that the bioavailability of an element can differ markedly when supplied from different feedstuffs and within the same element from feed in different diets. Many factors can influence the bioavailability of minerals. These include the intake level of the nutrient, its chemical form, the digestibility of the diet that supplies the element, the particle size, interactions with other nutrients, chelators, inhibitors, physiological and pathological states of the animal, the water chemistry, the type of feed processing, and the species of animal being tested.

The biological availability of an element in a diet can differ depending on the molecular form in which the element is present, its valence state, and the ligands present when the element is ingested from different diets. Mechanisms that involve the formation of insoluble and nonabsorbable substances in the gut may either hinder or facilitate the mucosal uptake, transport, and metabolism of an element in the body. Certain inorganic elements may compete with the test element for important binding sites during these processes. Factors influencing the bioavailability of zinc and iron in terrestrial animals are well documented (O'Dell, 1969; Forbes and Erdman, 1983; Benito and Miller, 1998). Zinc is better absorbed from animal protein supplements than from plant protein sources. Cereals and other plant feedstuffs contain a number of substances, particularly phytate, which can bind zinc, making it unavailable for absorption. The bioavailability of iron is influenced not only by its chemical form, but also by interactions between iron and other dietary components. Ascorbic acid enhances iron absorption, whereas phytate and tannic acid may decrease it. The availability of individual mineral elements is discussed in later sections. Apart from these dietary components, several environmental factors can also influence mineral bioavailability.

5.1.8. Effect of Mineral Supplements in Experimental Diets

Although fish have the ability to derive certain elements from the surrounding water, both practical and purified diets require mineral supplementation. Purified diets without mineral supplements result in a loss of appetite, growth depression, hypochromic anemia, a high mortality, and cranial deformities (Nose, 1972; Ogino and Kamizono, 1975). The addition of mineral supplements to these diets improved growth and survival. However, early work by Wolf (1951) indicated that rainbow trout fed a purified diet containing no mineral supplement grew as well as those receiving a 4% mineral mixture in a 23-week experiment. It is probable that fish were able to utilize calcium, phosphorus, and other trace elements from

the commercial casein in the diet and also from the hatchery water supply. Ogino and Kamizono (1975) found that common carp (*Cyprinus carpio*) did not show any clinical symptoms of mineral deficiency on a diet devoid of a mineral supplement in a 50-day experiment. The addition of mineral supplements resulted in only a slight improvement in growth rate.

The importance of mineral supplements in practical diets has also been emphasized. Ketola (1975a) reported that mineral supplementation of a soybean meal-based diet improved the growth and bone mineralization of rainbow trout. Similarly, Arai *et al.* (1975) found that the addition of calcium to a diet containing yeast resulted in an increase in growth. Commercial fish feeds contain a significant proportion of fish meal in the diet. Although fish meal is considered an adequate source of dietary minerals, supplementation of fish meal-based diet with certain trace elements may be necessary for optimum growth and bone mineralization (Watanabe *et al.,* 1980b; Arai *et al.,* 1974; Cuplin, 1969; Murakami, 1970). Murakami (1970) observed that the addition of phosphorus to commercial carp diet improved growth and prevented cranial deformities. Excessive amounts of minerals, particularly Ca and P, reduce zinc bioavailability and have also been linked to cataract formation in juvenile salmonid fish (Ketola, 1975a; Richardson *et al.,* 1986). It is essential to supplement diets containing high levels of total minerals (ash), particularly Ca and P, with trace elements. An imbalance of dietary minerals in certain diets predisposes the Atlantic salmon to bacterial kidney disease (BKD) under specific environmental conditions (Lall *et al.,* 1985). Supplementation of the diets with I, F, Fe, Cu, Co, Mn, and Zn resulted in a lower incidence of BKD infections.

Trace metal supplements are essential in practical broodstock diets. Takeuchi *et al.* (1981b) demonstrated the effects of maternal trace element deficiency on rainbow trout egg quality. The trace element-deficient group showed a reduction in egg production, hatchability of eggs, Mn content of eggs, and female body weight. The deletion of Mn from fish meal-based practical diets of Atlantic salmon and brook trout caused a reduction in the hatchability of eggs and a decrease in the Mn concentration of seminal fluid and eggs (Lall, 1985).

5.2 Essential Minerals for Finfish

5.2.1. Calcium and Phosphorus

Calcium and phosphorus are closely related to the development and maintenance of the skeletal system and participate in several physiological processes including the maintenance of acid–base equilibrium. Vertebrae

maintain their rigidity from a solid phase of calcium phosphate. Fish scales are an important site of Ca metabolism and deposition. The Ca exchange rate of fish scales is three times that in bones (Berg, 1968). The amount of Ca in scales of tilapia ranges from 19 to 24% of the dry weight (Garrod and Newell, 1958). The amount of Ca in the scales of tilapia, goldfish (*Carassius auratus*), carp, and salmon decreases during spawning migration and starvation (Crichton, 1935; Ichikawa, 1953; Yamada, 1956; Garrod and Newell, 1958). The ratio of calcium to phosphorus in scales and bones ranges from 1.5 to 2.1 for common carp, pike (*Esox lucius*), Nile tilapia, sea bream, rainbow trout, and chum salmon (Van Oosten, 1957; Ogino and Takeda, 1976; Ogino *et al.*, 1979b; Sakamoto and Yone, 1979a; Watanabe *et al.*, 1980a,b). The ratio of calcium to phosphorus in the whole body of several fish species ranges from 0.7 to 1.6, while the level of phosphorus in the whole body is about 0.4 to 0.5% of the fresh wet weight (Lall, 1991).

No attempt is made in this section to discuss the regulation of Ca in any detail, since this has been considered in recent reviews (Feinblatt, 1982; Copp, 1982; Hunn, 1985; Taylor, 1985; Flik *et al.*, 1993). Attention is directed to the nutritional requirement of phosphorus, since calcium is readily derived from the water and occurs in adequate amounts in most diets consumed by fish.

5.2.1.1. Functions and Metabolism

5.2.1.1.1. Calcium. Calcium is one of the most abundant cations in the body of a fish. Despite the importance of calcium in bone formation and maintenance of skeletal tissues, calcium ions are widely distributed in soft tissues. Other functions of calcium include muscle contraction, blood clot formation, nerve transmission, maintenance of cell membrane integrity, and activation of several important enzymes. In the cell membrane, calcium is closely bound to phospholipid, where it controls the permeability of membrane and thus regulates the uptake of nutrients by the cell.

The calcium requirement of fish is met in large part by their ability to absorb these ions directly from the aquatic environment. Unlike terrestrial animals, bone is not the major site of Ca regulation in fish. Gas exchange across gills in fish provides them with continuous access to an unlimited Ca reservoir. Thus, regulation of Ca influx and efflux occurs at the gills, fins, and oral epithelia. These structures are all actively involved in marine fish, however, the gills are the most important site for Ca regulation in both freshwater and marine fish. On a comparative basis, Ca ion transport across the gills appears to be more efficient in freshwater. The gut is not a major site of Ca absorption in marine fish, which drink copiously (Simmons, 1971). Some minerals (Mg, Sr, Zn, and Cu) reduce Ca absorption (Podoliak, 1970). The endocrine control of Ca metabolism in fish is not fully

understood (Wendelaar Bonga and Flik, 1995). Calcitonin inhibits Ca influx across salmon gills (Milhaud *et al.,* 1977). Prolactin stimulates Ca uptake by tilapia (Wendelaar Bonga *et al.,* 1984). Vitamin D metabolites do not affect Ca homeostasis (Hayes *et al.,* 1984). There are major differences in the hormonal mechanisms that regulate Ca and P balance in fish and terrestrial animals. In terrestrial vertebrates, three hormones generally control the metabolism of Ca as well as P: parathyroid hormones (PTH), calcitonin, and an active metabolite of vitamin D_3, 1,25-dihydroxycholecalciferol [1,25-$(OH)_2$D]. These hormones control the absorption of Ca and P from the gastrointestinal tract, influence their deposition and resorption in bone, and influence the extent of their excretion in urine and feces. PTH elevates the blood Ca concentration by increased mobilization of Ca from bones, increased tubular resorption of Ca, and enhanced production of 1, 25-$(OH)_2$D. Calcitonin acts as a physiological antagonist of PTH and 1, 25-$(OH)_2$D. When the blood Ca level is high, calcitonin prevents the mobilization of Ca into serum, thus inhibiting the resorption of Ca ions in the kidney and rapidly decreasing blood Ca to normal levels. The concentration of PTH is inversely proportional to the plasma Ca concentration, whereas that of calcitonin is directly proportional. PTH is also involved in the conversion of vitamin D into its active metabolite 1, 25-$(OH)_2$D, which in turn stimulates Ca and P absorption from the intestine.

The endocrine control of Ca and P metabolism in fish is also regulated by hyper- and hypocalcemic hormones, however, they lack PTH. Teleosts possess two hormones with hypocalcemic action: calcitonin, secreted by the ultimobranchial gland, and stanniocalcin (STC), secreted by the corpuscles of Stannius. The role of calcitonin (Wendelaar Bonga and Pang, 1991) and vitamin D metabolites (Vielma *et al.,* 1999a) in Ca and P homeostasis is not clearly established. STC is the predominant hormone that regulates Ca and phosphate metabolism in freshwater and seawater salmon as well as several freshwater fish (Verbost *et al.,* 1993; Wagner *et al.,* 1998). A rise in the plasma levels of ionic calcium (Ca^{2+}) stimulates STC secretion, and this hormone acts on the gills, gut, and kidney to restore normocalcemia. The level of dissolved Ca in the environment also acts as a stimulus for hormone synthesis and secretion. The environmental Ca effect is mediated by its diffusion across the gills and skin, as well as absorption via the gut as marine fish ingest large amounts of seawater. On account of these factors, STC cells in marine fish are metabolically more active and secrete more hormones than freshwater fish. Most of the research on Ca metabolism and the endocrine control in fish is based on freshwater species.

The absorbed Ca is deposited in bone and skin. Generally, the rate of uptake, the deposition pattern, and retention by the skeletal tissues appear to be similar in freshwater and marine species and also independent of bone

types, i.e., cellular or acellular bone. The chemical composition of the bony layer of scales is similar to that of other skeletal tissues but differs physiologically in Ca metabolism. An extensive study by Berg (1968) on Ca and strontium metabolism showed that the Ca exchange rate of fish scales was three times that in bones. The scales are also the site of labile Ca storage. In fish with smooth skin, such as eels and catfish, the cellular bones must also play an important role in Ca turnover. Resorption of scales occurs in fasting salmon during spawning migration. Calcium is excreted principally by the kidneys or through the gills. Feces also contain endogenous Ca secreted in the gut.

5.2.1.1.2. Phosphorus. Phosphorus exists naturally as inorganic phosphate, denoted P_i, and as organic phosphate, bound in molecules such as sugars, proteins, and other components of the cell. Hydroxyapatite, $Ca_5(PO_4)_3(OH)$, has the important role of being the principal crystalline material of bone. In addition, phosphorus is located in every cell of the body. Phosphate occurs in equilibrium with H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}. The predominant form at a neutral pH is HPO_4^{2-}, whereas phosphoric acid (H_3PO_4) is the major form at a low pH. In soft tissues, phosphate plays roles as (a) a structural component, (b) a factor in intermediate metabolism, and (c) a component of genetic material. Phospholipids are a major constituent of cell membranes and intracellular organelles. In DNA and RNA, phosphate is an essential part of the nucleic acids. Phosphate is a constituent of highly active intracellular compounds. Release of high-energy phosphate by hydrolysis of adenosine phosphate (ATP) provides the main source of energy for metabolic processes and for muscle contraction. Phosphate plays an important role in carbohydrate, lipid, and amino acid metabolism and in muscle and nervous tissue metabolism, as well as various metabolic processes involving buffers in body fluids.

The regulation of phosphate is considered more critical than that of Ca because fish must effectively absorb, store, mobilize, and conserve phosphate in both freshwater and seawater environments. Dietary phosphorus supplies most of the phosphate required for growth and metabolism. Urinary loss of phosphate is much higher in freshwater than seawater fish because of the large volume of urine produced in freshwater fish. In seawater-adapted eels, P excretion was 40 times lower than in eels held in freshwater (Chester Jones *et al.,* 1969). Approximately 90% of the phosphate lost from the body is excreted renally (Tomiyama *et al.,* 1956). To replace this loss and to supply the various structural and physiological needs of the body, phosphate must be absorbed from both food and water. The uptake of ^{32}P from water has been well documented (reviewed by Lall, 1979, 1991). In carp, the phosphorus uptake increases with a rise in water temperature and with any decrease in the Ca content of the water (Solomalina and Arsan, 1979).

Food is the main source of phosphate for fish because freshwater and seawater are low in phosphate. The amount of phosphate absorbed from the food is affected by the level of phosphate in the blood (Kudriavetz and Pora, 1958; Phillips, 1962). The absorbed phosphorus accumulates in soft tissues (heart, liver, kidney, muscle, and blood, etc.) and deposition in skeletal tissues is relatively low. When fish were exposed to water containing ^{32}P and then transferred to water free of this isotope, there was a rapid loss of ^{32}P from soft tissues; hard tissue ^{32}P remained unaffected (Tomiyama *et al.,* 1956; Asano and Ito, 1957).

The mechanism of phosphorus absorption and transport in fish has not been well studied. In higher vertebrates, intestinal phosphorus absorption is dependent on a Na gradient caused by the active transport of Na, and thus the transport of phosphorus from the lumen into the cells through the brush border membrane is secondary to that of Na. A Na-dependent absorption of inorganic PO_4^{3-} in carp intestine has been reported (Nakamura, 1985a,b). The control of intestinal absorption of PO_4^{3-} in most fish has not been studied with the exception of eels, where pituitary hormone is involved (Nakamura and Hirano, 1986). It was pointed out before that pituitary hormones are involved in the Ca regulation of fish but the specific hormone responsible for the effect is not known. It is also not clear whether the effect is mediated by vitamin D metabolites as in terrestrial vertebrates (Wendelaar Bonga and Pang, 1989). Although intraperitaneal injection of vitamin D metabolites influences P homeostasis (Fenwick and Vermette, 1989), the dietary intake of cholecalciferol had no clear effect on P absorption and retention in rainbow trout (Vielma *et al.,* 1999a). The removal of pituitary hormones caused a net decrease in phosphate absorption in freshwater eels (Nakamura and Hirano, 1986). However, the pituitary plays a limited role in the regulation of plasma phosphate in fish.

Bone may be a reservoir for phosphate rather than for Ca (Fleming, 1967). The level of blood Ca is similar in bony and cartilaginous fish, however, the blood phosphate level is much lower in cartilaginous fish (Fontaine, 1932). It is possible that the mechanism for regulating phosphate is similar in both freshwater and seawater because the concentration of this ion is low in both environments. The Ca level is high in the urine of seawater fish, whereas the phosphate concentration is low. A phosphorus deficiency lowers the plasma and urine phosphate concentrations, however, the total-body phosphorus is significantly depleted when fish are fed a phosphorus-deficient diet for a long period (Vielma and Lall, 1998b).

The fertilization of fish ponds to augment the production of natural food organisms is a common practice. The amount of inorganic phosphorus present in the water may directly affect the cellular energy metabolism and the physiological adaptation of fish to environmental conditions

(Arsan *et al.,* 1984). When phosphorus concentrations of freshwater ranged between 0.3 and 0.6 mg/liter, several physiological indices of carp were affected. These included an increase in alkaline phosphatase activity in kidney and a decrease in the activities of Na^+- and K^+-dependent ATPase and Mg^+-dependent ATPase in gill and kidney (Solomalina and Arsan, 1979). In addition, in recirculation systems used for rearing fish, the higher amount of P accumulation in freshwater and seawater may also affect P homeostasis and nutrient utilization (Lall, unpublished).

5.2.1.2. Deficiency

5.2.1.2.1. Calcium and Phosphorus. The uptake of Ca from seawater is not sufficient to meet the dietary Ca requirement of red sea bream (Sakamoto and Yone, 1973, 1976b). Calcium deficiency has not been detected in carp and catfish in freshwater (Andrews *et al.,* 1973; Ogino and Takeda, 1976) or in Atlantic salmon in seawater (Lall and Bishop, 1977). Generally, Ca from the feed ingredients of practical diets supplies sufficient Ca to meet the requirements of most finfish. Signs of phosphorus deficiency are listed in Table 5.2**.** Murakami (1967) reported cranial deformity associated with other skeletal abnormalities occurring in carp reared on commercial feeds. This problem was successfully overcome with the addition of calcium phosphate or 5% McCallum salt mixture in the diet. Phosphorus deficiency signs have been produced experimentally in several fish species and include poor growth, reduced feed efficiency, and poor bone mineralization. Other signs of deficiency in carp are an increase in the activity of certain gluconeogenic enzymes in liver, an increase in carcass fat with a decrease in carcass water content, reduced blood phosphate levels, a deformed head, and deformed vertebrae (Ogino and Takeda, 1976; Onishi *et al.,* 1981; Takeuchi and Nakazoe, 1981). A reduction in the hematocrit level of catfish may also occur (Andrews *et al.,* 1973). A low phosphorus intake by red sea bream also causes curved, enlarged vertebrae; increased serum alkaline phosphatase activity; higher lipid deposition in muscle, liver, and vertebrae; and a reduction in liver glycogen content (Sakamoto and Yone, 1980). A significant reduction in operculum and scale P concentration occurs in fish fed low-P diets (Skonberg *et al.,* 1997; Vielma and Lall, 1998a).

5.2.1.3. Requirement

5.2.1.3.1. Calcium. The Ca requirement of fish is affected by the water chemistry, the phosphorus level in the diet, and species differences. In large part, the requirement of most fish is met by absorption of Ca through gills and skin in freshwater and by drinking of seawater. A low concentration of Ca (0.34% or less) is required in the diet of carp, eel, red sea bream, tilapia, and catfish for optimum growth (Ogino and Takeda, 1976; Lovell,

Table 5.2

Mineral Deficiency Signs Reported in Certain Finfish

Mineral	Deficiency signs (fish species)[a]
Calcium	Reduced growth and poor feed conversion (1,8–10), anorexia (8), reduced bone mineralization (10)
Phosphorus	Reduced growth (1,3,6–9), anorexia (8), poor feed conversion (1,3,5,6,9), reduced bone mineralization (1,3,6,7,9), skeletal deformity (1,3,7), cranial deformity (7), curved and enlarged spongy vertebrae (9), increased visceral fat (7)
Potassium	Anorexia (5), convulsion (5), tetany (5), mortality (5)
Magnesium	Reduced growth (1,6–8), anorexia (1,6–8), sluggishness (1,6,7), nephrocalcinosis (1), convulsions (7), cataracts (1,7), degeneration of muscle fibers and epithelial cells of pyloric cecum and gill filaments (1), skeletal deformity (1), reduced bone mineralization (1), reduced bone (1,3,6,7,10), body (3), and serum (3) Mg concentration, mortality (1,6,7)
Iron	Reduced growth and poor feed conversion (6), hypochromic microcytic anemia (2,3,7–9), low hematocrit and hemoglobin levels (1,3,6), reduced plasma Fe and Fe transferrin saturation (3,6)
Zinc	Reduced growth (1,3,6,7), anorexia (6,7), short-body dwarfism (1), cataracts (1,7), fin erosion (1,7), skin erosion (7), reduced body zinc (3), bone zinc (1,6), and bone Ca (6) concentrations, low serum Zn level (6), mortality (1,7)
Manganese	Reduced growth (1,7,11), loss of equilibrium (11), short-body dwarfism (1,7), cataracts (1,7), high mortality (3,11), reduced bone (2,3) and body (3) Mn concentration (2,3), poor hatchability of eggs (1,2,3), abnormal tail growth (1)
Copper	Reduced growth (7), cataracts (7), reduced liver Cu/Zn-superoxide dismutase (3) and heart cytochrome *c* oxidase activity (3,6)
Selenium	Reduced growth (6,7), anemia (7), cataracts (7), muscular dystrophy (3), exudative diathesis (1), reduced glutathione peroxidase activity (1,3,6)
Iodine	Thyroid hyperplasia (1,2,5)

[a] *Key to fish species:* (1) Rainbow trout (*Oncorhynchus mykiss*); (2) brook trout (*Salvelinus fontinalis*); (3) Atlantic salmon (*Salmo salar*); (4) chum salmon (*Oncorhynchus keta*); (5) Chinook salmon (*Oncorhynchus tshawytscha*); (6) channel catfish (*Ictalurus punctatus*); (7) common carp (*Cyprinus carpio*); (8) Japanese eel (*Anguilla japonica*); (9) red sea bream (*Chrysophrys major*); (10) blue tilapia (*Oreochromis aureus*); (11) Mozambique tilapia (*Oreochromis mossambica*).

1978, Arai *et al.,* 1975; Sakamoto and Yone, 1973; Robinson *et al.,* 1984). Catfish and tilapia reared in water with a low Ca concentration (<1 mg Ca/liter) require 0.45 and 0.7% calcium in the diet, respectively (Robinson *et al.,* 1986, 1987). Atlantic salmon absorbs Ca from seawater, thus making dietary supplementation unnecessary (Lall and Bishop, 1977). The uptake of Ca from seawater is not sufficient to meet the dietary Ca requirement of red sea bream (Sakamoto and Yone, 1973, 1976b). Calcium deficiency has not been detected in carp and catfish in freshwater (Andrews *et al.,* 1973;

Ogino and Takeda, 1976) or in Atlantic salmon in seawater (Lall and Bishop, 1977). Generally, the feed ingredients of practical and purified diets supply sufficient Ca to meet the requirements of most finfish. High concentrations of dietary Ca and P interfere with the absorption and retention of certain trace elements and Mg. This is discussed in subsequent sections related to the specific elements.

5.2.1.3.2. Phosphorus. The phosphorus requirements for various fish are listed in Table 5.3. Dietary phosphorus requirements ranging from 0.5 to 0.8% have been reported for rainbow trout (Ogino *et al.,* 1979b), Atlantic salmon (Ketola, 1975; Lall and Bishop, 1977; Aasgard and Shearer, 1997; Vielma and Lall, 1998b), chum salmon (Watanabe *et al.,* 1980a), carp (Ogino *et al.,* 1979b), and red sea bream (Sakamoto and Yone, 1979a). The phosphorus requirement of Atlantic salmon is not significantly influenced when they are reared in either freshwater (Ketola, 1975b; Asgaard and Shearer, 1997) or seawater (Lall and Bishop, 1977). Andrews *et al.* (1973) reported the phosphorus requirement of catfish as 0.8% of the available phosphorus in a practical diet. Lovell (1978) and Wilson *et al.* (1982) reevaluated the phosphorus requirement using a purified diet and estimated it to be approximately 0.4% available phosphorus. The phosphorus requirement of Japanese eel is relatively low (0.29%) compared with that of other finfish (Nose and Arai, 1979).

Many studies with monogastric animals have shown that an optimum calcium-to-phosphorus ratio is important. Increasing the Ca-to-P ratio of the diet interferes with the absorption of P, and conversely, a high P-to-Ca ratio may restrict Ca absorption. Although the magnitude of the effect changes with the species and forms of Ca and P present in the diet, such studies on the Ca-to-P ratio in fish diets are limited. An optimum Ca-to-P ratio is important in the diet of red sea bream, 1:2 (Sakamoto and Yone, 1973), and eel, 1:1 (Nose and Arai, 1979).

5.2.1.4. Sources

Most salts of alkali metals present in foods are found in a freely soluble ionized form. However, the salts of alkali earth metals, such as Ca and Mg, are less soluble and form complex organic salts with varying degrees of solubility in the digestive tract. Certain naturally occurring Ca salts such as calcium oxalate are more readily degraded during the digestion process. Feedstuffs of animal origin show the highest concentration of Ca and P. Among common feedstuffs used in fish feed formulation, fish meal (1.5 to 3.2% P) and meat and bone meal (3.5 to 5.5% P) are the richest source of Ca and P. Skeletal tissue contributes a significant proportion of P in fish meal and meat meal. The major proportion of P in these feedstuffs is found in an inorganic form, whereas the remainder is present as phosphate complexes

Table 5.3

Mineral Requirements of Certain Finfish [a,b]

Species	Calcium (%)[c]	Phosphorus (%)[d]	Potassium (%)	Magnesium (%)	Iron (mg)	Copper (mg)	Manganese (mg)	Zinc (mg)	Iodine (μg)[e]	Selenium (mg)
Rainbow trout	—	0.6	R[f]	0.05	R	3	13	15–30	1.1	0.15–0.3
Atlantic salmon	—	0.6	R	0.04	30–60	5	10	37–67	R	R
Pacific salmon	—	0.6	0.8	R	R	R	R	R	0.6–1.1	R
Tilapia	—	0.9	R	0.06	R	3.5	12	20	R	R
Channel catfish	—	0.45	R	0.04	30	5	2.4	20	1.1	0.25
Common carp	—	0.6–0.7	R	0.05	150	3	13	15–30	R	R
Japanese eel	0.27	0.3	R	0.04	170	R	R	R	R	R
Red sea bream	0.34	0.7	—	NR[g]	R	R	R	R	R	R

[a] Percentage or amount per kg feed. Requirements were determined using purified or semipurified diets. Factors affecting the bioavailability of these elements and nutrient interactions must be considered when formulating fish feeds.

[b] Requirement for other elements reported: guppy, 0.054% Mg, 80 mg/kg Zn; red drum, 20–25 mg/kg Zn; channel catfish, 0.1 mg/kg Co.

[c] Requirement below the level of detection under normal rearing conditions. The calcium requirement of fish reared in low-calcium freshwater ranges from 0.03 to 0.65% of the diet.

[d] Inorganic phosphorus.

[e] The estimated iodine requirement of salmonids is 1.1 mg/kg (NRC, 1993).

[f] Essential in the diet but the quantitative requirement not reported.

[g] No dietary requirement demonstrated under experimental conditions.

of protein, lipid, and carbohydrate. In these forms, P is more readily available to fish than from forms present in plant protein supplements.

Phosphorus in cereal grains and vegetable protein concentrate may range from 0.3 to 0.4 and 0.5 to 1.4%, respectively. Plants store phosphate in seeds as phytates, i.e., salts of phytic acid (inositol hexaphosphoric acid). The term phytin implies the Ca–Mg salt of phytic acid and feedstuffs of vegetable origin primarily contain phytin. Distillers, dried corn with solubles contains a lower percentage of P in the phytate form because of a breakdown of P during the fermentation process. In contrast to other feed ingredients of plant origin, alfalfa meal P is nonphytate in nature. The average phytic acid content of cereal and oilseed products is approximately 1 to 2%, by weight, however certain varieties may contain higher amounts (3–7%). Phytate contributes approximately 60–90% of the total P in mature plant seeds. In soybean meal, phytic acid, phosphatides, and inorganic P constitute about 75, 12, and 4% of the total P, respectively. Phytate is unavailable to most finfish and monogastric animals because of the lack of an endogenous enzyme (phytase) in the gastrointestinal tract that catalyzes the breakdown of phytic acid to its moieties.

5.2.1.5. Bioavailability of Phosphorus

The bioavailability of P may differ markedly among feed ingredients and inorganic P supplements (Lall, 1991). Digestibility and growth measurements have frequently been used to determine the bioavailability of P to fish from selected feed ingredients. To some extent, apparent P digestibility data are of limited use unless a correction is made for endogenous P excretion in the feces. Bone ash is the most sensitive practical criterion for evaluating dietary P utilization and can provide a more accurate indication than values based on body weight or apparent digestibility. Plasma alkaline phosphatase assays are the least sensitive method for predicting P bioavailability.

The bioavailability of dietary P is influenced by several factors including chemical form, digestibility of diet, particle size, interaction with other nutrients, feed processing, and water chemistry (Lall, 1991; Vielma *et al.,* 1999b). There are significant differences in the availability of P from a variety of inorganic salts: the more soluble the salt, the higher the availability of P, thus P is more readily available from mono- or dicalcium phosphates than from tricalcium phosphate (Lall, 1991). Salmonids and tilapia utilize P present in fish meal more efficiently than do carp and channel catfish (Lovell, 1978; Ogino *et al.,* 1979b; Watanabe *et al.,* 1980a). The differences in the bioavailability of P to salmonids and carp or tilapia is probably due to the limited secretion of gastric juices by these warmwater species (Ogino *et al.,* 1979b). The digestibility of P in fish meals ranges between 40 and 60% in salmonids and other fish (Lall, 1991; Riche and Brown, 1996; Sugiura *et al.,* 1998).

Phytate present in either plant proteins or added to the diet is unavailable to most finfish. The intestinal mucosa of many fish does not secrete the enzyme phytase that is able to hydrolyze phytic acid to its moieties. Phytic acid also forms insoluble salts with free Ca in the digestive tract. Supplemental microbial phytase is very effective in improving P bioavailability of plant feed ingredients in the feed of monogastric animals (Simons *et al.,* 1990). Although supplemental phytase effectively hydrolyzes phosphate from phytates in rainbow trout (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Storebakken *et al.,* 1998; Vielma *et al.,* 2000), the beneficial effects of this enzyme in diets containing high levels of soybean products on growth and bone mineralization has not been demonstrated. Phytase enzymes have a potential for improving the biological availability of several plant proteins currently evaluated for their use in fish feeds, however, their stability during feed processing must be maintained.

5.2.1.6. Phosphorus Excretion and Strategies to Minimize P Output from Aquaculture Operations

The form in which P is excreted by fish has a direct influence on the enrichment of the aquatic environment and the growth of algae. Generally P is excreted in soluble and particulate forms: the soluble forms of P consisting of organic P and PO_4^{3-}, affect the water quality directly, whereas the particulate form settles to the bottom of the tank or accumulates in the sediment. Soluble P is readily available as a nutrient for plant growth. A significant amount of the phosphate fraction consists of free inorganic orthophosphate. The amount of particulate P excreted by fish maintained in experimental tanks or fish farm conditions ranges between 30 and 64% of the total excreted P. Under farm conditions excreted P settles in sediment and is gradually released as the soluble form during anaerobic or other related biological processes. Obviously, the form of P consumed by fish will affect both the amount of soluble and the particulate P excreted as well as the quantity of P that could subsequently be biologically degraded from the sediment. Persson (1988) reported that 30% of the total P from feed readily dissolves in water and neutral salt solution and was not detected in sludge collected from the bottom of sea cages or experimental tanks. Organically bound phosphorus was approximately 55 and 33% in feed and feces, respectively. The remaining P was found in sludge, it was bound to calcium and considered inert. The breakdown of organically bound P from feed and feces varies considerably due to differences in the chemical characteristics of feed ingredients used, the environmental conditions (temperature, oxygen, pH, salinity, etc.), and the type of microorganisms in natural waters. Persson (1990) estimated that the average amount of organically bound P fraction in feed and feces released to the water was approximately 80 and 60%, respectively.

It is important that the P content of effluents from land-based fish farms, fish hatcheries, and sea cages be minimized. The following strategies have been recommended to reduce P output from aquaculture operations (Lall, 1991): (a) estimation of the correct P balance; (b) reduction of P levels in feeds without affecting growth, feed efficiency, health, reproduction, etc.; (c) selection of feed ingredients, phytase supplementation of plant-based feeds and P supplements with a high P bioavailability; (d) selection of P supplements with a high P absorption and low solubility in water; (e) development of diets with a better feed efficiency; and (f) minimization of feed wastage.

5.2.2. Magnesium

5.2.2.1. Functions and Metabolism

Magnesium is an essential cofactor in many enzymatic reactions in intermediary metabolism. These include those that transfer phosphate groups (phosphokinase), hydrolyze phosphate and pyrophosphate groups (phosphokinase and pyrophosphatase), oxidize fatty acids involving acetyl coenzyme A (thiokinase), and activate amino acid synthesis (amino acid synthetase). Enzymatic functions of Mg are well documented. Magnesium is also required in skeletal tissue metabolism, osmoregulation, and neuromuscular transmission. It plays an important role in the respiratory adaptation of freshwater fish (Houston, 1985).

Most of the Mg in fish is located in the bone. The remainder is found within the cells of soft tissues. The red blood cells of fish contain significantly higher levels of Mg and lower levels of Ca than found in humans. The problems with Mg in fish are similar to those with Ca. Freshwater fish derive Mg ions either by active uptake from the environment or from dietary sources. There is no evidence of branchial uptake of Mg. In freshwater, excess Mg is excreted renally (Oikari and Rankin, 1985). In seawater, fish obtain Mg by drinking and excrete it exclusively via the kidneys (Hickman and Trump, 1969; Evans, 1979).

5.2.2.2. Deficiency

Magnesium deficiency in carp (Ogino and Chiou, 1976; Satoh *et al.,* 1983b), catfish (Gatlin *et al.,* 1982), eel (Nose and Arai, 1979), rainbow trout (Cowey *et al.,* 1977; Ogino *et al.,* 1978b; Knox *et al.,* 1981a; 1983), Atlantic salmon (El-Mowafi and Maage, 1998), and guppies (Shim and Ng, 1988) generally includes one or more of the following deficiency signs (Table 5.2): anorexia, reduced growth, sluggishness, a high mortality, and a reduced bone and body Mg content. In rainbow trout, Mg deficiency also causes calcinosis of kidney, vertebrae deformity, and degeneration of muscle fibers

and epithelial cells of the pyloric cecum and gill filaments (Cowey *et al.,* 1977; Ogino *et al.,* 1978b). Catfish and rainbow trout fed Mg-deficient diets show a flaccid appearance of their muscle (Knox *et al.,* 1981a; Gatlin *et al.,* 1982). Carp fed on a low-Mg diet also develop convulsions and cataracts (Ogino and Chiou, 1976). Atlantic salmon and red sea bream do not show signs of Mg deficiency in a seawater environment, where the Mg concentration is much higher than in freshwater and where they obtain Mg by drinking the seawater. However, it is not uncommon to find nephrocalcinosis in rainbow trout reared in seawater. Poor water quality, particularly low oxygen and high carbon dioxide levels, during the freshwater rearing period of salmonids and other factors may induce early signs of nephrocalcinosis but the clinical signs develop after seawater transfer. Cowey *et al.*(1977) observed that a Mg-deficient diet caused renal calcification at Ca levels of 2.6% (Ca:P ratio of 1:1) or more. A lower dietary Ca level (1.4%) or Mg supplementation (0.1%) produced trout with normal Ca levels. Thus dietary Ca levels of 2.6% were not detrimental to trout provided that sufficient Mg was present in the diet. An interaction between dietary protein and Mg level has been demonstrated in tilapia (Dabrowska *et al.,* 1989), where excess Mg (0.32%) in a low-protein diet (24%) produced toxicity signs and where Mg deficiency in a high-protein diet (44%) caused whole-body hypercalcinosis.

5.2.2.3. Requirement

The quantitative dietary Mg requirements of several fish are listed in Table 5.3. Magnesium requirements of rainbow trout (Ogino *et al.,* 1978b; Knox *et al.,* 1981a; Shearer, 1989), carp (Ogino and Chiou, 1976), channel catfish (Gatlin *et al.,* 1982), eel (Nose and Arai, 1979), and guppy (*Poecilia reticulata*) (Shim and Ng, 1988) have been estimated to range from 0.04 to 0.06% of the diet. A dietary Mg content of 0.06 to 0.08% is required for tilapia (Dabrowska *et al.,* 1989). Generally the Mg concentration in freshwater is too low (1 to 3 mg/liter) to meet the metabolic demand of fish and it must be supplied in the diet. In the marine environment, where the Mg concentration is higher (1350 mg/liter), Mg supplementation of the diet may not be necessary (Lall and Bishop, 1977; Sakamoto and Yone, 1979b). The Mg requirement of rainbow trout is not influenced by an increase in the dietary Ca and P levels (Knox *et al.,* 1981a). In terrestrial animals, the Mg requirement is affected by the Ca as well as the P level in the diet (O'Dell, 1960).

5.2.2.4. Sources

The Mg concentration of freshwater is approximately 200 μM. In seawater, it is the third most abundant ion, with a concentration higher than 50 mM (Rankin and Davenport, 1981). Among food sources, cereal grains contain less Mg (0.15–2%) than other common feedstuffs. In cereals, most

of the Mg is found in the bran fraction. Vegetable protein may contain 0.4 to 0.6% Mg. The skeletal tissues present in meat and fish meals contribute to the variable amount of Mg in these rich sources of Mg. In plant products, Mg is present as the chelated metal in the porphyrin moiety of chlorophyll. Little is known about the form of Mg in other feedstuffs. Magnesium present as magnesium acetate is more efficiently used by tilapia than either magnesium oxide or magnesium sulfate (Dabrowska *et al.*, 1989).

5.2.3. Sodium, Potassium, and Chloride

5.2.3.1. Functions and Metabolism

Sodium, potassium, and chloride are the most abundant electrolytes in the body of living organisms. Sodium and chloride are the major cation and anion, respectively, of extracellular fluids of the body, whereas potassium and magnesium are the major intracellular cations. They serve a vital function in controlling osmotic pressures and acid–base equilibrium. The osmotic pressure of the intracellular and extracellular fluids is rigidly controlled, largely through energy-dependent regulatory mechanisms that determine the rate of absorption of sodium ions and water by the epithelial membranes of the gill, gut, integument, and kidney.

Plasma membranes contain an energy-dependent Na^+ pump, which actively transfers Na^+ from the intracellular to the extracellular environment. As Na^+ exists, K^+ enters, because the membrane is fully permeable to K^+ and these ions are very similar in properties to the Na^+ they are replacing. These events result in a high concentration of Na^+ extracellularly and high K^+ intracellularly. Chloride ions remain mainly extracellular, as intracellular fluid already contains sufficient anions (HPO_4^{2-}, protein, etc.) to balance the K^+ and Mg^+. To maintain the osmotic balance across the cell membrane, divalent ions within the cell with intracellular protein particles balance the monovalent electrolytes extracellularly.

Chloride is the major anion of gastric juice and blood. In gastric juice it is accompanied by hydrogen rather than sodium ions, as in plasma and other extracellular fluids of the body. Another specific function of the chloride ion is the "chloride shift" in blood during the transport of carbon dioxide and carbonate.

5.2.3.2. Deficiency

Deficiencies of sodium, potassium, and chloride are difficult to produce because fish derive these elements from their aquatic environment. In terrestial animals, Na deficiency causes growth retardation, softening of bones, corneal keratinization, gonadal inactivity, adrenal hypertrophy, changes in

cellular function, and impairment of food utilization. Potassium deficiency causes overall muscle weakness, resulting in intestinal distention, weakness of cardiac and respiratory muscles, and their ultimate failures. Chloride deficiency resembles Na deficiency.

The supplementation of high levels (1.5–12% of the diet) of sodium chloride (NaCl) to the diet of young coho salmon (Zaugg and McLain, 1969) and rainbow trout (Salman and Eddy, 1988) inhibits growth and lowers feed utilization. There are no beneficial or detrimental effects of dietary NaCl supplemetation (<1%) on the performance of channel catfish and Atlantic salmon reared either in freshwater or seawater. Supplementation of NaCl to the diet of the eurohaline fish red drum reared at low salinities (<6‰) resulted in increased growth (Gatlin *et al.,* 1992). Feeding salt-enriched diets to Atlantic and coho salmon for a short period prior to seawater transfer reduces mortalities in these anadromous fish (Zaugg and McLain, 1969; Basulto, 1976). This effect is due to the elevation of Na^+, K^+-stimulated ATPase activity of gill microsomes by dietary salt supplementation, which makes saltwater adaptation easier physiologically. The specific activity of this enzyme is higher in the gills of seawater fish than in those of freshwater fish, and this difference has been attributed to the increased load of Na^+ in marine forms which must be pumped across the gills from blood to the external environment. The supplementation of dietary salt may not be necessary in Atlantic salmon smolts that are physiologically ready to enter seawater.

5.2.3.3. Requirement

Studies on the quantitative requirements of sodium and chloride have not been undertaken because fish readily absorb these elements from the surrounding water. The abundance of these elements in common feedstuffs used in fish diets means that they need not be supplemented in most diets formulated with natural feed ingredients. However, K supplementation was found necessary in purified diets for Chinook salmon. Juvenile Chinook salmon reared in freshwater required 0.8% K in their diet for maximum growth, and whole-body K saturation was reached at a K concentration between 0.6 and 1.2% of the diet (Shearer, 1988). Fish reared in seawater, where the K concentration is much higher than in freshwater, do not require K supplementation (Sakamoto and Yone, 1978a; Lall and Bishop, 1977; Lall, unpublished).

5.2.3.4. Sources

Most animal protein concentrates(particularly fish meal)are rich in sodium (0.5 to 1%), whereas plant protein concentrates contain relatively low levels (0.01 to 0.07%). The major proportion of Na in common feedstuffs

occurs in a free ionizable form as chloride, sulfate, or phosphate or as ionizable salts of carboxylic acids in plant and animal tissues. Thus Na in most feed ingredients is freely soluble and assumed to be highly available biologically. The availability of Na in bone and meat meal varies with the species because of differences in the rate of solubilization in the digestive tract.

The distribution of potassium in feed ingredients shows a pattern significantly different from that of sodium (Thomson, 1972). Of the protein supplements, corn gluten meal and fish meal are relatively poor sources of K, while soybean meal, cottonseed meal, dehydrated alfalfa meal, and cane molasses are good sources, containing 2% or more of this element. Whole cereal grains contain between 0.1 and 0.5% K. Like Na, the greater proportion of K in feed ingredients is present in a freely soluble ionic form and considered to be highly available biologically. The chloride distribution in feedstuffs shows a pattern similar to that of Na and appears to be equally available.

5.2.4. Iron

5.2.4.1. Functions and Metabolism

Iron is an essential element in the cellular respiratory process through its oxidation–reduction activity and electron transfer. It is found in the body mainly in the complex form bound to proteins such as heme compounds (hemoglobin and myoglobin), heme enzymes (mitochondrial and microsomal cytochromes, catalase, peroxidase, and so on), and nonheme compounds [transferrin, ferritin, and iron-containing flavoproteins (ferredoxins, dehydrogenases)]. Iron metabolism has been reviewed extensively by Bernat (1983).

A great deal of information in the area of Fe absorption and transport has resulted from studies on humans. Myoglobin found in muscle differs from hemoglobin only in the nature of the protein component of the molecule. Iron occurs in the blood as hemoglobin in erythrocytes and as transferrin in plasma. Small quantities of Fe as ferritin are also found in the serum. Transferrin serves as the principal carrier of Fe in blood and therefore plays an important role in Fe metabolism. Storage Fe proteins, ferritin and hemosiderin, occur widely in liver, spleen, and bone marrow. The two proteins are chemically different although intimately related in function.

The absorption of Fe is influenced by the age, state of health, iron status, and conditions within the gastrointestinal tract of the fish. The amount and chemical form of Fe ingested and the amount and proportion of both inorganic and organic components present in the diet can also affect the absorption of Fe in fish. Iron occurs in foods mainly in the organic form in combination with proteins such as hemoglobin, myoglobin, and other

complexes. Iron present in inorganic forms or as iron–protein complexes must be reduced to the ferrous state to be available for absorption. These changes are accomplished by gastric juices and other digestive secretions. The presence of reducing substances in the diet (e.g., ascorbic acid) can enhance the ability of fish to absorb iron. The mechanism of Fe absorption and transport is complex and is reviewed elsewhere (Wienk *et al.,* 1999).

There is relatively little information on the absorption and metabolism of iron in fish and other aquatic organisms. It appears that mechanisms of iron absorption from the digestive tract, and of storage and excretion, may be similar to those in other vertebrates. Some absorption of Fe takes place across the gill membrane, however, the intestinal mucosa is considered the major site of Fe absorption. Food is considered as the major source of Fe for metabolic purposes. However, the addition of ferrous sulfate to water may also enhance growth and hemoglobin level in certain warmwater fishes *(Xiphophorus helleri* and *X. maculatus)* (Roeder and Roeder, 1966). The absorbed Fe is probably transported in the blood by transferrin, which has been identified in several fish species. Aisen *et al.* (1972) found a protein in hagfish that closely resembles human transferrin. In rainbow trout Fe is absorbed from the peritoneal cavity and stored at higher concentrations in the liver, spleen, and head kidney (Walker and Fromm, 1976). There is little endogenous iron lost in the urine or feces.

5.2.4.2. Deficiency

Iron deficiency is not commonly observed in fish cultured under practical conditions, however, it may be readily produced experimentally in certain fish fed low-Fe diets (Table 5.2). Iron deficiency causes characteristic microcytic anemia or low hemoglobin levels in brook trout (Kawatsu, 1972), rainbow trout (Desjardins, 1985), Atlantic salmon (Bjornvic and Maage, 1993; Andersen *et al.,* 1996; Lall, unpublished data), red sea bream (Sakamoto and Yone, 1976a, 1978b), yellowtail (Ikeda *et al.,* 1973), eels (Nose and Arai, 1979), and carp (Sakamoto and Yone, 1978c). In most cases, the growth of fish was not influenced by the Fe deficiency. The normal liver color changes to yellowish-white during Fe deficiency in carp (Sakamoto and Yone, 1978c). In catfish, Fe deficiency suppresses hemotocrit, hemoglobin, and plasma iron levels and causes transferrin saturation (Gatlin and Wilson, 1986b). Sakamoto and Yone (1979c) found that ferrous chloride and ferric chloride were equally effective in prevention of anemia in red sea bream. However, a somewhat higher concentration of ferric citrate was required.

Dietary Fe toxicity signs develop in rainbow trout fed more that 1380 mg Fe kg^{-1} (Desjardins, 1985). The major effects of Fe toxicity include reduced growth, poor feed utilization, feed refusal, increased mortality, diarrhea, and histopathological damage to liver cells.

5.2.4.3. Requirement

The Fe requirement of certain fish has been established quantitatively (Table 5.3). The Fe requirements for catfish (Gatlin and Wilson, 1986b), red sea bream (Sakamoto and Yone, 1976a, 1978b), and eel (Nose and Arai, 1979) are 30, 150, and 170 mg/kg of diet, respectively. The reported dietary Fe requirement of Atlantic salmon ranges from 33 to 100 mg/kg (Bjornvic and Maage, 1993; Andersen *et al.,* 1996; Lall, unpublished). Iron supplied from a purified diet (39 mg of iron/kg of diet) may not be sufficient to avert Fe deficiency in rainbow trout (Desjardins, 1985), indicating a need for dietary inorganic Fe supplement.

5.2.4.4. Sources

The concentration of Fe in common feedstuff is highly variable and greatly influenced by the degree of contamination from ferrous metal during processing. Feeds of animal origin, other than milk by-products, are rich sources of iron. Fish meal and meat meal contain approximately 150 to 800 mg Fe/kg. The Fe content of cereal grain varies from 30 to 60 mg/kg, whereas oil seed protein may contain 100 to 200 mg Fe/kg. Certain feed-grade calcium phosphates and limestones may contain 2000 mg Fe/kg.

Little is known about the form of Fe found in common feedstuffs and their bioavailability to fish. In animal protein supplements, Fe may be present as iron–porphyrin, myoglobin, and hemoglobin. In cereal grains, a small proportion of Fe occurs as an iron–phytin complex. The utilization of ferrous sulfate and ferric chloride was essentially the same. In red sea bream, ferrous and ferric chloride are more efficiently utilized than ferric citrate (Sakamoto and Yone, 1979c). A hemoglobin regeneration assay in Atlantic salmon showed that the relative biological availability of Fe from ferric chloride, ferric oxide, blood meal, and herring meal was 98.8, 17.8, 52.3, and 47.1%, respectively (Naser and Lall, 1997). However, Anderson *et al.* (1997) observed that heme iron from blood meal was more efficiently utilized than ferrous sulfate in Atlantic salmon using liver Fe concentration and hemoglobin slope ratio methods. It is well known that in animals the bioavailability of iron is affected by the composition of test diets, processing methods used to produce blood meal, Fe concentration in the diet, age and species of test animals, intake of iron relative to the need, chemical form in which iron is supplied from other dietary components, and amount and proportion of other dietary components with which Fe interacts metabolically (Hallberg, 1981; Forbes and Erdman, 1983; Wienk *et al.,* 1999).

5.2.5. Copper

5.2.5.1. Functions and Metabolism

Copper (Cu) is an essential trace element for all animals including fish (O'Dell, 1984; Mertz, 1986; Lall, 1989). It is a vital component of several enzymes (Table 5.1) that are involved in oxidation–reduction reactions and occurs tightly bound to proteins in the cell rather than as free ions. The Cu metalloenzymes are involved in cellular energy production (cytochrome *c* oxidase), protection of cells from free radical damage (superoxide dismutase), brain neurotransmitters (dopamine hydroxylase and peptidyl α-amidating monooxygenase), collagen synthesis (lysyl oxidase), and melanin production (tyrosinase). Copper is also bound to the protein, ceruloplasmin, which occurs in the cell and plasma and is involved in iron utilization. Ceruloplasmin transports Cu as well as helping convert iron into a form that can be transported to other tissues. The ceruloplasmin differs from the iron protein, transferrin, in that it shows oxidative activity, especially toward *p*-phenylenediamine. The function of ceruloplasmin appears to be enzymatic but its specific role is unknown. Apart from the enzymatic function, Cu proteins and chelates are also involved in other metabolic roles.

Copper metabolism of fish is not clearly defined, despite numerous investigations concerned with toxic effects. The distribution of Cu and Cu-dependent enzymes is similar in a marine fish, plaice, and in mammals (Syed and Coombs, 1982). The highest concentrations occur in the brain, heart, liver, and eyes. High levels of Cu are also found in the iris and chloroid of eyes, where Cu is generally associated with melanins and largely bound to protein. Marine invertebrates, especially mollusks, accumulate large quantities of Cu. Hemocyanin, a blue-colored Cu-containing complex, is found in the hemolymph of mollusks and crustaceans. Copper serves as an oxygen carrier in the hemolymph of these organisms.

5.2.5.2. Deficiency and toxicity

The clinical signs of Cu deficiency have not yet been reported for fish. Gatlin and Wilson (1986a) observed reduced heart cytochrome *c* oxidase and liver copper–zinc superoxide dismutase activities in Cu-deficient catfish. In a long-term study, carp fed diets containing white fish meal without Cu supplement showed reduced growth and cataract formation (Satoh *et al.*, 1983b). Copper is widely distributed in feeds and the aquatic environment, therefore its deficiency would occur in fish only under extreme conditions. A low level of Cu is found in Atlantic salmon suffering from Hitra disease (Poppe *et al.*, 1986), which is a coldwater bacterial disease caused by *Vibrio salmonicida*. Copper deficiency is well recognized in domestic animals.

Symptoms include anemia, bone disorders, depigmentation of the hair, fur, wool, or feathers, demyelination of the spinal cord, fibrosis of the myocardium, gastrointestinal disturbances, and aortic rupture.

Copper toxicity arising from pollutants in natural water or produced experimentally may cause severe damage to the gills and necrotic changes in the liver and kidneys. The oral toxicity of this element has been induced in rainbow trout fed 730 mg of Cu/kg of diet (Lanno *et al.,* 1985). The toxicity signs include reduced growth and feed efficiency and elevated liver Cu levels. However, a diet of up to 665 mg of Cu/kg does not yield any toxicity signs (Knox *et al.,* 1982; Lanno *et al.,* 1985).

5.2.5.3. Requirement

The dietary Cu requirements (Table 5.3) of selected fish species have been reported as follows (mg/kg of diet): 3 for rainbow trout and carp (Ogino and Yang, 1980), 5 for catfish (Gatlin and Wilson, 1986a), and 5 for Atlantic salmon (Lall and Hines, 1987; Lorentzen *et al.,* 1997). The dietary Cu requirement also depends on the physiological state of the animal, the Cu concentration in the water, and probably the level of elements that are metabolic antagonists of copper, such as iron, zinc, cadmium, and molybdenum. In rainbow trout, antagonistic effects of zinc and copper have not been observed (Knox *et al.,* 1982).

5.2.5.4. Sources

Processed feed ingredients of plant and animal origin show a variable Cu content resulting from contaminants. Moisture during processing of materials rich in protein provides a favorable condition for the uptake of Cu by the protein fraction. High concentrations of Cu (>85 mg/kg) have been detected in condensed fish solubles and whey products. The Cu content of cereal grain ranges from 5 to 20 mg/kg. Most animal and vegetable protein supplements contain 5 to 30 mg Cu/kg.

5.2.6. Manganese

5.2.6.1. Functions and Metabolism

Essentiality of manganese in experimental animals is well established, and the importance of this element is now being recognized in fish nutrition. Manganese is widely distributed in fish and animal tissues. The highest concentration is found in bone, however, significant amounts are also present in liver, muscle, kidney, gonadal tissues, and skin. In tissues, Mn is more concentrated in the mitochondria than in the cytoplasm or other cell organelles. Manganese functions either as a cofactor activating

a large number of enzymes that form metal–enzyme complexes or as an integral part of certain metalloenzymes in carbohydrate, lipid, and protein metabolism. Since the chemistry of Mn is similar to that of magnesium, many enzymes can be activated by either Mn or Mg ions. Nonspecific Mn-activated enzymes include kinases, transferases, hydrolases, and decarboxylases. Certain enzymes, e.g., glycosyltransferase, are highly specific for Mn activation. Manganese-containing metalloenzymes include arginase, pyruvate carboxylase, and superoxide dismutase (Table 5.1). The enzymatic function of Mn in lipid and carbohydrate metabolism and brain function has been reviewed extensively by Keen *et al.* (1984).

Uptake of Mn from freshwater has been demonstrated (Miller *et al.,* 1980; Srivastava and Agrawal, 1983), however, the mechanism of Mn uptake from either gills or the gastrointestinal tract is poorly understood. It is well recognized that Mn is efficiently absorbed from food but the absorption may be reduced by high levels of calcium and phosphorus.

5.2.6.2. Deficiency

Manganese deficiency causes reduced growth and skeletal abnormalities in rainbow trout, carp, and tilapia (Ishak and Dollar, 1968; Ogino and Yang, 1980; Yamamoto *et al.,* 1983). Other deficiency signs in rainbow trout and carp include cataracts and dwarfism associated with disturbances in bone metabolism (Satoh *et al.,* 1983a, b). In rainbow trout, a low Mn intake decreases the activities of Cu, Zn-superoxide dismutase and Mn-superoxide dismutase in cardiac muscle and liver; it also suppresses Mn and Ca concentration of vertebrae (Knox *et al.,* 1981b). Liver Mn-superoxide dismutase activity was not influenced in catfish fed a diet containing 2.4 mg Mn/kg (Gatlin and Wilson, 1984b). In broodstock rainbow and brook trout, a fish meal-based diet without Mn supplement caused poor hatchability and low Mn levels of eggs (Takeuchi *et al.,* 1981; Lall, 1985).

5.2.6.3. Requirement

The Mn requirements (mg/kg diet) of the following fish have been demonstrated: 2.4 for channel catfish (Gatlin and Wilson, 1984b), 12 to 13 for carp and rainbow trout (Ogino and Yang, 1980; Satoh *et al.,* 1987), and 7.5 to 10.5 for Atlantic salmon (Maage *et al.,* 2000). Although the Mn requirement of juvenile fish may range between 2 and 15 mg/kg diet, broodstock salmon and trout require a considerably higher (>30 mg/kg diet) amount of this element (Lall, 1985). Undoubtedly, Mn supplementation of practical broodstock diet is necessary because feed ingredients often do not supply sufficient Mn. Higher amount of phosphorus in the diet may also lower the absorption of Mn and thus increase the need for supplemental Mn for fish.

5.2.6.4. Sources

Protein supplements of animal origin are generally low in Mn. Whole herring and capelin meal are particularly low (4 to 12 mg/kg). Manganese levels of 4 to 38 mg/kg are common in other fish meals. Wheat middlings, rice bran, alfalfa meal, and corn distillers' dried soluble are the richest plant sources of Mn. Corn is particularly low in Mn (4 to 11 mg/kg), whereas other cereal grains have contents ranging from 8 to 50 mg/kg.

The availability of Mn differs in various inorganic salt supplements. Manganese found in manganous oxide is poorly utilized by rainbow trout and Atlantic salmon (Satoh and Watanabe, 1985; Lall, unpublished). The availability of Mn is low in manganous carbonate for carp (Satoh and Watanabe, 1985).

5.2.7. Zinc

5.2.7.1. Functions and Metabolism

The essential function of zinc for living organisms is based on its role as an integral constituent of a number of metalloenzymes and as a catalyst for regulating the activity of specific Zn-dependent enzymes. Approximately 20 Zn metalloenzymes have been identified, including carbonic anhydrase, alkaline phosphatase, carboxypeptidases A and related peptidases, alcohol dehydrogenases, and cytsolic superoxide dismutase (Table 5.1). Thus Zn regulates many metabolic processes of carbohydrate, lipid, and protein metabolism. In addition to its role in enzyme function, Zn may have a structural role in nucleoproteins and is also involved in the metabolism of prostaglandins. Although the role of Zn in metabolic processes is evident, little is known of the relationship between the biochemical function and the pathological signs. Some of the clinical features of Zn deficiency may arise from disturbances of nucleic acid and protein metabolism.

Fish accumulate Zn from both water and dietary sources. Interest in environmental pollution and heavy metal contamination of human food has resulted in many investigations on the accumulation and distribution of Zn in aquatic organisms (Hogstrand and Wood, 1996; Alsop and Wood, 1999). In fish, the main sites of Zn uptake are the gills and the gastrointestinal tract (Pentreath, 1973; Part and Svanbert, 1981; Lovegrove and Eddy, 1982). However, dietary Zn is more efficiently utilized. In freshwater, even when dietary Zn levels are adequate, there is an active uptake of waterborne Zn (Spry *et al.,* 1988). Zinc absorption is relatively higher in rainbow trout fed a Zn-deficient diet (Spry *et al.,* 1988). The gills in rainbow trout also play a major role in excretion of dietary Zn (Hardy *et al.,* 1987).

Very little information is available on Zn absorption and mechanisms involved in its regulation. In mammals, Zn homeostasis is thought to be maintained by mechanisms operating at the sites of absorption and secretion in the gastrointestinal tract. In winter flounder, the entire digestive tract is capable of absorbing Zn, but the uppermost portion of the intestine has the highest capacity and the stomach has the lowest (Shears and Fletcher, 1983). Waterborne Ca is a competitive inhibitor of branchial Zn influx (Alsop and Wood, 1999). Zinc inhibits the influx of Ca across gills thus Ca in water protects fish against Zn toxicity. Rainbow trout and carp can tolerate 1700 to 1900 mg zinc/kg without any apparent signs of toxicity (Wekell *et al.,* 1983; Jeng and Sun, 1981). Common carp absorb and accumulate higher amounts of Zn in viscera, whereas the ability of trout to tolerate Zn in water is very limited (McKee and Wolf, 1963). It appears that excretory mechanisms and the control of gastrointestinal tract uptake may play important roles in maintaining Zn homeostasis. The zinc content of scales reflects the environmental metal concentrations. Zinc is normally excreted via the kidneys or by chloride cells of the gills (Bryan, 1976).

5.2.7.2. Deficiency

Although the essentiality of Zn and various deficiency signs for terrestrial animals and humans have been well recognized (Underwood, 1977; Prasad, 1985), its ubiquity in the environment and in feed ingredients makes it seem unlikely that a deficiency of Zn could cause a significant problem in fish. Between 1973 and 1974, a widespread occurrence of cataracts in rainbow trout fed practical diets in the United States ultimately led to the realization that Zn was unavailable in white fish meal (Ketola, 1979). Other signs of Zn deficiency have also been characterized (Table 5.2). In rainbow trout, Zn deficiency causes growth depression, high mortality, lens cataracts, erosion of fins and skin (Ogino and Yang, 1978a), and short body dwarfism (Satoh *et al.,* 1983a). Excess minerals (total ash) present in white fish meal may affect Zn absorption and retention, resulting in lens cataract (Ketola, 1979). Caudal fin Zn concentration is a good indicator of Zn status in rainbow trout (Wekell *et al.,* 1986). In catfish, diets low in Zn cause reduced growth, appetite, bone Zn and Ca levels, and serum Zn concentrations (Gatlin and Wilson, 1983). Broodstock diets low in Zn reduced the egg production and hatchability of eggs (Takeuchi *et al.,* 1981).

Elevated levels of dietary Zn (500 to 1000 mg Zn/kg) caused reduced hemoglobin, hematocrit, and hepatic copper concentrations in rainbow trout (Knox *et al.,* 1982, 1984). However, the Cu status of catfish was not impaired by diets containing 200 mg Zn/kg (Gatlin *et al.,* 1989). Common carp accumulate much higher concentrations of Zn in their tissues, particularly

in the viscera, than other fish studied, without any apparent toxicity signs (Jeng and Sun, 1981).

5.2.7.3. Requirement

A dietary requirement for Zn (mg/kg) has been reported for several freshwater fish fed semipurified diets (Table 5.2): rainbow trout and common carp, 15–30 (Ogino and Yang, 1978, 1979); Atlantic salmon, 37–67 (Maage and Julshamn, 1993); channel catfish, 20 (Gatlin and Wilson, 1983); blue tilapia, 20 (McClain and Gatlin, 1988); red drum, 20–25 (Gatlin *et al.,* 1991); and guppy, 80 (Shim and Lee, 1993). The minimum Zn requirement varies with age, sexual maturity, composition of diet, water temperature, and water quality. Among dietary factors, Ca and P levels, phytic acid, protein source, form of Zn, and Ca content affect Zn absorption and retention in fish (Takeda and Shimma, 1977; Gatlin and Wilson, 1984a; Hardy and Shearer, 1985; Richardson *et al.,* 1985; Wekell *et al.,* 1986; Satoh *et al.,* 1987, 1989; McClain and Gatlin, 1988).

5.2.7.4. Sources

The concentration of Zn in numerous marine invertebrates, vertebrates, and plants from several geographic locations worldwide is summarized by Eisler (1980). The richest source of Zn are filter feeding bivalve mollusks, especially oysters (>1200 mg Zn/kg). Most animal and fish tissues, when uncontaminated, contain approximately 30 mg Zn/kg of dry matter. Among feedstuffs, the common cereal grains contain 15 to 30 mg zinc/kg. Most of the Zn is found in the bran and germ fraction of grain. Typical vegetable protein concentrates may contain 40–80 mg Zn/kg. Levels of 80 to 100 mg Zn/kg are more common in fish meal. Egg albumin, because of its low Zn content (<3 mg/kg), is generally used in low-Zn experimental diets.

Zinc sulfate ($ZnSO_4$) and $ZnNO_3$ are effectively utilized by rainbow trout as Zn supplements (40 mg/kg) in diets containing white fish meal, and they also alleviate dwarfism and cataract problems (Satoh *et al.,* 1987). Some differences exist in Zn bioavailability from feedstuffs of plant and animal origin. As mentioned previously, plant protein contains phytates. Soluble phytates added to animal protein decrease Zn bioavailability and account for a large part of the low availability in oil seed protein (Oberleas, 1973). The bioavailability of Zn in fish meal is greatly affected by the tricalcium phosphate content (Satoh *et al.,* 1987). Higher levels of supplemental Zn should be included in the practical feeds to compensate for the reduced Zn bioavailability caused by dietary phytate, calcium, and phosphorus.

5.2.8. Iodine

5.2.8.1. Functions and Metabolism

Iodine metabolism and thyroid functions are closely related. Iodine is essential for the biosynthesis of the thyroid hormones, thyroxine and triiodothyronine, in fish, birds, amphibians, reptiles, and mammals. Thyroid hormones control cellular oxidation and influence growth, other endocrine glands, neuromuscular functions, circulatory dynamics, and also the metabolism of major nutrients. Eales (1979) and Higgs *et al.* (1982) have reviewed the literature related to thyroid hormones and iodine in fish.

Fish obtain iodine primarily from water via their branchial pumps (Leloup, 1970) and, to a lesser extent, from food sources. Rainbow trout derive 80% of their iodide from water, 19% from the diet, and less than 1% from recycling iodide originating from thyroid hormone degradation (Hunt and Eales, 1979). When dietary uptake is low or absent, fish are able to maintain their plasma iodide levels by uptake of iodide from water and mobilization of this element from tissue stores. Approximately 5% of the iodide intake is utilized by the thyroid.

Iodide trapped in the thyroid gland is oxidized to iodine by a process probably mediated by peroxide enzyme, which is followed by iodinization of tyrosine to form monoiodotyrosine and diiodotyrosine (DIT). The coupling of two iodotyrosines to form iodotyronines involves two possible routes. The two iodotyrosine molecules combine to form one molecule of thyroxine (T4) or one mono- and one diiodotyrosine combine to form one molecule of triiodotyronine (T3). T3 is the predominant hormone secreted by the thyroid gland and is regarded as an active precursor for T4. Major differences exist between fish and mammals in the handling of iodine and extrathyroidal metabolism of T4 and T3 (Higgs *et al.,* 1982).

Both T4 and T3 occur in blood. In trout, T3 binds more strongly to plasma proteins than T4, and consequently T3 turnover is slower in plasma than T4. They are both excreted extensively in bile, however, other routes (kidney and gills) may also be involved. Plasma iodide of freshwater teleosts ranges from 0.5 to 2244 μg per 100 ml. Several factors other than dietary and water iodide levels influence blood iodide levels, including an elevated water temperature, which causes greater iodide excretion and sexual maturation, and differences in the capacity of fish to bind iodide to plasma proteins.

5.2.8.2. Deficiency

Insufficient iodine in the diet is the most common cause of hypothyroidism in vertebrates. More than 70 years ago, the first element to prevent a specific mineral deficiency was iodine, used to prevent goiter in salmonid fish. Marine and Lenhart (1910, 1911) and Marine (1914) correctly

diagnosed simple thyroid hyperplasia from what was thought to be thyroid carcinoma in brook trout (Gaylord and March, 1914). These workers conclusively demonstrated that this disease could be controlled by the administration of iodine. Experimental thyroid hormone deficiency, created by glucosinalates present in the diet by the administration of thiocarbamide derivatives such as thiourea and propylthiouracil or by radiothyroidectemy, results in growth depression of salmonids (Higgs and Eales, 1978). Accumulation of ^{131}I by the thyroid gland is significantly reduced in the scorbutic *Channa punctatus* (Agrawal and Mahajan, 1981).

5.2.8.3. Requirement

The minimum dietary iodine requirement of most fish species has not been established. The requirement is likely to be influenced by growth, sex, age, physiological status, environmental stress, disease, and the iodine content of the water. Goitrogenic substances present in the diet also influence iodine requirement. Woodall and LaRoche (1964) reported a higher iodine requirement for advanced parr compared to fingerlings due to increased thyroid activity during smoltification. Lall *et al.* (1985) observed that 4.5 mg of iodine/kg of diet was essential to protect Atlantic salmon from bacterial kidney disease infections.

5.2.8.4. Sources

Iodine is widely distributed in nature. It is found in relative abundance in marine plants and animals, deposits of organic origin such as coal, certain natural mineral waters, sedimentary phosphate rock, and, particularly, the large nitrite deposits of Chile. This Chilean ore contains nearly 0.1% iodine and serves as the chief commercial source of this element. Certain seaweeds also contain 0.1% iodine. Marine fish have much higher iodine contents than freshwater fish (Lall, 1995).

Atlantic white fish meal may contain 60 to 90 mg iodine/kg, whereas herring and capelin meal contains only 5 to10 mg iodine/kg. Substantial amounts of iodine are lost during fish meal processing. The level of iodine in common feedstuffs in highly variable. Protein sources of animal origin other than fishery by-products contain nutritionally insignificant quantities of this element. The iodine content of protein concentrates of plant origin ranges from 30 to 100 μg/kg.

5.2.9. Selenium

5.2.9.1. Functions and Metabolism

The essentiality of dietary selenium for several animal species and its close metabolic relations have been well recognized. The biochemical role

of Se puzzled workers until the discovery that Se is an integral part of glutathione peroxidase (Rostruck *et al.,* 1973). Glutathione peroxidase activity in erythrocytes, plasma, and other tissues decreases in direct proportion to the reduction in Se intake and is believed to account for the manifestation of Se deficiency in terrestrial animals. Glutathione peroxidase destroys hydrogen peroxide and hydroperoxides by using reducing equivalents from glutathione, thereby protecting cells and membranes against oxidative damage. The exact physiological role of this enzyme is not clear because catalase and non-Se-dependent glutathione peroxidase also remove hydroperoxides. Selenium also has other biochemical roles (Shamberger, 1984). The protective effects of Se and Se compounds against the toxicity of heavy metals such as cadmium and mercury are widely recognized.

Prior to the discovery of the essential role of Se, its toxic properties were well recognized. Certain geographic areas are seleniferous and produce plants with a high Se content. Animals grazing on these alkali pastures show several toxicity syndromes such as blind staggers. The signs of Se toxicity are extensively described by Underwood (1977). High levels of Se exert their toxic effects in animals probably through competition with sulfur compounds or a strong affinity for sulfur in the formation of sulfur–selenium complexes. However, the precise biochemical mechanism involved in Se toxicity remains to be established. Diets high in sulfur or protein provide some protection against Se toxicity. A review by Shamberger (1984) provides a detailed account of developments in Se metabolism.

The absorption, distribution, and excretion of dietary and waterborne Se in salmonids have been investigated. Low concentrations of Se are found extensively in aquatic ecosystems. The uptake of Se as selenite across gills is very efficient at low waterborne concentrations (Hodson *et al.,* 1980). The toxicity of both dietary and waterborne Se has been experimentally produced (Hodson and Hilton, 1983). Liver and kidneys play important roles in the excretory process of Se in trout, in which the major excretory routes appear to be the gills and urine. A correlation between liver Se and Cu has been observed in rainbow trout and Atlantic salmon (reviewed by Hilton, 1989). Selenium and Cu interactions reduce the metabolically active form of these elements by the formation of Se–Cu complexes.

5.2.9.2. Deficiency

Selenium deficiency causes growth depression in rainbow trout (Hilton *et al.,* 1980), carp (Satoh *et al.,* 1983b), and catfish (Gatlin and Wilson, 1984c), but the Se deprivation alone does not produce any pathological sign in these fish. Both Se and vitamin E are required to prevent muscular dystrophy in Atlantic salmon (Poston *et al.,* 1976) and exudative diathesis in rainbow trout (Bell *et al.,* 1985). Glutathione peroxidase activity in plasma

and liver decreases during Se deficiency (Poston *et al.,* 1976; Hilton *et al.,* 1980; Bell *et al.,* 1985; Gatlin and Wilson, 1986c).

Selenium toxicity occurs in rainbow trout and catfish when the dietary Se exceeds 13 and 15 mg/kg dry feed, respectively (Hilton *et al.,* 1980; Gatlin and Wilson, 1984c). Reduced growth, poor feed efficiency, and high mortality are the major effects of Se toxicity. Trout reared on high-Se diets (10 mg/kg) also show renal calcinosis (Hilton and Hodson, 1983).

5.2.9.3. Requirement

The minimum selenium requirement of fish varies with the form of Se ingested, availability of Se in the diet, vitamin E content of the diet, and concentration of waterborne Se. The Se requirement determined on the basis of optimum growth and maximal plasma glutathione peroxidase activity is estimated to be 0.15 to 0.38 mg Se/kg diet for rainbow trout and 0.25 mg Se/kg diet for channel catfish (Hilton *et al.,* 1980; Gatlin and Wilson, 1984c). The Se requirements of other finfish are not known, but coho salmon reared on diets containing 5–8 mg/kg selenium had better seawater survival than those fed only 1 mg/kg in the dry diet (Felton *et al.,* 1996).

5.2.9.4. Sources

Selenium is widely distributed at low concentrations in freshwater (0.2–10 μg/liter) and seawater (approximately 0.09 μg/liter) (National Research Council, 1976). It also occurs naturally in foods and feedstuffs in organic complexes, primarily in the form of selenomethionine, selenium–methylselenomethionine, selenocystine, and selenocysteine. The selenium content of feedstuffs of plant origin varies according to the level and biological availability of Se in the soil at the various geographical locations. A detailed list of the Se content of food and feedstuffs is summarized by Lo and Sandi (1980) and Scott (1973). Fish meals and marine by-products represent the best natural sources of Se among the common feedstuffs for fish. However, certain fish meals, e.g., tuna may have a poor biological availability because of heavy metal complexing of Se. Selenium present in fish meal has a low digestibility, whereas selenomethionine is highly digestible (Bell and Cowey, 1989).

Common Se supplements include selenite, selenate, selenomethionine, selenium–methylselenomethionine, selenocystine, and selenocysteine. Monogastric animal nutrition studies have demonstrated the following relative availability of Se in pure compounds: selenite > selenate > selenomethionine > selenide > elemental selenium. In several countries there are regulations to limit Se supplementation in fish and animal feeds, and generally the limit is about 0.1 mg Se/kg in aquatic animal feeds.

5.2.10. Cobalt, Chromium, and Other Trace Elements

5.2.10.1. Cobalt

The biological function of cobalt relates to its role as a component of vitamin B_{12}. Approximately 4.5% of the molecular weight of B_{12} (cyanocobalamin) is contributed by elemental cobalt. Cobalt is of particular significance in ruminant nutrition, where it is used solely in the synthesis of the vitamin B_{12} molecule by rumen microflora. Most animals depend on microorganisms for their supply of vitamin B_{12}. Certain algae also contain Co (reviewed by Castell *et al.,* 1986). The marine rotifer *Branchionus plicatilis* is reported to have a definite requirement for vitamin B_{12} (Scott, 1981).

Extensive Russian studies on pond culture of carp have demonstrated that the addition of cobalt chloride and/or cobalt nitrate to the feed or cobalt chloride to the water of fish ponds enhances growth and hemoglobin formation in carp (reviewed by Castell *et al.,* 1986). Kashiwada *et al.* (1970) isolated bacteria from the intestinal tract of carp that produced vitamin B_{12} *in vitro*. Removal of Co from the diet of catfish significantly reduces intestinal synthesis of vitamin B_{12} (Limsuwan and Lovell, 1981). Most of the Co detected in maturing Atlantic salmon ovaries is present as part of vitamin B_{12} (Julshamn and Braekkan, 1975). Some uptake of Co occurs in rainbow trout eggs during embryonic development (Kuenze *et al.,* 1978).

5.2.10.2. Chromium

Chromium is an essential element for animals and humans. It exists commonly in the oxidation states Cr(II), Cr(III), and Cr(VI). Chromium(III) is required for normal carbohydrate and lipid metabolism. The ability of Cr to form coordination compounds and chelates is an important chemical characteristic that makes this essential metal available to living organisms. Chromium is found in foods as inorganic Cr(III) and as part of a biologically active molecule. Although the exact structure of the biologically active form(s) is not fully characterized, the active molecule appears to be a dinicotinatochromium(III) complex, stabilized with glutathione or its constituent amino acids (Toepfer *et al.,* 1977).

The biological function of Cr is closely related to that of insulin. Most chromium-potentiated reactions are also insulin dependent. In humans, Cr potentiates the action of insulin *in vitro* and *in vivo*; maximal *in vitro* activity requires a chemical form termed the glucose tolerance factor and tentatively identified as a Cr–nicotinic acid complex (Mertz, 1993). Chromium supplementation in carp diet improved the glucose utilization, probably by modulation of endogenous insulin activity (Hertz *et al.,* 1989), however, glucose utilization was not affected by dietary Cr in channel catfish (Ng and Wilson, 1997). Supplementation of the tilapia diet containing glucose

with chromic oxide improved growth, energy retention, and liver glycogen depostion (Shiau and Chen, 1993).

To date, the only study with fish on the effects of dietary Cr(III) did not show any deficiency signs or a change in tissue distribution of rainbow trout fed a low-chromium purified diet (Tacon and Beveridge, 1982). No attempts have been made to demonstrate the dietary toxicity of Cr(III). Toxicological effects of Cr(VI) in brook trout have been observed (Benoit, 1976). Common sources of Cr feed supplements include chromic chloride, high-Cr-yeast, Cr nicotinate, and Cr picolinate.

5.2.10.3. Other Trace Elements

Information on the dietary requirement of other trace elements is limited. Page *et al.* (1978) found that sulfate failed to promote growth or provide the requirements of sulfur amino acids in rainbow trout. George (1970) indicated that boron and/or molybdenum supplementation of the carp diet improved growth and survival. Increased dietary intake of fluoride enhances fluoride accumulation in the vertebrae of rainbow trout (Tiews *et al.,* 1982). There is minimal evidence that boron is essential to organisms other than vascular plants. A role of boron in embryonic growth of rainbow trout has been demonstrated (Eckhert, 1998). Deficiency signs and requirements of these elements remain to be established.

5.3 Concluding Remarks

Undoubtedly significant progress on the mineral requirements of aquatic animals has been made in the past two decades; overall developments in this field of fish nutrition have been relatively slow. Many gaps still exist in the knowledge of the quantitative requirements of inorganic elements and their physiological functions in most fish. In particular, limited information has been published on trace element metabolism of aquatic organisms. This creates great difficulties in the characterization of deficiency or toxicity symptoms even under controlled environmental conditions.

Standard mineral mixtures used in warm-blooded animal experiments have not been effective in supporting optimum growth or preventing nutritional deficiencies in studies designed to investigate the nutrient requirements of finfish and crustaceans. Several pathological conditions and nutritional deficiency signs of unknown etiology have been observed in hatcheries, and aquaculture operations may in fact be due to a dietary mineral imbalance and either limited or excessive uptake of trace elements from the water. Wide differences exist among freshwater, eurohaline, and marine

fish species in the absorption and utilization of certain dietary minerals. There are also indications that the requirement and toxicity of inorganic elements may be influenced by acidic water (low pH). In feed formulations for aquatic animals, one must consider the requirement of the individual element, potential interactions with other inorganic elements and nutrients in the digestive tract and their metabolic level in various tissues, and minerals supplied from the aquatic environment, as well as the species, age, and sex of the fish.

In the past, fish meal was regarded as an adequate source of minerals for commercial feeds. With the increased use of plant protein in recent years and environmental concerns to reduce the P discharge in aquaculture effluents, there is a greater need to improve the bioavailability of organic P and to evaluate the utilization of trace elements in fish feeds. In recirculation systems, problems associated with the biological effects of high mineral concentrations arising from feed and excretory material in the water are also widely recognized.

Defining the role of inorganic elements in immune response, disease prevention, and reproduction of fish is an important and interesting area of research. In recent years, vitamin E and selenium have been implicated in the prevalence of several infectious diseases. The involvement of zinc and manganese in the reproduction of fish is becoming apparent. Fish may also have requirements for other ultratrace elements known to be required by terrestrial animals. Recent advances in molecular biology, microscopy, and analytical chemistry now provide us with many opportunities to explore the trace element metabolism and regulation in fish. I hope that the importance of inorganic elements emphasized in this chapter will receive wider consideration in future fish nutrition studies.

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6

Intermediary Metabolism

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6.1
Introduction: Metabolic Circuitry and Control Mechanisms

6.1.1. Organization

Intermediary metabolism seems complex to the uninitiated eye and to students with bad memories of memorizing the intermediates of the Krebs cycle. However, this apparent complexity is markedly reduced when metabolic pathways are viewed in a functional context in which pathways are classified according to their catabolic or anabolic nature (Fig. 6.1). Metabolic pathways are multienzymatic sequences in which the product of one enzyme is the substrate of the next, leading to the accumulation of many small chemical changes in the original molecule. In the case of catabolic sequences, these changes serve to transfer part of the chemical energy of the substrate to ATP (or its equivalents) or to NADPH for use in other cell functions. In anabolic sequences, precursor molecules are converted into compounds from which macromolecules, including proteins, complex carbohydrates, nucleic acids, and lipids, are built. Most of the pathways in intermediary metabolism are linear, although certain important processes are circular.

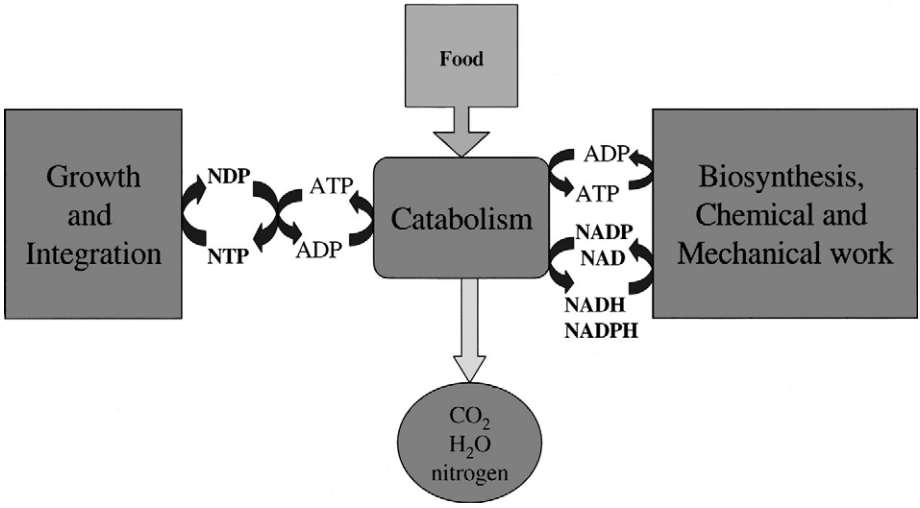

FIG. 6.1

General outline of metabolic processes, showing the central role of the coupling agents, ATP/ADP, NADH/NAD, and NADPH/NADP. Based on Atkinson (1977).

The central pathways of metabolism are comparatively few in number and their organization is highly conserved. The metabolic machinery in fish is much the same as that in mammals. The important functional differences between intermediary metabolism in fish and the more completely studied mammals lie in the means by which control is exercised, in the sensitivity of metabolic demand to biotic and abiotic factors, and in the exact roles of tissues and organs. Among abiotic factors, temperature is particularly central in its impact on intermediary metabolism in fish, given that the majority of fish are in thermal equilibrium with their environment. Its pervasive impacts on protein structure and function, in both the cytosolic and the membrane fractions of the cell, are the subjects of an excellent, comprehensive review (Somero 1997). Fish, living in water, have the advantage of an excellent medium into which their nitrogenous wastes can be excreted. Thus fish eliminate most of their excess nitrogen as ammonia from the gills, thereby simplifying their intermediary metabolism. Finally, many aspects of muscle metabolism are better understood in fish than in mammals, given that fish muscles are separated according to fiber type.

6.1.2. Mechanisms of Metabolic Control

Flux through metabolic pathways can be controlled in many fashions, all of which modify the rate at which the enzymes in the pathway catalyze their reactions. Enzymes will increase their catalytic activity when substrate and cofactor levels rise, up to concentrations at which the enzyme is saturated with substrate. Allosteric modulators can modify the activity of some enzymes by dramatically shifting the substrate affinity curves. Shifts in the intracellular binding of enzymes may modify their catalytic activity or the efficiency of transfer of substrates between enzymes. Phosphorylation–dephosphorylation reactions catalyzed by intracellular protein kinases and phosphatases change the activity of certain enzymes. Rapid hormonal control of metabolic pathways is generally exercised through such posttranscriptional modifications.

Classical studies of metabolic control emphasize the role of key regulatory sites, such as in glycolysis in which the "nonequilibrium" reactions catalyzed by glycogen phosphorylase, phosphofructokinase, and pyruvate kinase are thought to be major sites of control. Control of flux through these "key" control sites is postulated to occur by changes in metabolic signals, such as outlined above. The comparative studies of Newsholme and co-workers were based on the concept that the maximal activity of these enzymes set the maximal capacity for flux through the pathways in which they participate (Newsholme and Crabtree, 1986). Whereas the complex allosteric and posttranslational control mechanisms regulating these enzymes certainly

confer considerable potential for modulating metabolic flux, their activities are only closely related to maximal pathway flux in muscles of organisms which have specialized high metabolic rates such as hummingbirds, tuna, and honeybees (Suarez *et al.*, 1997). Therefore, tissue activities of regulatory enzymes are best used to indicate the metabolic specialization of a tissue or organ (i.e., carbohydrate or lipid catabolism, aerobic or glycolytic), rather than to measure the capacity for flux in a given pathway.

Metabolic flux may also be controlled by loci which catalyze near-equilibrium reactions. Such reactions are typically catalyzed by enzymes with maximal capacities 2–3 orders of magnitude higher than the net flux through the pathway. This "excess" capacity was explained by Haldane (1930) when he demonstrated that net forward flux at such reactions is possible only when their maximal capacity greatly exceeds the pathway flux. Therefore, modification of the capacities of these loci can lead them to assume greater importance in metabolic control than "apparently" warranted by either their maximal capacities or their kinetic properties. A clear indication of the importance of kinetic changes in the properties of equilibrium reactions is provided by the functional impact of the lactate dehydrogenase allozymes in the *Fundulus heteroclitus* system on the eastern seaboard of the United States (Powers and Schulte, 1998). Application of the Haldane equation to phosphoglucoisomerase from honeybee flight muscle indicates that although its maximal capacity is 20-fold higher than its maximum pathway flux, under intracellular conditions it supports a maximum flux only 5% above the maximum pathway flux (Suarez and Staples, 1997). The use of metabolic control theory to establish the relative contributions of different components of a series of reactions to metabolic control has been particularly successful with mitochondrial physiology (Brand *et al.*, 1993), although less has been done with fish systems than could be desired.

Few of the above control mechanisms modify the maximal capacity of the enzymes or pathways. Changes of enzyme concentrations through longer-acting control mechanisms or by modifications of the microenvironment in which the enzymes operate (i.e., membrane lipid composition) are means by which the overall capacity of a metabolic pathway can be changed. Such changes occur during development and growth or in response to shifts in environmental conditions. For example, changes in food availability lead to marked changes in the metabolic capacities of fish muscle (see below). Oxidative (red) fibers conserve their metabolic capacities during starvation, whereas glycolytic (white) fibers undergo marked decreases in metabolic capacities (Loughna and Goldspink, 1984). Thermal change modifies tissue metabolic capacities in many fish species, with cold acclimation/acclimatization leading to increases in tissue aerobic capacity. Within fish species, increases in size typically enhance the glycolytic capacity of white muscle

while tissue aerobic capacity decreases with increases in size (Somero and Childress, 1980, 1990). For white and red muscle, the allometric patterns vary with the longitudinal position (Martinez *et al.,* 2000). Therefore the metabolic capacities of tissues and organs in fish are dynamic, changing with the functional requirements and habitat conditions faced by the fish.

In many situations it is desirable to know the energetic status of a cell or tissue and many indicators have been proposed. As the adenylates are involved in the vast majority of energy-producing and energy-dependent reactions, Atkinson (1977) proposed the use of the energy charge, i.e., the proportion of the total adenylate pool which is available in the form of ATP, as a means of assessing energetic status. Examination of the changes in intracellular metabolites during major shifts in ATP use and production indicates that the levels of ATP and free ADP undergo only limited changes (Hochachka and McClelland, 1995). Although these findings underscore one of Atkinson's central tenants, that of the central importance of the maintenance of relative adenylate levels, only extreme decreases in the energetic status of fish and mammalian tissues are reflected in the energy charge. In tissues, such as fast glycolytic muscle, that use phosphocreatine to fuel initial contractile activity, phosphocreatine levels are linearly related to tissue energetic status (Arthur *et al.,* 1992). More comprehensive parameters, such as measures of tissue VO_2, heat production, mechanical work, or ion pumping, would provide a clearer indication of a tissue's capacity for energetic expenditures.

In the following sections we examine the metabolism of carbohydrates and proteins. The metabolism of lipids is covered in Chapter 4, by Sargent *et al.*

6.2 Carbohydrate Metabolism

6.2.1. Digestibility

Carbohydrates are excellent sources of energy and carbon, one of the major elements of which living organisms are composed. The breakdown of carbohydrates is the primary means by which animal tissues obtain their chemical energy. However, dietary carbohydrates are not the principal source of energy or carbon for most fish. Polysaccharides tend to be repeating polymers of simple sugars, making the conformation of links between monomers fairly easy to predict. Proteins are composed of ~20 types of amino acids of markedly differing structures and specific proteases are needed to recognize the bonds between specific amino acids. Therefore the digestive breakdown and absorption of carbohydrates are simpler than those of proteins. Despite

the apparent simplicity of carbohydrate degradation, animals typically can only digest glycogen and starches with endogenous enzymes and lack the enzymes required to degrade cellulose, chitin, and lignin. The strategy for overcoming such deficiencies is illustrated by the ruminants that have been famously successful in exploiting diets composed virtually only of plants rich in cellulose but poor in other nutrients. The success of ruminants is due to a symbiosis with microorganisms which digest and ferment the plant material, and not to the ruminant's capacity to produce cellulase. As a major obstacle in breaking down plant material is mechanical, herbivory requires structural adaptations for grinding plant material. Fish are no exceptions to the general rule that animals are best at breaking down starches and glycogen and that digestion of cellulose and chitin generally requires microbial assistance and specific mechanical adaptations to break down plant structures.

Because most fish are primarily carnivorous or omnivorous, carbohydrates are not the major components of their diets. Nonetheless, some fish species, in both freshwater and marine environments, have specialized for herbivory and many species, including species of interest for aquaculture, are able to ingest and digest significant quantities of plant material. Amylase, disaccharidases, cellulase, and chitinase have been extracted from fish stomachs (Jobling, 1995), but considerable species differences occur in the capacities of fish to digest and absorb polysaccharides (Stickney, 1994). Thus, channel catfish handle starch well and their diets can contain up to 40% starch (Wilson and Poe, 1987), whereas trout have only a limited ability to digest starch (Hilton *et al.,* 1983). The intestines of herbivorous fish are longer relative to organismal size than are those of omnivorous or carnivorous fish. On the intraspecific level, carp, *Cyprinus carpio,* roach, *Rutilus rutilus,* and grass carp, *Ctenopharyngodon idella,* fed large quantities of indigestible fiber have longer intestines than their conspecifics fed animal prey (Jobling, 1995).

The fish that opt to feed at a lower trophic level have several strategies to facilitate the digestion of plant materials that have their parallels in other vertebrates. The creation of a highly acidic environment in a thin-walled stomach allows the lysis of plant cell walls. Use of this strategy is facilitated by selective ingestion of plant food (i.e., separated from inorganic material). A thick-walled muscular stomach or a pharyngeal mill can be used to grind and rupture the plant cell walls. When species, such as parrotfish and mullets ingest sediment particles with algal food, grinding the inert particles with the food particles facilitates the mechanical breakdown of algal cells. Typically these fish need to feed copiously to obtain sufficient food from the mixture of sediment and algae. Alternately, microbial fermentation in a cecum of the hindgut can be employed to digest plant material. Such strategies are

likely central in the particular feeding habits such as those of wood-eating fish. In the wood-eating catfishes of the genus *Panaque,* a consortium of microorganisms seems to be required for cellulose breakdown (Nelson *et al.,* 1999). Whereas the digestibility of carbohydrates for herbivorous fish poses no problem, more carnivorous fish, in particular, salmonids, are less able to benefit from the nutritional value of carbohydrates.

The carbohydrates typically found in fish diets include chitin (from crustacean and insect exoskeletons), cellulose (plant material), glycogen (animal tissues), and starch (plant materials). The ability to digest these products parallels their presence in the diet, although in many cases it is not clear whether the digestive enzymes arise in the fish tissues or in microorganisms in the fish intestine. Chitinases and cellulases most likely originate in the intestinal flora (Stickney and Shumway, 1974; Fagbenro, 1990; Nelson *et al.,* 1999), whereas amylases are pancreatic in origin. The breakdown of carbohydrates to monosaccharides is completed by enzymes located in the brush border. Enzymes required for the breakdown of the specific linkages present in algal storage carbohydrates have been isolated from herbivorous marine fish. The capacity for carbohydrate digestion shows a certain plasticity, particularly in omnivorous fish. Thus, both cyprinids and tilapia modify their secretion of digestive enzymes when their diet is varied (Jobling, 1995). The capacity for absorption of the sugar monomers also parallels the relative importance of carbohydrates in the diet and changes with ontogenetic switches in dietary preferences.

6.2.2. Carbohydrate Storage and Breakdown

6.2.2.1. Responses to Starvation and Refeeding

Glycogen is the major carbohydrate storage form in fish and major deposits typically occur in the liver and muscle. Both oxidative (red) and glycolytic (white) muscle contain significant concentrations of glycogen, but given that white muscle makes up the bulk of the musculature, it stores most of the body's glycogen. The reliance on tissue glycogen stores during periods of food limitation differs from the patterns observed in mammals, in which starvation quickly leads to breakdown of liver glycogen so that blood glucose levels remain constant. In cod (*Gadus morhua*), carp (*Cyprinus carpio*), and roach (*Rutilus rutilus*), if hepatic lipids are present in significant amounts, they are the first reserves used during starvation (Black and Love, 1986; Lim and Ip, 1989; Blasco *et al.,* 1992; Méndez and Wieser, 1993; Böhm *et al.,* 1994). Muscle lipids are next to be used, followed by liver and muscle glycogen. In these species, muscle protein is the last "reserve" to be mobilized during starvation. In contrast, during the long spawning migrations of Pacific

salmon, muscle protein is degraded, while hepatic glycogen is conserved as a fuel for spawning itself (Mommsen *et al.,* 1980). During the salmon's migration, muscle protein seems to serve both as a fuel and as a source of carbon skeletons required for the maintenance of hepatic glycogen levels. Similarly, the mudskipper (*Boleophthalmus boddaerti*) favors muscle over liver glycogen during starvation (Lim and Ip, 1989). These changes in tissue glycogen levels with starvation and refeeding are particularly pronounced when tissue size is taken into account.

Feeding after a period of starvation leads to rapid recovery and particularly high growth rates (compensatory growth). During the beginning of feeding after starvation, liver and muscle glycogen levels in cod, roach carp, and mudskipper are quickly replenished (Black and Love, 1986; Lim and Ip, 1989; Blasco *et al.,* 1992; Méndez and Wieser, 1993; Böhm *et al.,* 1994). Recovery of tissue protein stores occurs more gradually, and once this process is well advanced, lipid reserves begin to be deposited (Black and Love, 1986; Böhm *et al.,* 1994).

6.2.2.2. Hepatic Glycogen Metabolism and Its Hormonal Control

6.2.2.2.1. Glycolysis. Hepatic glycogen is broken down both to provide glucose for export to the blood and to channel glucose into glycolysis, oxidative phosphorylation, or other ATP-yielding metabolic conversions (Fig. 6.2). Most glycogen mobilization is accomplished by glycogen phosphorylase. Glycolysis involves the gradual oxidation of glucose derived from glycogen or from the blood. It proceeds via two initial phosphorylation steps (catalyzed by hexokinase and phosphofructokinase), which serve primarily to increase the equilibrium constant of the glycolytic pathway and to commit the carbon skeletons to their breakdown via this pathway. Subsequently, the six-carbon sugar is broken into two triose molecules, which are then oxidized before two-substrate level phosphorylation reactions (at phosphoglycerate kinase and pyruvate kinase) provide the limited ATP yield which can be obtained in the absence of oxygen. During the oxidation of the sugar, an NAD is reduced to an NADH. As NAD concentrations are no higher than 0.5 m*M*, the NAD supply must be renewed for glycolysis to continue. In fish and mammalian muscle, this is typically the role of lactate dehydrogenase that converts pyruvate into lactate while oxidizing the NADH to produce the required NAD. As liver typically functions in aerobic mode, it does not need to form lactate to maintain redox balance. Rather, the NADH produced during glycolysis is oxidized in mitochondria, through the action of the electron transport system that allows the generation of a proton gradient which can then be used for oxidative phosphorylation.

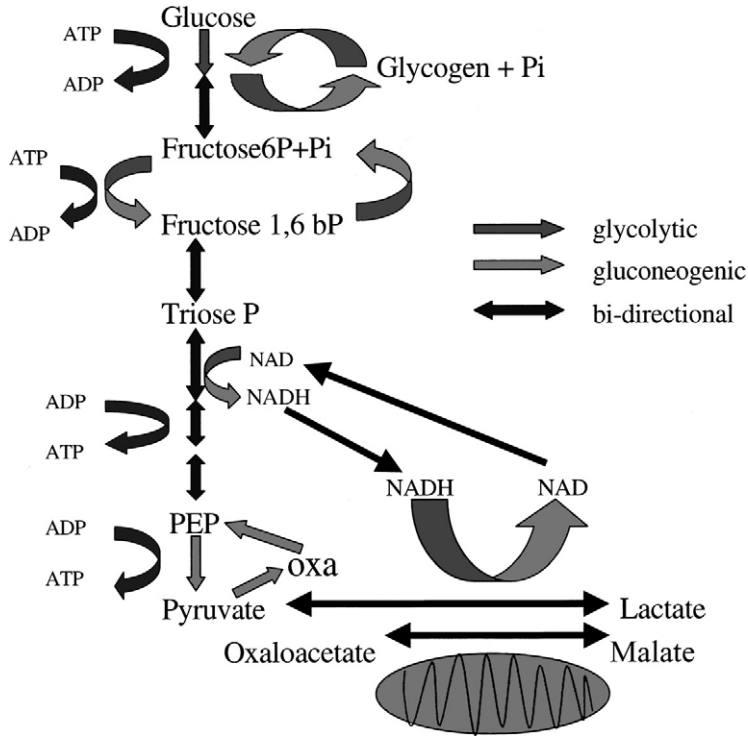

FIG. 6.2

Glycolysis and gluconeogenesis: two opposite pathways sharing all but two enzymes. Bypass reactions, with greater effective ATP investments, allow gluconeogenesis to be thermodynamically favorable without radically altering intracellular metabolite concentrations.

6.2.2.2.2. Pentose Phosphate Pathway. This metabolic sequence is initiated at the level of glucose 6-phosphate and has two major physiological roles: the primary role is the production of NADPH for biosynthetic purposes (Fig. 6.1); a secondary role is the production of the ribose sugar moiety required for nucleotide synthesis. Beyond its role in reductive biosynthesis, NADPH also protects cells against damage from oxygen radicals (Pelster and Scheid, 1991, 1992). The swim bladder of fish can contain high levels of oxygen, leading to considerable potential for free radical damage to its cells. Flux through the pentose phosphate pathway in the swim bladder of the toadfish virtually doubled under hyperoxic conditions, strongly suggesting

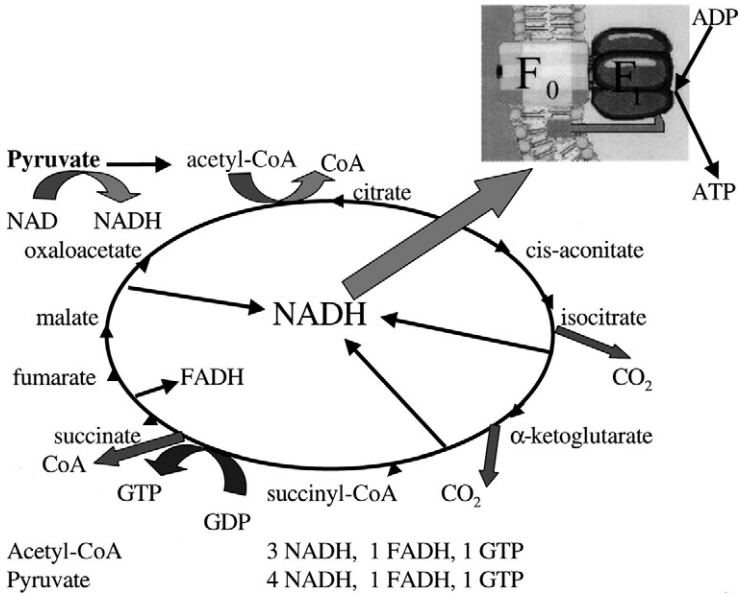

FIG. 6.3

The organization of the Krebs cycle, showing sites of NADH, FADH, and GTP generation.

that the high levels of the pentose phosphate shunt enzymes in this tissue are related to the protection of the tissue from free radical damage (Walsh and Milligan, 1993).

6.2.2.2.3. Krebs Cycle and Oxidative Phosphorylation. The pyruvate produced by glycolysis is fully oxidized to CO_2 and H_2O in the mitochondria through the combined action of Krebs cycle and the electron transport system (ETS) (Figs. 6.3 and 6.4). Again, the basic principles of the functioning of mitochondrial substrate oxidation and oxidative phosphorylation are similar to those in mammals, although the specific conditions under which fish function have led mitochondrial design to diverge somewhat from the mammalian model. Pyruvate is first converted into acetyl-CoA, through the action of pyruvate dehydrogenase. The acetyl-CoA is condensed with oxaloacetate through the action of citrate synthase and, thereby, enters into the Krebs cycle; the functioning of the cycle is fairly straightforward. Essentially the two carbons of the acetyl-CoA are gradually split off (as CO_2), while the six-carbon compound formed at the start of the cycle is gradually

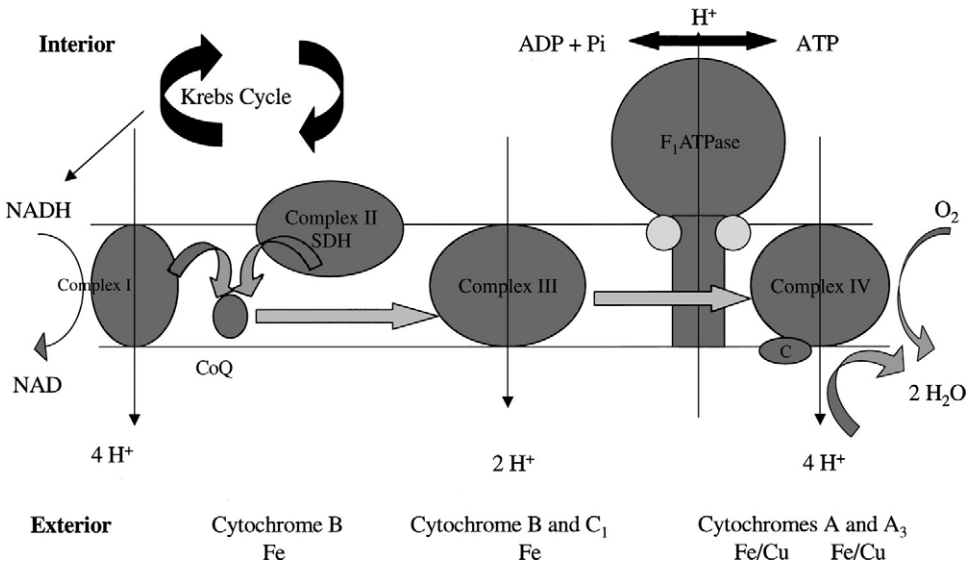

FIG. 6.4

Mitochondrial electron transport, showing sites of proton flow across the inner mitochondrial membrane as well as the cytochromes associated with the different inner membrane complexes.

oxidized, allowing the formation of three NADHs, one FADH, and one GTP per acetyl-CoA which enters the cycle. The formation of acetyl-CoA from pyruvate forms an additional NADH. The NADH is converted to NAD by NADH dehydrogenase, the first step in the electron transport chain. As the electrons are passed along the different cytochromes and electron carriers in the ETS (complexes 1–4 in Fig. 6.4), protons cross the inner mitochondrial membrane, creating a proton and an electrical gradient between the inner mitochondrial matrix and the intermembrane and cytosolic compartments. This electrochemical gradient, i.e., protonmotive force, provides the energy for the phosphorylation of ADP into ATP by the F_1-ATPase situated in the inner mitochondrial membrane. Effectively, protons are thought to pass through this membrane-spanning enzyme, providing it with the conformational energy required to transform ADP + P_i into ATP.

There is not an obligate stoichiometry between the number of electrons which are passed among the cytochromes (or protons which are translocated across the membrane) and the number of ATP molecules produced. Textbooks typically suggest that three ATP molecules are synthesized for each NADH molecule oxidized by the NADH dehydrogenase. However,

not all proton flow from the outside to the inside of the inner mitochondrial membrane is linked to oxidative phosphorylation. Proton leak across this membrane is considerable and reduces the coupling between oxygen consumption and ATP synthesis. Thus, electron transport can occur with no concomitant ATP synthesis. Current estimates for the stoichiometry between mitochondrial electron transport and oxidative phosphorylation range from 1.4 to 2.5 ATP per oxygen consumed and are all considerably lower than the theoretical value of 3 (Brand *et al.,* 1993). The genes for UCP-2 (uncoupling protein 2) have been sequenced in carp and zebrafish, indicating that the proteins implicated in this proton leak are as present in fish as in other vertebrates (Stuart *et al.,* 1999). Thus, while the efficiency of carbohydrate oxidation is higher than that of anaerobic glycolysis, it is lower than the 36 molecules of ATP per molecule of glucose that is traditionally presented.

6.2.2.2.4. Glycogen Synthesis and Gluconeogenesis. Hepatic glycogen synthesis is based both on the incorporation of bloodborne glucose into glycogen and on gluconeogenesis from lactate and amino acids. Glucose incorporation into glycogen occurs via production of UDP glucose from glucose 1-phosphate via the glycogen synthase reaction. Gluconeogenesis from lactate or amino acids requires the reversal of many glycolytic reactions (Fig. 6.2) and follows enzymatic bypasses for the pyruvate kinase (PK) and phosphofructokinase (PFK) reactions. The bypass for PK requires two enzymes. The first reaction is catalyzed by pyruvate carboxylase, which converts pyruvate into oxaloacetate (the functionally equivalent reaction can be catalyzed by malic enzyme, which converts pyruvate into malate, which can then be converted to oxaloacetate via the malate dehydrogenase reaction). Next the oxaloacetate is converted into phospho*enol*pyruvate by phospho*enol*pyruvate carboxykinase. The complete PK bypass requires two ATP equivalents. The bypass enzymes may be located either in the cytosol or in the mitochondria. The precise location influences the regulation of the reactions. The second bypass reaction requires fructose bisphosphatase (FBPase), which converts fructose 1,6-bisphosphate (F1,6BP) into fructose 6-phosphate (F6P) (Fig. 6.2). An additional enzyme produces fructose 2,6-bisphosphate from fructose 6-phosphate (Fig. 6.5). The sole apparent role of this compound is to stimulate the activity of PFK and inhibit that of FBPase.

6.2.2.2.5. Hormonal Control Mechanisms. As in mammals, the enzymes involved in glycogen metabolism are sensitive to hormonal controls, via phosphorylation and dephosphorylation reactions, as well as responding to intracellular metabolite signals. In general, the hormones that regulate glycogen mobilization and storage are similar to those that are active in

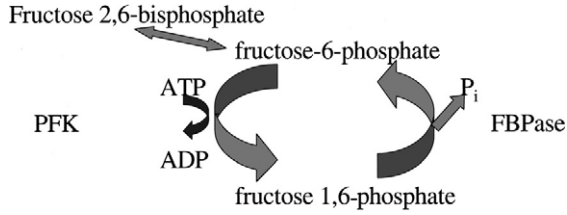

FIG. 6.5

The intermediates involved in the phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FB Pase) reactions.

mammals, although the precise regulatory patterns found in mammals are not necessarily present in fish (Fig. 6.6). This may be partly because the well-studied fish (trout) tend to be more carnivorous than the well-studied mammals (rat). Accordingly, gluconeogenesis occurs at considerable rates, even in fed fish, possibly reflecting the paucity of carbohydrate in the normal piscine diet.

Catecholamines, glucagon, and glucagon-like peptides and glucocorticoids are the major hormones stimulating glucose liberation from glycogen, while insulin and the insulin-like growth factors are the major hormones stimulating glycogen storage (Fig. 6.6). Glucagon, glucagon-like peptides,

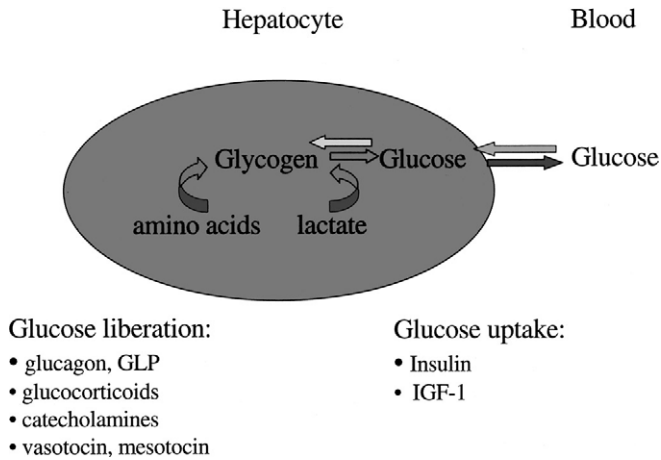

FIG. 6.6

Hormones implicated in the control of glucose liberation and uptake by fish hepatocytes.

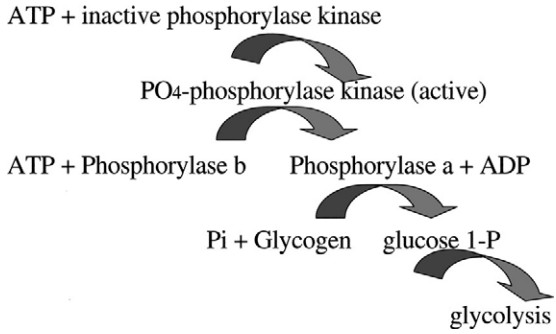

FIG. 6.7

Amplification of metabolic signaling allowed by the activation of glycogen breakdown by sequential phosphorylation reactions.

and cortisol can also stimulate hepatic gluconeogenesis from amino acids. The glucoregulatory hormones affect hepatic enzyme activities both through short-term modification of kinetic properties and through changes in gene expression. The regulation of glycogen phosphorylase and synthase is controlled largely by phosphorylation–dephosphorylation reactions (Fig. 6.7). The hormonal activation of hepatic gluconeogenesis by glucagon and epinephrine occurs via phosphorylation of PK and of PFK2, the enzyme that produces fructose 2,6-bisphosphate. Several enzymes in glycolysis and gluconeogenesis show changes in their kinetic properties shortly after exposure to insulin or glucagon (Wright *et al.,* 1989; Foster and Moon, 1990). The reader is referred to some excellent, recent reviews on insulin, glucagon, and glucagon-like peptides for details concerning the specific peptides and their actions (Mommsen and Plisetskaya, 1991; Duguay and Mommsen, 1994; Plisetskaya and Mommsen, 1996).

Glycogen synthesis in trout hepatocytes occurs at least partly via direct incorporation of glucose into glycogen (i.e., glucose → glucose 6-phosphate → glucose 1-phosphate → UDP-glucose → glycogen) (Pereira *et al.,* 1995) and does not necessarily involve a partial glycolytic breakdown of glucose and subsequent gluconeogenesis, as occurs in mammals. The incorporation of glucose into glycogen is influenced by the relative activities of phosphorylase and synthetase. When isolated hypatocytes were incubated with insulin, glycogen phosphorylase activities decreased, whereas glycogen synthase activities did not increase. However, when both insulin and higher glucose levels (10 m*M*) were present in the incubation medium, glycogen synthase activities increased. Thus, as both glycogen phosphorylase and

synthetase activities seem to function simultaneously in the fish hepatocyte, shifts in the ratio of their activities are likely to dictate cellular glycogen contents.

6.2.2.2.6. Receptors and Signaling Systems. During the last decade, considerable effort has been directed toward elucidating the receptors and signaling systems involved in cellular responses to glucoregulatory hormones in fish. Moon and co-workers have made considerable progress in identifying the signals implicated in cellular responses to the hormones stimulating glycogen breakdown. Thus, glucagon acts on eel and bullhead hepatocytes through both the cAMP and the inositol triphosphate (IP_3) signaling pathways (Moon *et al.,* 1997). Epinephrine actions on hepatocytes from these species also involve both the cAMP and the IP_3 pathways (Fabbri *et al.,* 1995). Changes in intracellular Ca^{2+} levels caused by epinephrine may be mediated by its effects on α-adrenergic receptors as demonstrated for catfish, *Ictalurus melas* (Zhang *et al.,* 1993), bullhead, trout (*Oncorhynchus mykiss*), and eel (Moon *et al.,* 1993). Species differences can exist in the receptor types implicated and in the strength of the responses to a given hormone. The vasoactive peptides, vasotocin and mesotocin, stimulate gluconeogenesis only in eel hepatocytes, where they act via a V_2-type receptor (Moon and Mommsen, 1990). Chronic increases in cortisol levels in trout raised hepatocyte β_2-adrenoceptors, suggesting an enhanced sensitivity to adrenergic stimulation, which could facilitate hepatic glucose production during periods of chronic stress (Reid *et al.,* 1992). Gutiérrez and co-workers have detected the receptors for insulin and insulin-like growth factor in fish liver, glycolytic and oxidative muscle, heart, ovary, and adipose tissue (Planas *et al.,* 2000). The receptors are similar to those present in mammals and change in their cellular levels with physiological status (Navarro *et al.,* 1999).

Molecular techniques are being increasingly used to assess how the regulation of the expression of hepatic enzymes is as modified. Feeding trout with carbohydrates did not modify the expression of phospho*enol*pyruvate carboxykinase, which catalyzes the conversion of oxaloacetate into phospho*enol*pyruvate (Fig. 6.2) (Panserat *et al.,* 2001), or that of glucose-6-phosphatase (Panserat *et al.,* 2000a). Similarly, carbohydrate feeding did not change the expression of fructose bisphosphatase in Atlantic salmon (*Salmo salar*) and Eurasian perch (*Perca fluviatilis*) (Tranulis *et al.,* 1996; Borreback and Christophersen, 2000). On the other hand, dietary carbohydrate induces the expression of the glucokinase gene in trout, carp, and sea bream (Panserat *et al.,* 2000b). Given that the natural diet of these fish is low in carbohydrate, they may lack mechanisms by which dietary carbohydrates decrease the expression of gluconeogenic enzymes.

6.2.2.2.7. Glucosidic Pathways of Glycogen Breakdown. In animal tissues, glycogenolysis can occur via both phosphorolytic (producing glucose 1-phosphate) and glucosidic (producing glucose) pathways. A variety of enzymes cleaves glucose units off glycogen; the forms with acidic pH optima are thought to function in lysosomal recycling of the products of cellular autophagy. Whereas the physiological significance of the phosphorolytic pathway is well established, little is known of the physiological regulation of the glucosidic pathways. In mammals, defects in the glucosidic pathway lead to massive accumulation of glycogen in the lysosomes (type II glycogenosis). In frogs, the glucosidase pathways liberate significant amounts of glucose from muscle glycogen after exhaustive exercise (Fournier and Guderley, 1992), leading to a marked postexercise hyperglycemia. Whereas few studies have examined the roles of the glucosidic pathways in fish, three forms of α-glucosidase were found in trout liver, and one of these differed in kinetic properties with the exercise status of the trout (Mehrani and Storey, 1993).

6.2.2.2.8. Regulation of Blood Glucose. A major difference between mammals and fish, which may extend to all ectothermal vertebrates, is the considerably greater tolerance which fish show to wide fluctuations of blood glucose levels. Thus, many fish species survive periods, caused by either natural or experimental treatments, during which blood glucose levels are undetectable (as reviewed by Mommsen and Plisetskaya, 1991). Nonetheless, under most physiological conditions, blood glucose levels are fairly stable and respond to hormonal treatments: rising with increases in cortisol, catecholamines, and glucagon and decreasing with insulin administration.

Because fish erthryocytes are nucleated and possess mitochondria, they can oxidize their fuels. Blood glucose is their most probable fuel, but the actual fuel use by blood cells is not entirely clear. Glycolytic and Krebs cycle enzymes are present in fish blood cells (Sephton *et al.,* 1991; Ferguson and Storey, 1991). When the oxidation of [6-^{14}C]glucose is followed, the calculated rates are 1000-fold lower than those of glucose disappearance, as such (Sephton *et al.,* 1991). Lack of knowledge about the exact fuels which are available in plasma complicates the analysis of fuel use by fish blood cells (Guppy *et al.,* 1999).

6.2.2.3. Muscle Carbohydrate Metabolism

6.2.2.3.1. Metabolic Characteristics of Different Fiber Types. Fish skeletal muscle is separated into distinct zones: oxidative (red) fibers are at the surface, usually underlying the lateral line, intermediate pink fibers lie directly beneath these oxidative fibers, and fast glycolytic (white) fibers form the bulk of the musculature. As suggested by their name, the oxidative fibers have a

high aerobic capacity, with a considerable portion of their fiber volume occupied by mitochondria (up to 45% of the fiber volume in cold-acclimated striped bass) (Egginton and Sidell, 1988). On the other hand, the glycolytic fibers have a low mitochondrial volume density, a high myofibrillar volume density, large fiber diameters, and a low capillary density (Johnston, 1981). The limited access to blood oxygen supplies coupled with the low mitochondrial abundance forces these fibers to rely on anaerobic glycolysis to support the high-intensity contractile activity of which they are capable. Lower-intensity metabolic activities, such as protein synthesis, ion pumping, and recovery metabolism, will of course require oxidative metabolism, even in the white fibers. The physical separation of the fiber types has allowed a greater understanding of the functional specialization of fiber types in fish than in most other vertebrates.

6.2.2.3.2. Patterns and Control of Muscle Carbohydrate Metabolism. While following the same pathways as in liver (Figs. 6.2–6.4), muscle carbohydrate metabolism has somewhat different regulatory properties. Furthermore, muscle carbohydrate metabolism reflects primarily the requirements of ATP generation (for myofibrillar ATPase activity, protein synthesis, or ion transport) and not the generation of intermediates for biosynthesis. This makes carbohydrate metabolism easier to analyze in muscle, particularly in fast glycolytic fibers, than in liver. Accordingly, a fairly complete understanding of the dynamics of carbohydrate metabolism in fast glycolytic muscle is available for several species, particularly rainbow trout.

During burst exercise (startle responses, sprints), fast glycolytic fibers can work for up to several minutes with minimal input from the remainder of the body. Essentially their contractile activity is fueled entirely by materials present in muscle at the start of activity. As in other vertebrates, these intense rates of contractile activity during burst exercise initially rely on ATP produced from phosphocreatine. After the first few tail flaps, phosphocreatine levels decline markedly and anaerobic glycolysis takes over ATP production (Dobson *et al.,* 1987). Startle responses, which last for seconds at most, would use only phosphocreatine as a metabolic fuel. Given a short recovery time, these responses can be repeated frequently, much as during interval training of humans. Thus, the aerobic capacity of white fibers can facilitate startle responses (Garenc *et al.,* 1998). Of course, anaerobic glycolysis leads to lactate production and intracellular acidification. Lactate concentrations in white muscle of exhausted fish can easily reach 30 m*M* or higher in large salmonids or tunas and the intracellular pH drops by approximately 0.5 pH unit (for excellent reviews see Moyes and West, 1995; Milligan, 1996; Kieffer, 2000). In salmonids, lactate accumulation increases with size until a length of approximately 30 cm, after which no further increases are noted

(Kieffer, 2000). Typically, the accumulation of lactate in white muscle can be fully accounted for by the decrease in muscle glycogen.

Recuperation of fish after exhaustive exercise requires both correction of the acid–base disturbance and restoration of muscle glycogen reserves. While both processes must be completed for full recuperation, they are not necessarily carried out in parallel (for a review see Milligan, 1996). Given the low density of capillaries in white muscle, and possibly linked with the unusual lactate transporter in white muscle (Laberee and Milligan, 1999), much if not most of the lactate produced during burst exercise is metabolized *in situ*. Some of this lactate is oxidized to support the costs of glycogen synthesis. As in other ectothermal vertebrates, much of the glycogen is regenerated from lactate directly in the muscle. The hepatic contribution to lactate removal is limited (Walsh, 1989; Milligan, 1996). Whereas the exact metabolic pathway used remains somewhat mysterious, given the absence of a complete gluconeogenic bypass for the pyruvate kinase reaction, reversal of this reaction remains the most likely means by which lactate glycogenesis proceeds (Moyes *et al.,* 1992a; Schulte *et al.,* 1992). As glucose uptake by exhausted white muscle accounts for less than 10% of glycogen repletion (West *et al.,* 1994), clearly the Cori cycle is not relevant to white muscle recovery. On the other hand, in oxidative muscle of exhausted trout, up to half of glycogen repletion can occur from blood glucose, implicating both the Cori cycle and lactate glycogenesis in its recovery (West *et al.,* 1994).

Full metabolic recovery from bouts of exhaustive exercise is typically a lengthy process in fish, with up to 24 hr being required for full replenishment of muscle glycogen stores and elimination of the lactate load (Wang *et al.,* 1994; review by Milligan, 1996). The elevated cortisol levels frequently observed during the recovery period delay the restoration of resting metabolic status (Pagnotta *et al.,* 1994; Eros and Milligan, 1996). As exhaustive exercise is typically stressful and as cortisol is frequently an indicator of stress, the type of exercise stimulus seemed a likely reason for the delayed recuperation. However, if exhausted trout are allowed to recuperate while swimming at an easily sustainable speed, their recuperation is markedly accelerated, the postexercise increase in plasma cortisol is eliminated, and that of plasma lactate is attenuated (Milligan *et al.,* 2000).

Size has a significant impact both on the extent to which lactate is accumulated during burst exercise and on the speed of metabolic recovery. Smaller fish typically have a lower glycolytic capacity, accumulate less lactate, and recuperate much more quickly from strenuous activity than larger fish (Franklin *et al.,* 1996; Garenc *et al.,* 1998). This may reflect the changing hydrodynamic constraints with size as well as the allometry of organismal oxidative capacity. Thus larval fish satisfy a considerable portion of their

oxidative requirements by diffusion across the body surface, rendering reliance on anaerobic glycolysis less necessary during strenuous activity.

Glycogen degradation and anaerobic glycolysis during burst activity are activated through signal metabolites and through phosphorylation of critical enzymes. Despite the much higher rates of carbohydrate breakdown in muscle than liver cells, glycogen breakdown is again initiated by the activation of glycogen phosphorylase. In vertebrate muscle, this activation occurs via increases in cytoplasmic Ca^{2+} during the initiation of contraction which stimulate a phosphorylase kinase, thereby converting phoshorylase into its active a form. The increased production of hexose phosphates stimulates glycolytic flux, largely through the activation of PFK by the increases in its substrate (F6P) and then its product (F1,6BP) (Dobson and Hochachka, 1987). Exhausting exercise changes the binding of glycolytic enzymes to the myofibrillar apparatus in white muscle (Brooks and Storey, 1988a,b), suggesting another means, albeit controversial, by which their activity may be regulated. In anoxia-tolerant cyprinids, ethanol can replace lactate as the terminal product of anaerobic carbohydrate metabolism (Shoubridge and Hochachka, 1980). In these cyprinids, lactate produced during anoxia elsewhere in the body (Dorigatti *et al.,* 1997; Lutz and Nilsson, 1997) is converted to ethanol in skeletal muscle.

6.2.2.3.3. Fuel Use During Sustained Activity. Some fish swim continuously, particularly those that must swim to ventilate their gills. Thus the physiology of oxidative skeletal muscle, particularly in the ram-ventilating fish, has much in common with that of cardiac muscle (Dickson, 1995). Even in fish that do not need to swim for gill ventilation, sustained activity can last for extensive periods. In definitions of fish swimming, sustained swimming is considered to last more than 200 min, burst swimming up to several (3) minutes, and prolonged swimming is anything between the two. While not unique in the animal world, the piscine capacities for sustained activity put humans, including athletes, to shame. In analogy with mammals, fuel use by oxidative muscles would seem to require a circulatory input. Nonetheless, fish oxidative muscle has extensive intracellular deposits of lipid and glycogen (Johnston, 1981; Egginton and Sidell, 1989; St. Pierre *et al.,* 1998). As many fish are highly carnivorous, the concept has arisen that fish rely extensively on protein metabolism, even to fuel activity. On the other hand, the order of reserve mobilization during the extensive spawning migration of salmon (Mommsen *et al.,* 1980) indicates that proteins are the last to be used.

During sustained swimming by rainbow trout, neither blood glucose nor protein is a quantitatively important fuel. In a study analyzing O_2 consumption, CO_2 production, and nitrogen excretion in juvenile rainbow trout

swimming at 5 and 15°C, protein use did not account for more than 30% of fuel use under any condition. Even in satiation-fed trout, protein use remained constant as swimming speed increased (Alsop and Wood, 1997), suggesting that it is a minor fuel for contractile activity. Lipids and carbohydrates were the most important fuels, with lipids generally playing a greater role than carbohydrates (Kieffer *et al.,* 1998). As lipids fill a considerable fraction of fiber volume in oxidative muscle, their role during sustained swimming is understandable. The limited hepatic glucose production during sustained swimming (Shanghavi and Weber, 1999) together with the low uptake of glucose by heart and red muscle (West *et al.,* 1993) indicates that circulating glucose is of limited importance as a fuel for sustained swimming by trout. Thus, the carbohydrate used during sustained swimming must be intracellular glycogen.

Mitochondria are extremely abundant in fish oxidative muscle, reaching volume densities far higher (45%) than those in mammalian oxidative fibers, particularly during cold acclimation of species such as striped bass, *Morone saxatilis* (Egginton and Sidell, 1989). Mitochondria from carp red and white muscle differ primarily in their capacity to oxidize lipids: both having an excellent capacity for pyruvate oxidation (Moyes *et al.,* 1989). The primary role of mitochondria during exercise clearly is the provision of ATP without the concomitant problems of proton accumulation. Mitochondria are found both in a layer directly below the fiber membrane (subsarcolemnal) and among the myofibrils (intermyofibrilar). In trout the properties of the mitochondria from these two populations do not differ (Moyes *et al.,* 1989). Increases in mitochondrial volume density decrease gradients in ATP and phosphocreatine within muscle fibers, with this effect being particularly pronounced in the range of mitochondrial volume densities found in white fibers (Hubley *et al.,* 1997).

Despite their high abundance in fish oxidative fibers, the oxidative capacities per unit mitochondria are as high in fish as in other vertebrates. When measured at a common temperature, similar substrate oxidation rates are found for mitochondria isolated from oxidative muscle of fish living at warm temperatures (carp and various species of tilapia) and mammalian oxidative muscle (for a recent review see Guderley and St. Pierre, 1996). The cristae surface densities for a variety of perciform fish (Johnston *et al.,* 1998) are similar to or higher than the values reported for mitochondria from mammalian muscle (20–40 $\mu m^2\ \mu m^{-3}$) (Hoppeler and Lindstedt, 1985; Schwerzmann *et al.,* 1989) and mammalian and reptilian heart and muscle (35–60 $\mu m^2\ \mu m^{-3}$) (Else and Hulbert, 1985) but are lower than those reported for hummingbird flight muscle (58 $\mu m^2\ \mu m^{-3}$) (Suarez *et al.,* 1991) or tuna red muscle (63–70 $\mu m^2\ \mu m^{-3}$) (Moyes *et al.,* 1992b). Certainly,

oxidative capacities and cristae surface densities indicate that fish muscle mitochondria have capacities similar to those in mammalian and avian muscles. Thus, the high mitochondrial volume density attained in fish muscle under some conditions is not simply a compensation for low functional capacities. Sidell proposes that the combination of high lipid and mitochondrial volume densities provides a mechanism for the rapid intracellular distribution of oxygen, facilitating aerobic metabolism throughout the rather large muscle fiber (Desaulniers *et al.,* 1996; Sidell, 1998).

6.2.2.3.4. Adjustment of Muscle Metabolic Capacities to Abiotic and Biotic Conditions. Given the extensive thermal equilibration during branchial gas exchange, most fish are in complete thermal equilibrium with their environment and change their operating temperature on diurnal and seasonal bases. The extensive impact of temperature on biochemical reaction rates and structures (for an excellent recent review see Somero, 1997) makes it imperative that tissue metabolic organization and regulatory properties be adjusted if a species is to maintain similar capacities in the face of such thermal change. Modifications in biochemical properties in response to thermal change have been observed on the level of enzyme, fatty acid, phospholipid, and cholesterol contents of membranes and organelles as well as on the level of muscle ultrastructure and function (Egginton and Sidell, 1989; Hazel, 1995; Guderley and St. Pierre, 1996; Somero, 1997).

The primary response of fish muscle to cold acclimation is an increased aerobic capacity, which improves their sustained swimming capacity (Sänger, 1993). This increased aerobic capacity can arise from a greater percentage oxidative fibers, from a greater mitochondrial content, or from increases in mitochondrial cristae density. Thermal change leads to a suite of responses, starting with expression of heat shock proteins (Somero 1997) and continuing with rapid changes in phospholipid head groups and more gradual shifts in fatty acid composition (Hazel and Landrey, 1988a,b; Hazel, 1995) and in levels of mitochondrial enzymes (Sidell *et al.,* 1973). Once these processes have stabilized (thought to require ~6 weeks), isolated mitochondria, muscle, and the fish have changed their oxidative capacity. The extensive remodeling of the oxidative capacities and regulatory properties of muscle mitochondria during cold acclimation has been well documented (Van den Thillart and Modderkolk, 1978; Guderley and Johnston, 1996; Guderley *et al.,* 1997; St. Pierre *et al.,* 1998). Examination of the molecular mechanisms coordinating this marked remodeling indicates a shift in the relationship between the level of mitochondrial messages and that of enzyme activity in response to cold acclimation (Battersby and Moyes, 1998; Hardewig *et al.,* 1999).

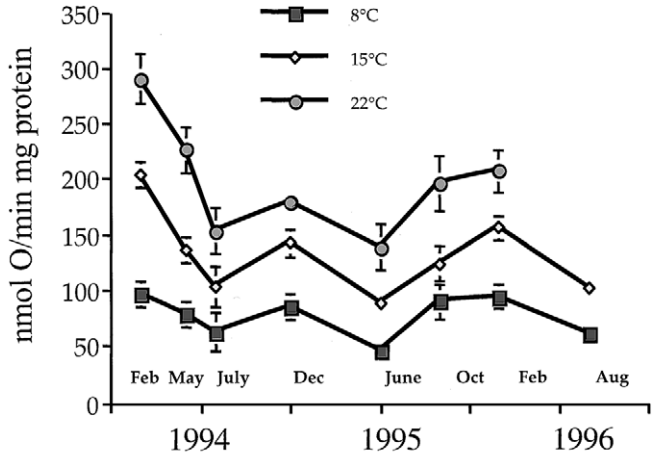

FIG. 6.8

Modification of the maximal oxidative capacity of mitochondria isolated from rainbow trout red muscle during seasonal acclimatization. Modified from Guderley and St. Pierre (1999). Seasonal cycles of mitochondrial ADP sensitivity and oxidative capacities in trout oxidative muscle. *J. Comp. Physiol B* **169,** 474–480.

Responses to thermal acclimation are likely to vary according to the season (or photoperiod) at which the acclimation occurs. In channel catfish, *Ictalurus punctatus* (Seddon and Prosser, 1997), positive acclimation of numerous enzyme activities is apparent in fall and winter but not in spring and summer. When the properties of mitochondria isolated from naturally acclimatized trout are examined over an annual cycle, the maximal oxidative capacities of the isolated mitochondria exhibit extensive thermal compensation during cold periods (Fig. 6.8). Nonetheless, winter rates at habitat temperatures do not attain the rates at habitat temperatures during warmer periods (Fig. 6.9) (Guderley and St. Pierre, 1999). The ADP affinities of the mitochondria undergo cycle modifications, with lower $K_{m_{app}}$ values typical for the colder periods (Fig. 6.10). In three-spined stickleback (*Gasterosteus aculeatu*) compensation of muscle oxidative capacity was apparent during cold acclimation in spring but not in fall, commensurate with the reproductive and migratory activities of the spring fish (Guderley *et al.,* 2000).

The interplay among temperature, growth, reproductive investment, and genetics determines the metabolic capacities of muscle. In fish muscle, temperature is a major determinant of aerobic capacity, whereas energetic status is a major influence on glycolytic capacity (Sullivan and Somero, 1983; Loughna and Goldspink, 1984; Martinez *et al.,* 1999; Dutil *et al.,* 1998; Guderley *et al.,* 1994).

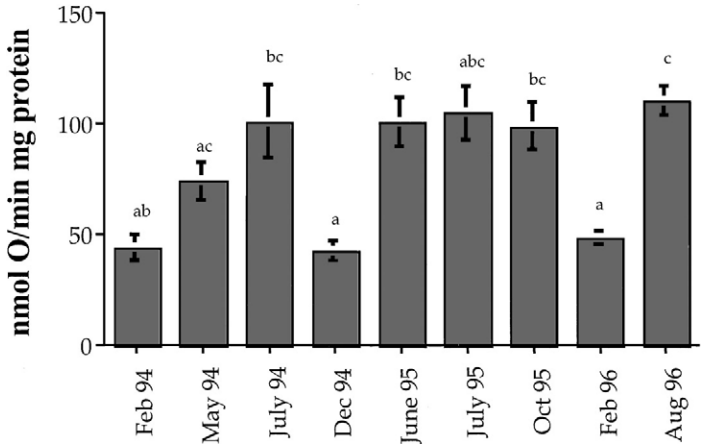

FIG. 6.9

Maximal oxidative capacities at habitat temperatures for mitochondria isolated from the oxidative muscle of seasonally acclimatized rainbow trout. Adapted from Guderley and St. Pierre (1999).

During fish growth, muscle fibers increase in size and number (Weatherley, 1990). In parallel, muscle metabolic capacities change, perhaps due to increases in fiber size or to changing locomotor requirements, (Pelletier *et al.,* 1993a,b; Guderley *et al.,* 1994; Dutil *et al.,* 1998). At reproductive maturity, male salmon show cardiac hypertrophy, with a parallel increase in mitochondrial abundance (West and Driedzic, 1999). In contrast, mammalian muscle maintains its metabolic profile with changing growth rate. In three-spined stickleback, the absolute speed during startle responses and the activities of phosphofructokinase (PFK) and lactate dehydrogenase (LDH) in glycolytic fibers increase dramatically as juveniles grow, but this positive allometry is lost in adults (Garenc *et al.,* 1999). As muscle glycolytic enzyme levels change with energetic status (Guderley *et al.,* 1994), the loss of muscle capacities with age may reflect a cost of reproduction. A certain genetic control of muscle metabolic capacities and swimming performance is apparent in sticklebacks (Garenc *et al.,* 1998). Full-sib heritability of burst swimming by three-spined stickleback is significant at 5 weeks of age, but not at 3 months, suggesting that genetic or parental effects are masked during growth. At 3 months, muscle metabolic capacities show significant full-sib heritability. At this age, white muscle cytochrome *c* oxidase (CCO) and PFK levels are correlated with burst speeds (Garenc *et al.,* 1999).

This discussion of the determinants of muscle metabolic capacities clearly demonstrates the multitude of factors that set the capacities for carbohydrate

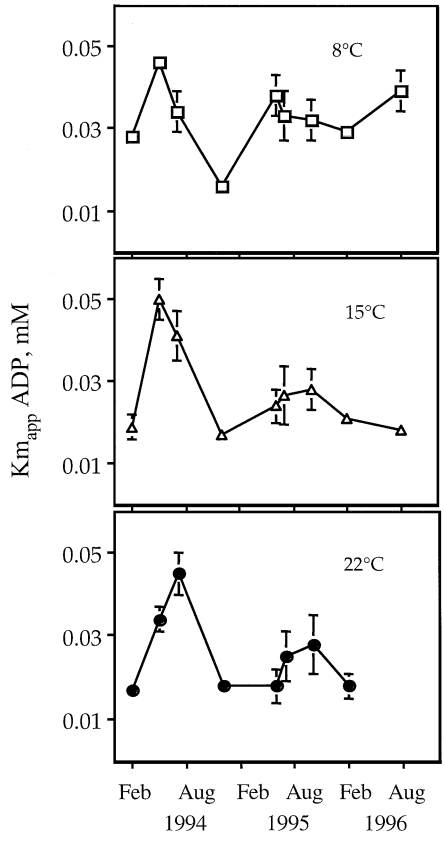

FIG. 6.10

Changes in the ADP affinity of mitochondria isolated from oxidative muscle of seasonally acclimatized rainbow trout. Adapted from Guderley and St. Pierre (1999).

metabolism. Similar analyses could be made for the metabolic capacities of other tissues. Fish, due to their thermal equilibrium with their habitat, may be particularly sensitive to this symphony of environmental factors. Alternately, their aquatic habitat may allow fish to change their tissue hydration more drastically than terrestrial animals that have to retain water at the same time that they must pay the cost of carrying the water. Finally, the great variety of fishes as well as the range of habitats and environmental conditions in which they occur requires prudence in the generalization of results reported here to species not yet studied. Rainbow trout, carp, and goldfish have been extensively studied, but they are unlikely to be representative of all fish species.

6.3
Protein and Amino Acid Metabolism

6.3.1. Protein Deposition

Body protein is in a continuous process of synthesis and degradation (catabolism). This is a fundamental fact in understanding how to quantify the processes of intake, digestion, circulation, synthesis, and hydrolysis, where protein/amino acids are in a state of turnover (Fig. 6.11). As this process seems energetically wasteful in the short run to a naïve observer, in the long term, multiple renewal and repair are required for the longevity of protein structures in living organisms. The process of turnover of amino acids, sequentially cycling through protein via synthesis degradation, is extremely specific at different tissue levels, but overall, fish catabolize 40–50% of body proteins to be returned to the pool of precursors and reused for synthesis (Cowey and Luquet, 1983). Based on leucine metabolism in rainbow

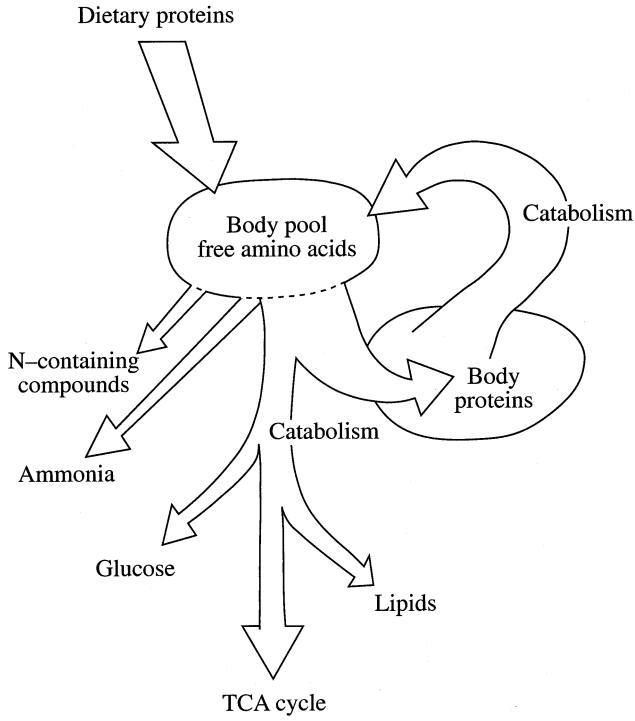

FIG. 6.11

Main pathways of protein and amino acid metabolism.

trout, Fauconneau and Arnal (1985) argued that 60% of this amino acid introduced directly by single flooding dose injection into the free amino acid pool was used for protein synthesis. The authors provided evidence that the concentration of the labeled amino acid remained stable in the whole-fish pool over the period of incorporation/synthesis measurement. Thus it was not apparent if other amino acids which may be used preferentially for the synthesis of other compounds of metabolic importance, such as hormones and neurotransmitters, may differ in their fate. In experiments quantifying protein synthesis, Owen *et al.,* (1999) used phenylalanine, a precursor of many substances of biochemical importance other than protein, and confirmed a linear increase in the protein incorporation and free pool stability of labeled phenylalanine in white muscle of trout (60% of the body mass in trout). This finding arguably satisfied the conditions required for using a stable isotope flooding technique as a reliable method for *in vivo* measurement of protein synthesis in fish.

Protein synthesis rate estimates based on labeled arginine appeared to meet the criteria of a stable concentration in tissues following a flooding effect, although high levels of this amino acid differed among tissues and could have been selectively catabolized or excreted (Fauconneau *et al.,* 1990). Thus, under such conditions, the rates of protein synthesis rate could be overestimated, but the net result of comparisons between diploid and triploid rainbow trout would hold true. However, the protein synthesis rate in the muscle and carcass of the triploids was lower than that of the diploids of the same gender. The large muscle mass in fish acts as the major reservoir of the free amino acid pool, although special storage proteins have also been suggested. Protein synthesis differed among the fractions of white muscle of rainbow trout. The myofibrillar fraction, which accounts for more than 60% of the total protein, had fivefold lower rates of protein synthesis than the mitochondrial or nuclear fractions (which together account for only 5–18% of the white muscle protein) (Fauconneau *et al.,* 1995). This suggests that the oxidative capacity of white muscle is maintained, although white muscle is clearly less oxidative than red muscles (see above). The adaptability of white muscles needs to be pointed out, as protein synthesis in the mitochondrial and postmitochondrial fractions will secure muscle response to acute physiological (exercise) or environmental (temperature) changes.

6.3.1.1. Influence of Fish Size

Small fish at larval stages would be expected to have high protein synthesis rates compared with larger fish, as the growth rate achieved in larvae is frequently over 100%/day (Dabrowski, 1986). Increased growth rates may result from an increased protein synthesis rate, a decreased protein degradation

rate, or a combination of both. Consequently it did not come as a surprise that in juvenile common carp (45 mg), the protein synthesis rate was estimated to be in the range of 60–600%/day (Fauconneau, 1984). In the study with small fish, infusion of labeled amino acids had to be replaced by an immersion technique. This resulted in an extremely slow labeling of protein, which did not reach a plateau during 30 hr, although the acid-soluble fraction, the equivalent of a precursor pool for protein synthesis, stabilized at 8–10 hr. Houlihan *et al.* (1992) used smaller juveniles of another species of cyprinid larvae, nase, weighing 11 mg, and the protein synthesis rate in the whole animal at the highest growth rate amounted to 35%/day. The rate of protein synthesis in fish larvae switching into exogenous feeding may differ depending on the species and inherent growth rate, and the efficiency of protein retention may not come close to that in the phase of endogenous feeding, when the digestive tract is not functional. The efficiency of protein synthesis [retention, over 50% (Houlihan *et al.,* 1992)] demonstrates that, despite the cost of protein synthesis being three times higher than the minimal theoretical costs in larval fish, a high growth rate is the paramount objective.

Protein synthesis rates in true larval fish 0.5–1 mg in body weight need to be analyzed, but the data already available for juveniles showed that the protein synthesis rate is more than 60 times higher in warmwater juvenile carp than in coldwater rainbow trout [4–5%/day (Fauconneau and Arnal, 1985)]. In catfish embryos utilizing yolk reserves through endocytosis and cathepsin hydrolysis, the whole-body protein synthesis rates amounted to over 130%/day and the major reason for high growth rates of embryos seems to be a high protein retention efficiency, 70% (Conaicao *et al.,* 1997). The cost of protein synthesis in endogenously feeding embryos was close to the theoretical minima.

6.3.1.2. Cost of Protein Deposition

Rainbow trout hepatocytes used 79.7% of their total oxygen consumption on cyclohexamide-sensitive protein synthesis (Pannevis and Houlihan, 1992). The authors interpreted this as a high value in comparison to other studies on mammals. If the energy expenditure for the maintenance of Na/K transport is added, only 17.5% of the oxygen consumption in liver is devoted to gluconeogenesis and lipogenesis. The measured cost of protein synthesis in trout hepatocytes at 10°C was 223–830 mmol ATP/g protein, whereas with cyclohexamide (inhibitor) it was 670–3500 mmol/g. With an increasing growth rate and protein synthesis, the energetic cost declines to a minimum value of 670 mmol ATP/g; that is still 10-fold higher than in mammals.

6.3.1.3. A New Approach to Protein Deposition Estimation

Meyer-Burgdorff and Gunther (1995) used ^{15}N-labeled protein and assumed that when nitrogen, excreted as ammonia, becomes constant (21 hr in warmwater common carp), the metabolic pool of amino acids is stabilized. When excretion rates are stable, no recycling will take place (ammonia released during transamination is used mostly in the reductive amination by glutamate dehydrogenase, a mitochondrial enzyme in the liver), and this period, 21–72 hr, can be used for measuring conditions of protein/energy utilization.

The efficiency with which synthesized protein was retained as protein gain in carp was fairly low, 23.8–26.6% at the optimal protein/energy ratio, in comparison to the 38 to 62% obtained by Mathers *et al.* (1995) in measurements in juvenile rainbow trout, which used a ^{14}C-labeled phenylalanine injection method. Meyer-Burgdorff and Gunther (1995) were the first who provided data on the actual protein intake and proportion of protein synthesized. In the case of a low or a high protein–energy (PE) ratio, common carp synthesized 300 and 200% of the protein intake, respectively. A low PE ratio enhanced protein synthesis, which doubled in comparison to that at the optimal PE, but proportionally increased protein degradation (90% of protein synthesized). This is the best demonstration thus far of the importance of providing fish with an energy source that spares the use of amino acids. This also demonstrates how imperfect the method of amino acid "flooding" is for measuring protein synthesis.

Meyer-Burgdorff and Rosenow (1995a) combined protein synthesis measurement using ^{15}N-labeled casein with protein absorption (direct method), oxygen consumption, and ammonia excretion. They concluded, based on data derived from both conventional N-balance measurement (indirect digestibility coefficient, carcass composition) and the ^{15}N technique, that fecal ^{15}N collection was unreliable, whereas other elements of the N budget corresponded consistently. According to these measurements, energy loss due to excretion (22%) and heat dissipation (40%) as a part of the digestible energy are very high, in contrast to claims made with respect to highly efficient ammoniotelic animals (Smith *et al.,* 1978). In the follow-up study Meyer-Burgdorff and Rosenow (1995b) estimated that heat dissipation attributed to protein synthesis in common carp varied between 23 and 52% depending on the PE ratio, with a clear indication that diet formulation based on low protein (possible deficiencies in essential amino acids) tends to drive the cost up.

The high proportion of protein degradation is most likely resulting in the selective loss of some essential ureo- or ammoniogenic amino acids, such as arginine and histidine, and an increased rate of protein turnover.

Arginase in salmonids, splitting arginine into urea and ornithine, has been marked as one of the most active amino acid metabolizing enzymes (Walton and Cowey, 1982). However, ammoniogenesis from arginine has to be monitored in both mitochondria and cytosol to account for the total metabolite production (Chew and Ip, 1987). In mudskippers examined by the latter authors, ammonia is not converted to glutamine by glutamine synthetase, whereas in juvenile rainbow trout this may be the important step in ammonia detoxification (Wright *et al.,* 1995). Urea synthesis in juvenile salmonids has been reported by the same authors, and this essentially means that arginine is a dispensable amino acid in the early ontogeny of salmonids and other ureogenic fish (Anderson and Walsh, 1995). It is certainly an area of major interest for nutritional biochemistry.

Amino acidemia and a higher rate of ammonia excretion were also felt to be responsible for a decrease in utilization of diets based on protein hydrolysates in comparison to a complete fish meal (Langar *et al.,* 1993). These authors were the first to demonstrate that replacement of 50% of fish meal with partially hydrolyzed or unbalanced proteins led to an increase in protein synthesis of 29 and 60.8%, respectively, in the juvenile sea bass (*Dicentrarchus labrax*). However, an even more interesting finding was an increase in whole-body protein degradation in fish fed modified dietary formulations, which resulted in a decreased efficiency of protein deposition and, consequently, a decrease in growth rates.

6.3.2. Dietary Effects on Amino Acid Utilization

Simultaneous availability of all essential amino acids, assuming an excess of nonessential amino acids, at the site of protein synthesis is required for efficient protein synthesis. The initiating enzymes of protein synthesis (acyl synthetases) have a high affinity for amino acid substrates (low K_m values; 16^{-3}–10^2 m*M*) in comparison to enzymes which catabolize amino acids (K_m, 1–10 m*M*) (Walton and Cowey, 1982). If these values are compared to the apparent K_m values for the major detoxifying enzyme in fish liver and muscle, glutamate dehydrogenase (100 and 35 m*M* NH_4^+) (Iwata *et al.,* 1981), the picture seems to emerge that as the postprandial level of amino acids increases [10–40 m*M* in blood plasma (Ogata and Arai, 1985)], massive deamination occurs with or without urea synthesis. Consequently, because of the mitochondrial location of GDH and highly efficient transfer of ammonia from the subcellular fraction to the cytosol (Chew and Ip, 1987), this process may differ considerably from the *in vitro* scenario, where 60% of the glutamate in trout liver mitochondria was metabolized to α-ketoglutarate (Walton and Cowey, 1982).

In vitro rates of amino acid absorption in Atlantic salmon intestinal preparations differed without any apparent trend in substrate or regional location, pyloric, mid-, or posterior intestine (Bakke-McKellep *et al.,* 2000). Furthermore, apparent affinity constants (K_m) ranged from 0.1 (leucine) to 27.3 (aspartate) m*M*/liter. More dramatic differences in absorption of individual amino acids become apparent when linear absorptions were calculated, ranging five orders of magnitude in value. This must be a reflection of the nutritional history of the fish, the characteristics of the amino acid, and, among other factors, probably most important, the number of transporters per unit of intestine.

The postprandial distribution and concentrations of amino acids vary with time, fish species, and individual amino acid, but in general the level in diets is highly correlated with the blood levels (Dabrowski, 1982). Consequently, blood or tissue concentrations of amino acids proved useful in determining the dietary requirement for essential amino acids. Walton *et al.* (1986) examined the effect of the concentration of supplemented synthetic amino acids on their level in tissues, oxidation as expired carbon dioxide (Fig. 6.12), and activities of major catabolizing enzymes in liver. The fish failed to increase activities of tryptophan pyrrolase, lysine α-ketoglutarate reductase, and arginase, respectively. This contrasted with the enhanced production of CO_2 as the enzyme activities optimal for maximum growth of fish were surpassed (Fig. 6.12). Changes in dietary levels of lysine and arginine resulted in only a trend of corresponding changes in tissue levels. In contrast to rainbow trout, large Atlantic salmon responded with an almost-linear increase in blood plasma and muscle concentrations of free arginine to a dietary increase in synthetic arginine from 0 to 2.08% (Berge *et al.,* 1997). Liver arginase activity did not indicate an increase in catabolism of the supplemented amino acid, although the product of hydrolysis, the ornithine concentration in plasma, increased 10-fold in fish supplemented with 2.08% arginine in comparison to controls.

Espe *et al.* (1992) examined the effect of the molecular weight of the protein source (the proportion of combined peptides 2–25 kDa in size amounted to 18.7, 43.5, and 62.8% of the total protein) on salmon growth and protein synthesis. As the proportion of small peptides (5–30 amino acids) increased, protein synthesis in muscle decreased by 1.5- to 2-fold, with a correspondingly significant decrease in growth rate. The authors concluded, based on the retention of lipids in the fish body, that utilization of hydrolyzed protein requires an increased level of dietary energy to prevent utilization of free amino acids to cover anabolic needs. Walton *et al.* (1982) stressed, however, that in rainbow trout fed intact proteins or an equivalent amount of free amino acids, a comparison of lipid and protein in the liver has to be made for an equal size of fish to be meaningful, and

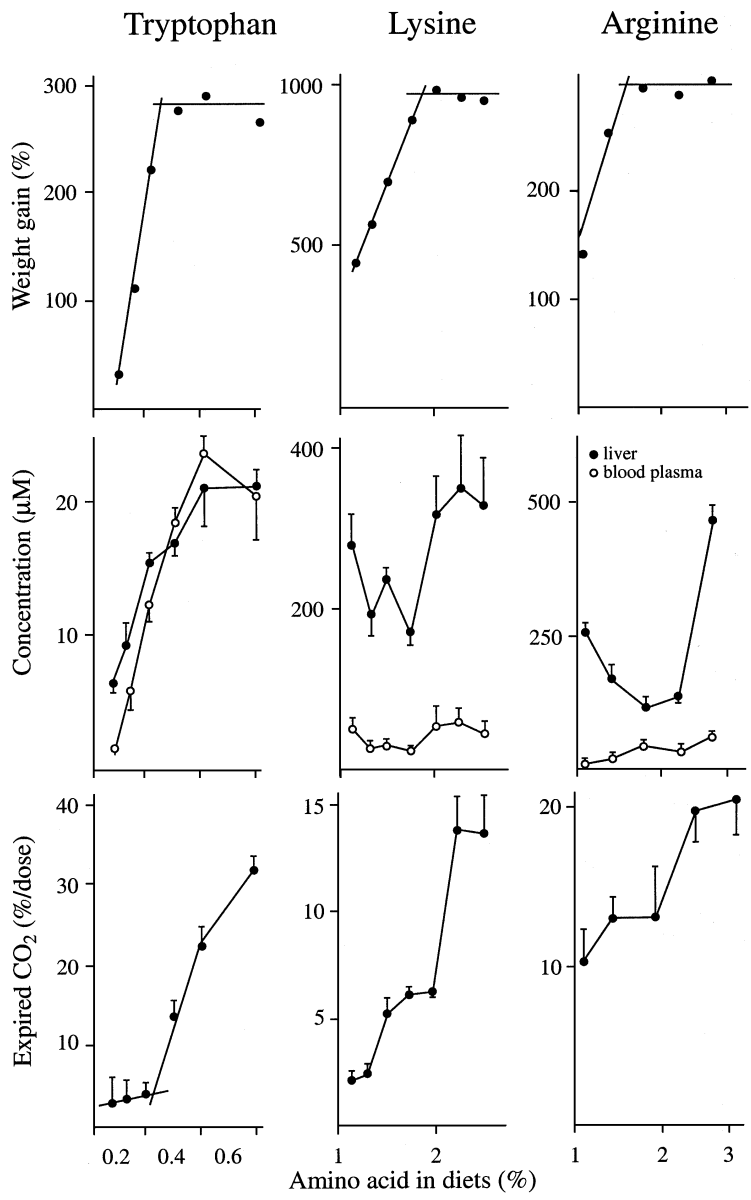

FIG. 6.12

Graphical presentations of the responses in fish weight gain, concentration of free amino acids in tissues, and expired CO_2 after injection of radiolabeled amino acids versus the dietary amino acid concentration (Adapted from Walton *et al.*, 1986, with kind permission from Kluwer Academic Publishers).

in this case larger livers in fish fed free amino acid-based diets will clearly indicate increased levels of glycogen.

6.3.2.1. A "Metabolically" Optimized Amino Acid Diet Formulation

In many instances, a comparison of interspecies differences of amino acid requirements in fish exceeded credibility. For example, tryptophan requirements of rainbow trout ranged from 0.5 to 1.4% and methionine requirements in salmonids were estimated as from 2.2% in rainbow trout to 4.0% in chinook salmon (Akiyama *et al.,* 1997). The questionable formulation of test diets to secure acceptability and maximize growth of fish is the single, most important factor resulting in discrepancies observed in published amino acid requirements. Therefore, we concentrated on a critical review of diet formulation used in the amino acid requirement experiments and attempted to determine the "metabolic causes" that resulted in growth responses to graded levels of particular indispensable amino acid.

Diet formulations for protein and indispensable amino acids requirement are based entirely, or in large proportion, on free, synthetic amino acids. These diets seek to maximize the growth rates of fish to be comparable to those attained on diets based on proteins. It was realized early that protein hydrolysates (Aoe *et al.,* 1974) and amino acid mixture diets (Aoe *et al.,* 1970b; Murai *et al.,* 1981) were greatly inferior in supporting growth in common carp and tilapia (Mazid *et al.,* 1978) in comparison to casein-based diets. Rainbow trout fed diets containing protein/amino acid mixtures grew more slowly than fish fed complete protein diets (Cowey and Luquet, 1983). Cowey *et al.* (1992) used a 20% amino acid mixture in the experimental diets formulated for a methionine requirement study in rainbow trout and reported markedly inferior growth in comparison to that on an all-protein, control diet. This study was exemplary because of the duration of the experiment and more than 10-fold increase in fish weight to the estimate optimum requirements for methionine. Less convincing is a variable amount of food eaten by different groups, resulting in a confounding effect on fish growth of food intake in the lowest methionine treatment.

Quantitative requirements for the 10 indispensable amino acids for growth in fish should be reported as percentage of the dietary protein and be representative of the near-maximum growth rate. However, many data sets used for comparisons of individual amino acid requirements among fish were collected under rather unfavorable conditions of inferior growth rates due to inadequacy of amino acid test diets. For instance, the amino acid requirement for common carp is based on data where in many cases fish only doubled their body weights and/or growth rates were less than 2% per day in individuals 0.5–4 g in body weight (Nose 1979). Ravi and Devaraj (1991) determined amino acid requirements in Indian carp (*Catla catla*) using a

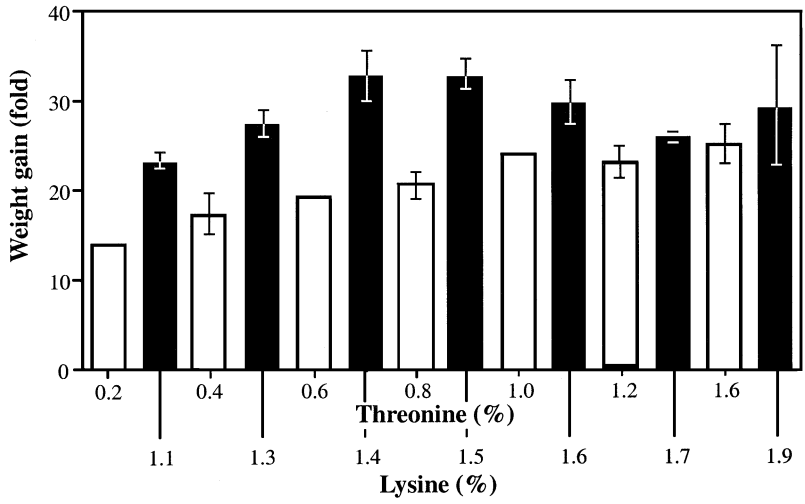

FIG. 6.13

Increase in body mass of juvenile Nile tilapia (40- to 50-mg individual weight) in response to varying dietary levels of threonine and lysine (Santiago and Lovell, 1988). Threonine diet (6.5% protein, 23.04% free amino acids); lysine diet (18.15% protein, 14.05% free amino acids).

diet containing a 40% amino acid mixture and weight gains were two- to fourfold the initial body weight. More importantly, weight gains abruptly declined after a maximum weight gain, clearly indicating that the fish "tolerance" for the particular amino acid was exceeded. The essential nutrient became toxic at increasing dietary concentrations.

Santiago and Lovell (1988) present an illustrious example of an amino acid requirement study in Nile tilapia where (1) the growth rate was nearly maximal, (2) the study was of sufficient duration, resulting in a body weight increase of 15- to 35-fold, and (3) no decline in weight gains was observed after a peak of response was reached (Fig. 6.13). However, the latter study was performed using diets containing 5.5–18.15% of a casein–gelatin base. The growth response seemed to be related partially to the increase in protein base.

Ng *et al.* (1996) added white sturgeon to a list of species almost-incapable of utilizing a diet based on 51% synthetic amino acids. Dietary pH adjustment to a neutral value was not helpful in sturgeon as demonstrated earlier in common cap (Nose *et al.*, 1974) and channel catfish (Wilson *et al.*, 1977). Diet neutralization in the sturgeon study, however, significantly decreased the urinary excretion of free amino acids. Although not directly

demonstrated in sturgeon, the authors concluded that amino acids absorbed were excreted via gills as shown earlier in common carp (Murai *et al.,* 1984). Increased catabolism of amino acids ingested in diets based on synthetic amino acid mixtures was clearly not the case when ammonia excretion rates were measured in comparison to those on a casein-based diet (Kaushik and Dabrowski, 1983).

It therefore becomes intriguing that amino acids infused into the dorsal aorta of rainbow trout (Kaczanowski and Beamish, 1996) or injected into the caudal aorta of channel catfish (Brown and Cameron, 1991a,b) induced increases in oxygen consumption mimicking an elevated metabolism. Thus, an elevated metabolism is not always associated with the amount or the composition of nutrients ingested. Mixtures which were deficient in essential amino acids or unbalanced with respect to optimum requirements, resulted in a higher oxygen consumption than infusions of amino acids of profiles equivalent to those of fish body proteins. Brown and Cameron (1991b) suggested, based on the use of the protein synthesis inhibitor, cyclohexamide, that an increase in oxygen uptake was correlated with the protein synthesis rate. In catfish, ammonia excreted following amino acid infusion constituted 21% of the amino acid nitrogen (Brown and Cameron, 1991a), whereas in common carp fed amino acid mixture diets, metabolic loss constituted only 6% of the dietary nitrogen source (Kaushik and Dabrowski, 1983). This comparison, with all interspecies limitations, demonstrates that amino acid catabolism is elevated in fish fed "parenterally."

Both studies with catfish and trout infused with amino acids suffered from several physiological artifacts which make the findings uncertain. First, infusion of only amino acids, without other nutrients, minerals, and vitamins, may profoundly affect transport and metabolism. Second, infusion of amino acid into the dorsal aorta (trout) or caudal artery (catfish) by bypassing receptors on the absorption site of digestive tract and liver (Holstein and Haux, 1982) must have sent some awkward neurosignals to the central nervous system, affecting the compartmentalization of blood flow (Axelsson and Fritsche, 1991; Thorarensen *et al.,* 1993), motoricity, and secretory functions of the digestive tract. Despite these shortcomings, experimentation on the whole fish, with further refinement of the method, should be used to address the metabolism of amino acids.

The strong statement by Aoe and Saito (1970) that "salmonids show no difference in growth between the casein-gelatin diet and corresponding amino acid test diet" requires further investigation. For instance, Barroso *et al.* (1999) observed that rainbow trout fed diets with a 15% amino acid mixture replacing fish meal on a weight basis showed a significant decline in body weights. An increase in the frequency of feeding from twice to four times daily improved fish growth on these experimental diets, whereas it did

not have any effect on the growth of fish fed controls diets. Rodehutscord *et al.* (1995a) declared that "free amino acids can replace protein-bound amino acids" in diets for rainbow trout. However, their conclusions do not seem to be warranted, as supplementation of essential amino acids at 2.6 and 4.0% did not restore weight gain to their respective controls (experiment A). The results of experiment A are also questionable because glutamic acid was withdrawn from diets with a lower protein content. Schuhmacher *et al.* (1995) demonstrated that the source of nonessential amino acids has a highly significant effect on rainbow trout growth. In experiment C of Rodehutscord *et al.* (1995a), where fish meal was completely replaced by wheat gluten and supplemented with essential amino acids, the level of protein was decreased to 30–31%. Only under these conditions of dietary protein levels below the optimum for growth did the fish final weight between treatments not differ significantly. Experiments with 12% wheat gluten replacement as a protein source for indispensable amino acids (4%) and dispensable amino acids (11.4%) resulted in body weight gains that did not differ among treatments. However, daily growth rates in this study, 0.8% per day, were inferior to those normally occurring in 40- to 50-g rainbow trout [4.1% (Bassompierre *et al.*, 1998)]. These findings are not sufficient to generalize that dietary free amino acids do not negatively affect the efficiency of utilization of amino acids for growth in comparison to protein-bound amino acids. In retrospect, the results obtained by Rodehutscord *et al.* (1995a) may be considered as disappointing and of limited use in practical or experimental diet formulations. Ogata *et al.* (1983) demonstrated that replacement of casein with 4.47% essential amino acids on a mass basis resulted in growth enhancement by 18–21% in two juvenile salmonids. Supplementation of a casein-based diet with six essential amino acids (total of 2.38%) highly reduced mortality and more than doubled the final body mass of juvenile Atlantic salmon (Rumsey and Ketola, 1975). In conclusion, it seems that more studies are required to optimize the ratio of synthetic amino acids to protein in high-nutrient diets aimed at examining requirements for individual amino acids.

Studies that address the mechanism of amino acid absorption and transport at the digestive tract level *in vivo* add substantially to the understanding of amino acid utilization and the way they must be presented in formulated diets. A unique feature of double capillarization of the venous system in teleost fish allowed Murai *et al.* (1987) to examine concentrations of amino acids in the hepatic portal vein, reflecting the level derived from intestinal absorption, and then in the hepatic vein when a decline due to absorption in liver can be demonstrated based on comparisons to the level in the previous location. They reported that concentration differences resulting from liver absorption (or protein synthesis) of essential amino acids were

consistently higher in fish fed a casein-based diet rather than a diet composed of 38% synthetic amino acids between 3 and 12 hr after force-feeding. This would actually mean that "flooding" of the major protein synthesis site in rainbow trout is much more severe in fish fed a casein-based diet. This would contradict a major argument harbored in the literature of impaired utilization for protein synthesis of dietary synthetic amino acids in comparison to dietary protein-bound amino acids. This difference between dietary treatments in liver absorption of circulating amino acids extends for many passages of blood through a portal liver system (assuming that 2 hr is needed for complete equilibration of the blood level). In effect, there was no evidence of extensive hepatic catabolism of amino acids leading to excretion of ammonia into the hepatic blood vessel in rainbow trout fed an amino acid mixture diet. In the arteriovenous blood collected in the caudal vessels, ammonia concentrations were also not different between the two diets (Murai *et al.,* 1987). Murai *et al.* (1984) reported that 36% of the total N excretion was in the form of free amino acids. Alternatively, Murai and Ogata (1990) found that the injection of exogenous insulin reduced free amino acid concentrations in tissues of common carp and suggested accelerated synthesis and deposition of protein. However, they did not provide evidence in terms of fish growth response to support this hypothesis.

These data demonstrate the high efficiency of amino acid uptake in the liver. It would not be unreasonable to assume that in fish, as in alligators (Coulson *et al.,* 1990), essential amino acid removal from body fluids is accomplished severalfold more rapidly by incorporation into synthesized proteins than by catabolism.

Ash *et al.* (1989) and McLean and Ash (1989) described another approach to analyze net absorption of amino acids in the digestive tract of fish, where the difference in concentrations between the dorsal aorta and the hepatic portal vein multiplied by the blood flow rate would provide a direct estimate of absorption. The decrease in nonessential amino acids (glutamic acid and glycine) was 10-fold between arterial blood and hepatic venous concentrations (see also Table 6.1), whereas the decreases in lysine, arginine and methionine concentrations were much more moderate, 2- to 4-fold. This method may provide additional information on postabsorptive handling of amino acids if strengthened by data related to changes in blood flow.

Espe and Njaa (1991) experimented with 0.88-g Atlantic salmon offered a diet based exclusively on free amino acids (50.2%) along with a diet based on fish meal and found that the body weight gain was fivefold lower on the former diet. They concluded that free amino acid leakage could be partly responsible for impaired growth. Yamada and Yone (1986) gave some more

Table 6.1

Concentrations of Amino Acids in Blood Plasma of Rainbow Trout[a]

Amino acid	(1)	(2)	(3)	(4)	(5)	(6) G	(6) AA
Lysine	747	121	824	457	318 (4)	331.26 (6)	361.3 (3)
Arginine	262	65	236	241	65 (4)	413.8 (24)	481.0 (9)
Methionine	252	10	121.1	202	328 (12)	234.5 (18)	264.8 (15)
Leucine	672	126	622.6	571	814 (12)	1,615.1 (18)	1,360 (15)
Hydroxyproline	—	69	42.4	—	—	—	—
Proline	587	34	810.3	—	760 (4)	—	—
Glycine	894	397	523.9	2.338	929 (12)	1,800 (12)	2,120 (9)

[a] Under different conditions after feeding and different diets: (1) Yamada *et al.* (1981)—dorsal aorta, 6 hr postprandially, amino acid (AA) mixture, 10°C; (2) Ogata and Arai (1985)—caudal vein–artery, commercial feed; (3) Murai *et al.* (1987)—hepatic portal vein, 3 hr postprandially, AA diet; (4) Ash *et al.* (1989)—hepatic portal vein, 3 hr postprandially; (5) Walton and Wilson (1986)—caudal vein, maximum postfeeding in parentheses (hr), casein; and (6) Schuhmaeher *et al.* (1997)—heart, gluten (G)- or AA-based diet (AA), maximum in parentheses (hr).

evidence to the "leakage hypothesis" with respect to the use of an amino acid mixture diet. In common carp, fish that masticate food by pharyngeal teeth, loss of water soluble amino acids may reach 60% and contribute to the loss of other dietary nutrients.

The research group of Berge *et al.* (1994) compared diets containing a 13.4% amino acid mixture with free lysine versus protein-bound lysine in Atlantic cod. Incorporation into muscle protein was threefold higher in the case of the latter diet, indicating that free amino acid supplements are catabolized extensively, in contrast to amino acids released after hydrolysis. This is most likely the same mechanism that governs incorporation of hydrolyzed algal protein ^{14}C-labeled amino acids into the systemic blood of rainbow trout (Cowey and Walton, 1988). These authors reported a higher activity and an earlier peak of amino acids in the acid-soluble fraction when delivered as hydrolyzed algal protein. Interestingly, the "dietary protein history" did not influence the rate of amino acid incorporation into protein from either protein- or free amino acid-containing diets. Therefore, it appears that (1) the proportion of free amino acid mixture to protein in a diet formulation and (2) the protein source are critical for utilization of amino acid supplements. Espe and Lied (1994) argued that the inclusion of 20–30% free amino acid in the total protein (or a 14% absolute amount)

was the optimal ratio for salmonid fish growth. However, this conclusion may be quite speculative. First, the protein source was fish muscle meal devoid of the peptide and free amino acid fraction. In this context, the original composition of fish meal was merely reconstituted in a diet supplemented with "amino acids mixtures of identical composition" (Espe and Lied, 1994). Second, the growth studies lasted only 2 weeks, with weight gains of 10–20% of the initial value. This reservation is reinforced by the results of Ogata *et al.* (1983), who demonstrated that salmon juveniles fed a casein diet supplemented with a mixture of 3.37–4.37% of essential amino acids performed significantly better than fish fed a fish meal-based diet of equal protein contents.

Schuhmacher *et al.* (1995a) addressed the question of possible advantages of supplying the dispensable amino acids in the "free" portion of the dietary protein over providing a single amino acid (e.g., glycine) which would satisfy all amino nitrogen needs for synthesis of other nonessential amino acids. In rainbow trout of 48-g individual weight, glutamine was proven to be superior to glycine and glutamic acid as a source of nitrogen. However, fish less than doubled the body weight during the 84-day experiment. Assessment of these results with less than adequate growth rates (0.13–0.3% per day) is somewhat difficult and most likely a consequence of using a pure synthetic amino acid mixture diet, in some cases supplemented with 30% glutamic acid. Glutamate incorporation into the protein fraction in rainbow trout is severalfold less than that of acetate (Fauconneau *et al.,* 1989), and in fact, glutamate dietary toxicity was suggested in these fish (Hughes, 1985). In tilapia diets, supplements of 9.5% glutamate or an equivalent mixture of six amino acids did not result in significant improvements in the growth rate (Mambrini and Kaushik, 1994). Therefore, other nonessential amino acids which are at high concentrations in the postprandial state and low during fasting, such as alanine, glycine; and proline, need to be examined in diet formulations.

6.3.3. Pools of Free Amino Acids

The function of free amino acids in aquatic animal behavior, communication, and metabolism begins in their sensory organs, where amino acids serve as chemical signals (Saglio *et al.,* 1990). For instance, proline was the most effective gustatory amino acid in rainbow trout at an estimated threshold of 10^{-7} M (Kohbara and Caprio, 2001). In marine fish, particularly during embryonic stages with endogenous nutrition, amino acids provide stability in body fluid osmolality and serve as substrates for protein synthesis and/or aerobic catabolism (Ronnestad and Fyhn, 1993; Ronnestad *et al.,* 1993; Sivaloganathan *et al.,* 1998). Extraoral utilization of protein and amino

acids in fish embryos through endocytosis and cellular proteases activity is markedly different from that found during exogenous feeding, and therefore, comparisons must be drawn with caution. Conceicao *et al.* (1998) quantified differences in amino acid contents between the time of fertilization and the time of complete yolk protein absorption and suggested a retention efficiency of 50–80% for essential amino acids, with the free amino acid pool being quantitatively unimportant (not exceeding 5%).

Body proteins cannot be stored in major quantities and are continuously renewed through degradation and synthesis. The free amino acid pool changes in its profile (composition) and concentrations depending on the tissue (Carter *et al.,* 1994), frequency and time after feeding (Tantikitti and March, 1995), temperature and food (Knapp and Wiser, 1981), and salinity (Dabrowski *et al.,* 1996; Auerswald *et al.,* 1997).

Free amino acid concentrations have frequently been used to monitor the postprandial response in fish, however, the relative distribution between plasma and red blood cells was not measured. It became evident that essential amino acids tend to be concentrated in the plasma compartment (more than 55%), with some, such as lysine, showing a significant decrease in concentration during the postabsorptive stage (Fig. 6.14). Nonessential amino acids tended to be concentrated in the red cell compartment of the blood. However, aspartic acid has shown an increase in blood plasma partitioning from 10 to 85% in the postabsorptive stage. Therefore, these observations indicate the significance of separating plasma and cellular free amino acids in the circulation for transport and metabolic purposes.

Differences in amino acid availability are reflected in the plasma amino acid concentrations (Tantikitti and March, 1995), however, feeding at 3- to 6-hr intervals significantly eliminates fluctuations. Maximum plasma concentrations for essential amino acids were obtained in, for instance, in salmonids, between 4 and 24 hr, with dietary proteins as varied as fish meals, casein, corn gluten, and soybeans (Walton and Wilson, 1986). Besides the effect of diet, the location of blood sampling has a profound effect on amino acid concentrations in blood plasma (Table 6.1). Schuhmacher *et al.* (1997) emphasized the nutritional history and duration of fasting prior to an experimental meal to follow postfeeding changes in plasma free amino acids. Plasma concentrations attained their peak at 9 hr postfeeding for most essential amino acids in trout fed a synthetic amino acid diet, earlier than the 12–18 hr in fish fed wheat gluten as the intact protein source (Table 6.1).

The best correlation between essential free amino acids in the hepatopancreas or blood plasma and dietary amino acids occurred 4 hr after feeding ($r = 0.914$ and 0.896, respectively) (Ogata, 1986). There was no correlation between dietary levels and concentrations of free amino acids in the liver

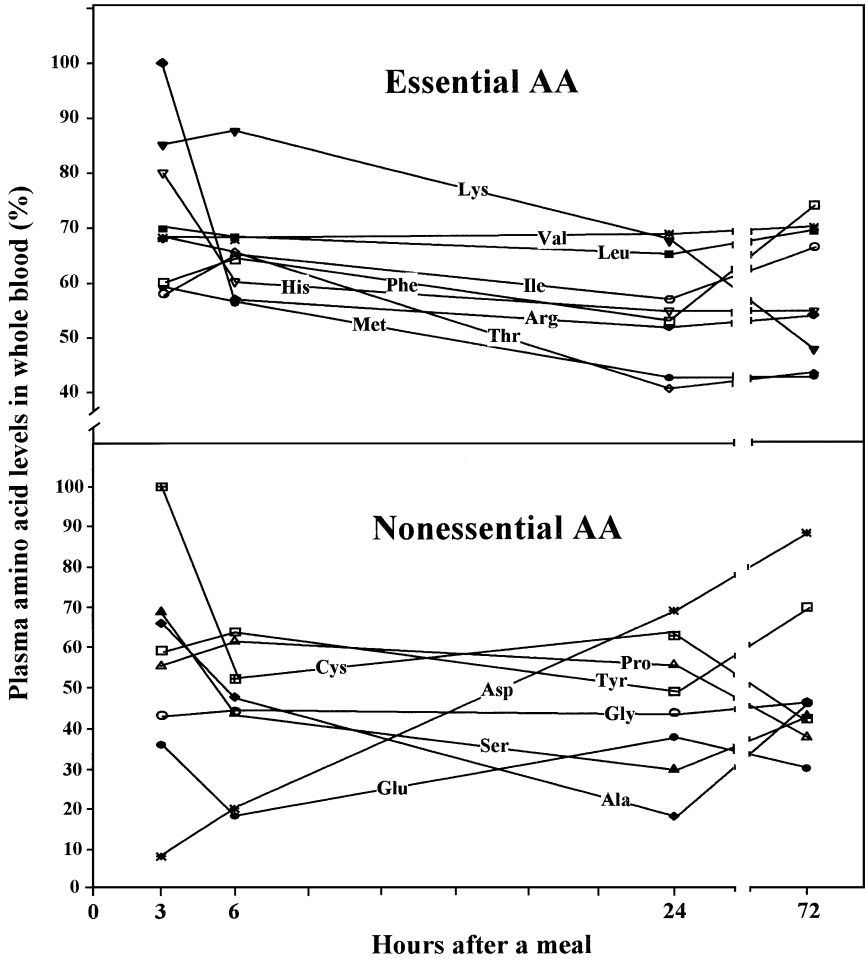

FIG. 6.14

Postprandial changes in the blood plasma free amino acid levels expressed as a percentage of the whole-blood levels (Dabrowski, 1982).

of rainbow trout (Murai *et al.*, 1987). On the contrary, in rainbow trout the best correlation ($r = 0.849$) between the total free amino acid concentrations in the liver and the dietary consumption of amino acids was found 4 hr after a meal (Carter *et al.*, 1994). However, essential free amino acids in the liver did not correlate at all. The authors calculated that over 83% of amino acids were recycled to the general pool after protein breakdown,

therefore in starved rainbow trout only a portion of the free amino acid pool (53%) is used for protein synthesis. Someone may argue that a single meal after 7 days of fasting in rainbow trout is probably weak evidence of a free amino acid pool in fish which otherwise feed actively. Thus, it seems hazardous to conclude that there is a "consistency of free amino acid concentrations in tissues following feeding" (Carter *et al.,* 1994). It appears of great interest to find out how "the anabolic drive," an increase in protein synthesis following an influx of absorbed free amino acids, is regulated in light of a possible "shutting-off effect" which may occur with the "flooding" of orally (or injected) administered free amino acids.

6.3.4. Ammonia and Urea as End Products of Protein Catabolism

Production of ammonia requires little energetic expenditure but potentially results in accumulation of a toxic compound. Release of ammonia into the surrounding water or detoxification at the site of liberation of aminonitrogen from amino acids must follow shortly after absorption of amino acids in the digestive tract and delivery to the liver. Mommsen and Walsh (1992) provided some indications that ammonia elimination from organs other than the liver in eel may be as important. The ratio of urea production via the ornithine–urea cycle (OUC) to uricolysis is about 100 to 1 in fish more ancient than teleosts, whereas bony fish form most urea via uricolysis (Randall and Wright, 1987). There are, however, an increasing number of exceptions to this generalization. Production of urea in teleosts as a compound less toxic than ammonia is limited, therefore, ammonia is typically eliminated from the blood during its first passage through the gills. As over 70% of ammonia is produced from protein catabolism in the teleost liver (Cowey and Walton, 1989), multiple mechanisms operate at the gill site to accelerate elimination. (1) The endogenously produced ammonia in gill tissues is eliminated via nonionic diffusion (Avella and Bornacin, 1989). Mommsen and Walsh (1992), however, suggested that the "gill merely serves as the prevalent excretory site." (2) The ionic diffusion of NH_4^+ allows the fish to maintain a large negative gradient against passive diffusion of ammonia. And (3) active $NH_4^+H^+$ exchange in gills in freshwater fish and NH_4^+/Na^+ exchange, predominantly, in seawater fish (Wilson and Taylor, 1992) are quantitatively the most important means of elimination of ammonia. Fish reared in water low in Na (1–1.6 μg/liter) and K (0.4–0.7 μg/liter) displayed signs of ammonia toxicity, edema, and fusion of gill lamellae in comparison to controls reared in water with higher sodium and potassium (20–30 and 5–5 mg/liter, respectively) (Bradley and Rourke, 1985). However, there are exceptions, as in the embryonic rainbow trout exposure to Na-free water did not alter the ammonia excretion rates. Exposure of the same embryos to 1.6 m*M* NaCl,

in comparison to 1 m*M* in controls, resulted in a twofold increase in ammonia excretion (Rahman-Noronha *et al.,* 1996).

As mentioned earlier, Wright *et al.* (1995) observed an active OUC in embryos and alevins of rainbow trout. Furthermore, as in early stages of rainbow trout ontogeny up to 26% of nitrogenous waste was excreted in the form of urea, this figure was not considerably different from those during embryonic or endogenously feeding larval stages of common carp (Kaushik *et al.,* 1982) and whitefish (Dabrowski *et al.,* 1984). In common carp evidence was presented that during hatching there was no concomitant increase in urea and ammonia excretion; the authors interpreted this as suggesting that urea production was not related to ammonia detoxification processes. Urea excretion rates frequently reached 50% of the total nitrogen excreted in common carp embryo, larvae, and juveniles, and clear diurnal changes were demonstrated (Kaushik *et al.,* 1982; Kaushik and Dabrowski, 1983a,b). The system with specially designed flowthrough metabolic chambers and continuous monitoring of metabolites used in the latter study has been immensely advantageous in comparison to closed systems, where water stagnation and frequent handling of embryos or larvae are required and may contribute to may artifacts. Chadwick and Wright (1999) used small-volume open chambers to monitor urea and ammonia excretion in Atlantic cod and their results suggest that a high proportion of nitrogen is excreted in the form of urea (50–100%). Curiously, no association was found among urea excretion and arginase and mitochondrial carbamoyl phosphate synthetase (CPS III) activity.

Pilley and Wright (2000) were able to demonstrate that urea excretion was inhibited by phloretin, a urea transport inhibitor, and by urea analogs such as thiourea and acetamide. This is strong evidence of facilitated urea transport in rainbow trout embryos and alevins. In adult rainbow trout fed diets supplemented with 1 or 3% urea, the compound was completely absorbed in the digestive tract and then 7–9 hr after a meal the peak urea excretion occurred. No metabolic cost was associated with absorption and/or excretion of urea, predominantly via gills, based on the postprandial oxygen consumption (Kaushik *et al.,* 1983).

Terjesen *et al.* (1997) measured urea excretion in a freshwater species, African catfish, and indicated a possible reliance on urea synthesis in the endogenous feeding phase as a means of eliminating toxic nitrogen products. In marine toadfish (Mommsen and Walsh, 1989, 1991) and freshwater tilapia, *Oreochromis alcalicus,* living in a very alkaline (pH 10) environment, *de novo* urea synthesis and elimination are the means of ammonia detoxification. Therefore, beside urea synthesis in OUC, the relative contribution of arginine degradation and the uricolysis pathway, present in all teleosts (Mommsen and Walsh, 1992), needs to be quantified. The concentration

of urea in nitrogen waste excretion was found to peak 4 and 8 hr postprandially in common carp juveniles fed zooplankton and formulated feed, respectively (Kaushik and Dabrowski, 1983a). High levels of lysine in the diet may reduce the degradation of arginine as indicated by blood urea concentrations (Berge *et al.,* 1998) and consequently affect urea excretion rates.

6.3.5. Metabolism of Individual Amino Acids

As demonstrated in Fig. 6.11, measurement of amino acid oxidation in the case of intraperitoneal injection frequently failed to identify a breakpoint in the dose–response curve for an increase in CO_2 production. Kim *et al.* (1992) argued that the fact that an increase in amino acid oxidation and the weight gain breakpoint response do not coincide is due to the delivery of amino acids intraperitoneally rather than fed in the diets.

6.3.5.1. Lysine

The lysine requirement was assumed to be high in fish based on the high concentration of this amino acid in fish muscle proteins (Berge *et al.,* 1998). However, estimates among species varied between 1.14% in hybrid striped bass (Keembiyehetty and Gatlin, 1992) and 2.22% in sea bass (Tibaldi and Lanari, 1991). Neither of these two studies can be considered conclusive, as only one treatment of five or six levels of supplemented lysine tested determined a breakpoint in the dose–response curve. In contrast to the "classical" response of lysine concentration in plasma of rainbow trout to dietary lysine levels (Fig. 6.12), the response in hybrid striped bass did not provide support for the requirement estimate. In the same hybrid, provided diets with identical protein levels (35%), fitting of weight gains to the appropriate model enabled determination of the requirement as 1.4% in hybrid striped bass (Griffin *et al.,* 1992). The serum lysine level pattern confirmed the dietary requirement estimate in this study. Ruchimat *et al.* (1997) also confirmed serum lysine concentrations to be a supplementary indicator of the lysine requirement (1.78%) in marine fish, such as the yellowtail.

Rodeshutscord *et al.* (2000) reported that rainbow trout fed diets with 55% crude protein grew more slowly than fish fed diets with 35% protein. Disproportionate levels of the essential compound, lysine, may have a more detrimental effect in a high-protein diet than in a low(er)-protein diet. Although these findings are intelectually stimulating, more detailed analysis of diet formulations revealed that two factors were changed simulataneously: first, the protein level and a proportion of synthetic amino acids. Second, an assumption was made that protein and free lysine are equally available. Based on fecal lysine concentrations, where almost all (free and protein-bound)

amino acid was absorbed (96–98%), however, trends in growth rates clearly indicated that diets formulated with a high proportion of purified gluten are not well utilized. The protein efficiency ratio, calculated as retention/intake, amounted to 40 and 23% for diets with 35 and 55% protein, respectively, for the same concentration of lysine (1.3% dry matter). The results in rainbow trout seemed to suggest that a greater proportion of free lysine in 35% protein diets allowed greater weight gains. This is exactly opposite to Zarete and Lovell's (1999) conclusions, where a comparison of free and protein-bound lysine in soybean meal was carried out in channel catfish. When channel catfish were fed to satiation two or five times a day in the latter study, weight gains were higher at the higher frequency, but utilization of free lysine did not improve significantly. In common carp, weight gains on free amino acid diets increased in proportion to the incremental frequency of feeding from 3 to 18 times per day (Yamada *et al.,* 1991b).

De la Higuera *et al.* (1998) used a supplement of free or albumin-coated lysine in the diet of common carp based on corn gluten, a protein naturally deficient in lysine. Fish fed with a coated-lysine supplement showed increased protein synthesis compared to fish fed a deficient diet. Coating lysine with albumin significantly improved the growth of common carp. In large Atlantic salmon, Berge *et al.* (1998) used a number of other biochemical indicators [subsidiary methods (Cowey, 1995)], such as blood and liver metabolites of lysine and antagonistic amino acid, arginine (ammonia, urea), and enzyme activities (arginase, ornithine decarboxylase). Berge *et al.* (1998) found an excellent response in blood lysine concentrations at a 1.8% dietary lysine level. Lysine supplement in feeds was also found to increase arginase activity in the liver. However, *in vitro* assay of salmon liver arginase activity indicated a linear inhibition of the enzyme by lysine concentrations up to 800 m*M*. This work needs to explore, however, the physiological range of arginine concentrations, which is much lower than that used in the assay (425 m*M*).

Forster and Ogata (1998) was one of the few reports confirming increased mortality in two species of marine fish fed diets with high protein levels (45-48%), deficient in lysine. No specific pathologies were described.

6.3.5.2. Arginine

Based on the relatively high activity of four of the OUC enzymes, Wright *et al.* (1995) concluded that urea synthesis in juvenile salmonids is an important mechanism of ammonia detoxification at this stage of fish ontogeny in dramatic difference to adults. A corresponding increase in arginase activity, however, may be responsible in large part for the increased urea excretion in juvenile rainbow trout after hatching. Adult trout, according to this study,

have markedly diminished activity of ornithine transcarbamylase (OTC) and glutamine synthetase (GSase) in the liver, which results in a lack of functional OUC (urea synthesis). However, juvenile rainbow trout of 4–10 g seemed to utilize ornithine or citrulline for growth as well as arginine (Chiu *et al.,* 1986). Arginine concentrations in blood plasma were severely depressed in fish fed arginine precursors (26–36 μg/ml) in comparison to fish fed a diet supplemented with arginine (60.6 μg/ml). Rodehutscord *et al.* (1995b) concluded that in rainbow trout fed a diet deficient in arginine, deposition of this semiindispensable amino acid exceeded the quantity provided in the diet. It is a reasonable assumption that other fish also have the ability to synthesize arginine. Buentello and Gatlin (2000) concluded that at 0.5% arginine in channel catfish diets, a glutamate supplement of 0.5% made a significant contribution to arginine synthesis and enhanced the growth rate of fish in comparison to a glycine supplement. However, without evidence of the presence and upregulation of OTC and mitochondrial carbamoyl phosphate synthetase (CPSase III) (Anderson and Walsh, 1995) activities in channel catfish liver in the condition of arginine deficiency, the hypothesis of arginine synthesis in catfish remains unproven. Alternatively, an improved lysine-to-arginine ratio in low-protein diets for catfish may explain the higher efficiency of arginine utilization and increase in blood concentrations observed by Buentello and Gatlin (2000).

Cho *et al.* (1992) performed two well-designed experiments (reference casein-based diets were included) of a long duration (12 weeks) with rainbow trout and concluded that weight gain and protein retention were maximized by an arginine concentration of 1.4% of the diet or 4.2% of the protein. The requirement derived from this work was substantially lower than those recommended thus far. In effect, this study may combine the range of rainbow trout sizes where synthesis of arginine is substantial (1–2 g) to that where it becomes negligible (20–30 g).

Lall *et al.* (1994) markedly expanded the number of biochemical indicators to derive the most physiologically sound arginine requirement value for large Atlantic salmon. In their study blood plasma concentrations of arginine, arginase activity in kidney, and CO_2 excretion following injection of radiolabeled arginine corroborated well with the growth rate data, further confirming the dietary requirement of 1.6% (4.1% of protein). Berge *et al.* (1997) also indicated, in large Atlantic salmon, that low levels of dietary arginine (1.13–1.68%) resulted in a significant increase in lysine and threonine in blood plasma and muscle. Although their study was of a relatively short duration and growth data were inconclusive, the arginine concentrations in blood plasma and muscle increased significantly when the dietary level of arginine was raised to 1.68%. The interactions of lysine and arginine during intestinal uptake strongly suggest dependence on a common carrier

(Berge *et al.*, 1999). A lysine concentration of 3.4 m*M* and an arginine concentration of 1 m*M* inhibited the uptake of each other by 60–80%. This is particularly relevant for synthetic amino acid diet formulations, where the concentration of lysine should be substantially higher to balance differences in absorption kinetics. Griffin *et al.* (1994) demonstrated, in hybrid striped bass, that with diets where lysine supplementation exceeded arginine supplementation by ratios of 1.35:1 to 2.26:1, there was no effect on blood serum arginine concentrations. However, lysine concentrations in serum were elevated in fish fed diets with an increasing proportion of dietary arginine, with no influence on fish weight gain. This does not seem to be sufficient to claim major interspecies differences in fish with respect to lysine–arginine antagonism.

Rodehutscord *et al.* (1995b) used diets supplemented with 14% glutamic acid (see the discussion of glutamate toxicity above) and, from the resulting low growth rates of rainbow trout (0.72%/day), estimated an arginine requirement of 1.16%. However, an inspection of their data on protein deposition suggested that only the group of fish fed the lowest arginine supplement, 1.5 g/kg, differed from the groups on the remaining 10 treatments (between 1.5 and 16.5 g/kg arginine supplements). In other words, there was no difference among 10 of 11 treatments. Therefore, it seems speculative to calculate an arginine requirement based on such a data set.

In juvenile hybrid striped bass, Griffin *et al.* (1994) estimated the arginine requirement as 1.5% (4.4% protein), and in a marine species, sea bass, Tibaldi *et al.* (1994) estimated 1.8% (3.9% of protein). The latter authors provided data corroborating a value of urea concentration in blood plasma regressed against dietary arginine content to determine the optimum level for growth and physiological status. There was no evidence, however, for a disparity of requirements for dietary arginine among freshwater and marine teleost fish. It is of interest that in larval diets containing 60% protein, Lopez-Alvarado and Kanazawa (1994) suggested a growth-promoting effect of 1.5% arginine supplement. This conclusion is difficult to reconcile with the fact that larval fish obtained an unspecified contribution of live rotifers in their daily ration and no appropriate control groups without live food were examined.

The dietary requirement for arginine among fish may differ in the same manner as in carnivorous mammals, where the metabolic and enzymatic efficiency results in the highest concentrations required in the cat, intermediate concentrations in the dog, and the lowest in the rat (Morris, 1985). Luzzana *et al.* (1998) reported the arginine requirement in coho salmon at a level of 2.4% for satiation-fed groups and 2.7% for restricted-fed groups, almost twice as high as in other salmonids. In fish such as coho salmon, supplied in their natural diets with a rich source of arginine, the production of

urea cycle intermediates, citrulline and ornithine, and an increase in ureogenesis after a meal may reduce the catabolism of dispensible amino acid, decrease ammonia load and improve nitrogen balance.

Plisetskaya *et al.* (1991) advocated a somatotropic effect with high arginine levels and suggested an increase in insulin production in salmonids. However, these results were contradicted in the following report (Lall *et al.,* 1994).

6.3.5.3. Methionine

Methionine is frequently the first limiting amino acid in fish diets (Nordrum *et al.,* 2000). Poston (1986), with all the elegance of design and consequence of a classical nutritionist, had difficulty matching the results of a 20-week study on the juvenile rainbow trout (1- to 2-g body mass) requirement for L- or D-methionine. He also concluded that cystine, another sulfur amino acid, did not promote growth or prevent pathologies linked to methionine deficiency. This conclusion reached, with respect to salmonids, is questioned in coolwater percid fish. Twibell *et al.* (2000) estimated that the dietary total sulfur amino acid requirement of yellow perch was 1.0–1.1% in the case of diets containing only traces of cystine (0.03%) and 0.85–1.0% when the cystine level was elevated to 0.4%. Therefore, in this species the requirement for methionine can be spared by cystine at 50% when diets contained at least 0.33% choline. The sulfur amino acid catabolic pathway provides precursors for choline synthesis.

Cataractogenesis of methionine deficiency in salmonids has not been explored in depth besides the higher efficiency of the L-form versus the D-form and descriptions of opacities originating in the anterior part of lenses and progressing inward (Poston, 1986). In contrast to that study, Cowey *et al.* (1992), with more accurate eye lens examination, concluded that the methionine requirement for cataract prevention is higher (0.96%) than that to maximize growth (0.76%).

Poston (1986) reported that, as the initial weight of trout doubled in his second experiment, low levels of added methionine were significantly better utilized. This suggests fish size-related differences. This is a significant finding because, as Cowey *et al.* (1992) used fish of similar size and a very diversified and balanced diet formulation (four protein sources), they were able to improve the growth rate of rainbow trout and avoid major differences in the food actually eaten. In contrast, it is apparent that methione added to experimental diets at 0.2–0.6% severely restricted their intake in comparison to diets with 0.9–1.1% methionine and, consequently, growth and protein deposition (Rodehutscord *et al.,* 1995c). It appears that differences among treatments are driven by intake, and in these circumstances pair-feeding on a restricted intake should address the metabolic requirement

for methionine. Contrary to the adoption of the method of satiation feeding in amino acid requirement studies advocated by Cowey (1995), restricted feeding would allow separation of the effects of preference from metabolic efficiency. Indeed, the difference in metabolic efficiency is the essence of nutrient utilization. It is equally important that a high intake of experimental, i.e., semipurified or purified, diets is secured to achieve growth rates approaching those in fish fed practical diets. As Cowey (1995) pointed out, numerous studies with proteins of unbalanced amino acid composition, such as zein, gluten, and soybean protein concentrate, then supplemented with a graded level of synthetic amino acid, did not meet this standard for the high growth rate. Rainbow trout offered simultaneously a casein-based diet with a balanced amino acid profile, a gelatin-based diet with an unbalanced composition, and a protein-free diet in self-feeders showed a higher preference for the balanced amino acid diets regardless of the protein level, 15 or 40% (Yamamoto *et al.,* 2000). Further study is needed to equalize intakes by feeding a stimulant(s) of the diets differing in a single essential amino acid.

Blood plasma concentrations of methionine were twice as high in hybrid sunshine bass fed diets with DL-methionine as in fish fed an L-methionine diet, although their growth was not significantly different (Keembiyehetty and Gatlin, 1995). In rainbow trout, a dietary supplement of 0.8% in comparison to 0.4% increased the blood level of methionine, however, the increase in taurine levels (55 and 301 μM) was more substantial. Also, Nordrum *et al.* (2000) noticed an increase in methionine and taurine concentrations in the plasma of Atlantic salmon when diets were supplemented with 0.6% methionine. Methionine concentrations in rainbow trout liver did not increase in line with the dietary intake, whereas taurine concentrations rose in the livers (0.8, 10.4, and 28.0 μmol/g) of fish fed diets with 0.4, 0.8, and 1.9% methionine, respectively (Cowey *et al.,* 1992). The same authors found a decline in ornithine decarboxylase activity in livers of rainbow trout fed a methionine-deficient diet. In yellow perch serum methionine concentrations were not significantly affected by dietary methionine contents (Twibell *et al.,* 2000), whereas Schwarz *et al.* (1998) suggested that the concentrations of some amino acids were significantly increased in the plasma of common carp fed methionine-deficient diets. However, sampling performed 18 hr after a meal in warmwater common carp did not directly reflect dietary effects.

Methionine has chelating and antioxidant properties. It could be involved in the detoxification of reactive thiols produced on glucosinolate degradation which are present in many common plant ingredients of fish diets. For instance, Schwarz *et al.* (1998) obtained negligible growth of common carp fed a diet with 37.2% pea meal and an overall 40% protein diet,

whereas on methionine supplementation growth was significantly improved. Although speculative, this probability needs to be explored as synthetic amino acids are added to a diet containing plant protein concentrates with antinutrients.

6.3.5.4. Tryptophan

Beside being an indispensable constituent of protein, tryptophan is a precursor of serotonin and nicotinic acid in fish. In tryptophan-deficient salmonids, lordoscoliosis was reported (Akiyama *et al.,* 1985). A study of the tryptophan requirement of rainbow trout was one of the most elegant, where growth responses (five fold weight gain) corresponded with tryptophan concentrations (breakpoint at 2.5 g/kg) in blood plasma and liver as well as with enhanced oxidation (breakpoint at 2.0 g/kg) (Walton *et al.,* 1984). The only relationship which was not consistent with dietary tryptophan levels was that of hepatic tryptophan pyrrolase activity. This enzyme is the first to initiate the tryptophan catabolic pathway. Tryptophan pyrrolase activities in liver of milkfish did not change as the dietary level increased from 0.9 to 6.1 g/kg (Coloso *et al.,* 1992). With the weight gain peaking at only 125–150% of the initial weight after 12 weeks of feeding, no gross pathologies were observed. Alternatively, tryptophan can be catabolized by tryptophan hydroxylase (5-monooxygenase) present in rainbow trout liver (Nagai *et al.,* 1995). This enzyme is the rate-limiting step in the biosynthesis of serotonin, however, its K_m value of 460 μM in rainbow trout seemed to suggest that at 20 μM (Walton *et al.,* 1984) catabolism would be suppressed.

Kim *et al.* (1987) used diets containing 30% synthetic amino acids and 7% of a casein–gelatin mixture supplemented with graded levels of tryptophan. The authors obtained a linear increase in fish body mass from 0.06 to 0.2% dietary tryptophan, however, their conclusion that the requirement was 0.2–0.25% does not seem warranted, as their reported findings included only one diet with 0.5% tryptophan to determine the breakpoint.

Akiyama *et al.* (1986) explored the mechanism involved in spinal deformities recognized in salmonids on a tryptophan-deficient diet. The authors eliminated spinal deformities by dietary supplementation of 5-hydroxytryptophan (5-HTP) and reported high concentrations of serotonin in the brain of these fish in comparison to control fish. However, the growth rates of fish fed tryptophan deficient and 5-HTP diets were severely depressed. The same authors further addressed the question of the optimum 5-HTP level in diets preventing scoliosis (0.1%) in chum salmon and reported major changes in other brain amino acids, such as glutamate, aspartate, and γ-aminobutyric acid (GABA), serving as neurotransmitters (Akiyama *et al.,* 1989).

In trout, as in mammals, Johnston and Glanville (1992) suggested that tryptophan competes for uptake into the brain with other large neutral amino acids, such as leucine, valine, and phenylalanine. This occurs because tryptophan and these aromatic amino acids share a common transporter at the blood–brain barrier. Johnston *et al.* (1990) extended these findings on the effects of dietary tryptophan based on an analysis of tryptophan and serotonin in the brain, which increased linearly with dietary tryptophan levels from 0 to 0.53%. The uptake of tryptophan into the brain is stereospecific (the L-form is preferably absorbed), reaches the saturation level at approximately 750 μM in blood plasma, and is inhibited by other aromatic amino acids, such as tyrosine (Aldegunde *et al.,* 1998). The value of this study, however, is of limited relevance, as concentrations in the blood plasma of fish injected with tryptophan were 100-fold higher than physiological levels. Aldegunde *et al.* (2000) argued that with intraperitoneal injection of 12–25 mg/kg, tryptophan oxidation was not inhibited by the end product and a dose response in 5-HTP concentrations was found. It is difficult to establish the following steps leading to serotonin synthesis and evidence of this "serotoninergic neurotransmission cascade" in fish. In mammals, variation in serotonin concentrations in the brain were associated with appetite and detection and selection of energy as protein or carbohydrate (Leathwood, 1987). Serotonin in the brain of chum salmon fed diets with different tryptophan and/or 5-HTP levels has shown a nonlinear response (Akiyama *et al.,* 1989). Akiyama *et al.* (1996) reported that tryptophan supplementation in a flounder diet suppressed aggressive attacks and cannibalism. The same authors suggested that tryptophan intake in reproductively active fish may be involved in the regulation of gonadotropin synthesis and secretion and, consequently, in maturation-inducing effects.

6.4 Conclusions

Despite the limited importance of carbohydrate in the natural diets of most fish, carbohydrate metabolism is the central pathway by which energy transactions occur in fish, as in most animals. Carbohydrate breakdown is initiated by glycolysis, which can proceed in the absence of oxygen, albeit with a reduced ATP yield compared to that provided by mitochondrial oxidation to CO_2 and H_2O. While fish have lower mass-specific metabolic rates than mammals do, mitochondrial volume densities in fish oxidative muscle can be far higher than in most mammalian muscles. Glycogen is stored in major quantities in fish liver, and muscle and blood glucose levels

are hormonally regulated. Hepatic carbohydrate metabolism is sensitive to control by corticoid, catecholamine, and peptide hormones. The receptors and intracellular coupling mechanisms implicated have been elucidated for several fish species. Muscle glycogen is a primary fuel for both sustained and burst swimming. The regulatory mechanisms operative during the activation of carbohydrate metabolism in white muscle have been characterized. Most glycogen resynthesis after exhausting exercise occurs within muscle, but its exact pathway remains elusive.

Whereas the metabolic circuitry operative in fish tissues resembles that in the better-studied mammals, the sensitivity of metabolic processes to abiotic and biotic factors and the exact roles of tissues and organs differ between fish and mammals. In contrast to mammals, fish show drastic increases in tissue hydration during starvation and show a marked plasticity of their metabolic capacities with abiotic conditions and growth. As a multitude of factors sets the capacities for carbohydrate metabolism in a given species, it is difficult to extrapolate results obtained for one species to another. This is particularly true given the systematic diversity within the fish.

In the years since the excellent review by Cowey and Walton (1989) was published, not only has the number of studies dealing with protein metabolism increased, but also new approaches have been tested (^{15}N) and methods refined, although some components of protein turnover, such as protein degradation rates, remain illusive. The number of fish species examined for their amino acid requirements has probably doubled, and several estimates have been refined; however, they are greatly disproportionate to investigations addressing metabolic pathways of indispensable amino acids and mechanisms of their interaction and/or degradation. Studies on amino acid requirements have used diets which did not maximize growth, and consequently some of the estimates are inflicted by substandard growth, insufficient durations, or simply limited numbers of replicates. The formulation of an optimum purified diet for even the most common species of interest to aquaculture, such as salmonids and cyprinids, remains a challenge.

Enormous strides have been made in understanding nitrogen metabolism, mechanisms of excretion of nitrogenous compounds, and enzymatic pathways related to the formation of protein catabolites. Not surprisingly the diversity of pathways and the need for an evolutionary perspective on metabolism in fish have been confirmed to be characteristic for this part of the animal kingdom. The area of research where the metabolism of individual amino acids and complete diets, with their link to nervous, endocrine, or gene expression mechanisms, can be addressed may be the focus of the coming decades.

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7

Nutritional Physiology

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7.11.5. Feeding and Diet Acceptability
Acknowledgments
References

7.1 Introduction

Opportunities for advancement in the field of fish nutrition in the next 20 years will differ somewhat from those in the past 20 years. While the determination of individual nutrient requirements for a growing number of species will still be important, there is an increasing need to find ways to meet these requirements using a greater diversity of feedstuffs. The global supply of fish meal and fish oil is finite and fully utilized. Alternative or nontraditional feedstuffs may differ in terms of taste, smell, texture, and color, as well as nutrient composition, from the traditional feedstuffs, which are produced largely from the natural prey of the fish being raised. Alternative feedstuffs may also contain compounds and antinutritional factors that affect digestive or sensory physiology (see Chapter 10, by Hendricks). To utilize alternative feedstuffs effectively, the physiological systems of fish that will interrogate and digest them must be better understood.

Another important area of fish nutrition for the next 20 years will be larval fish nutrition. Currently, the cost and difficulty of rearing a great number of species from the first feeding to the juvenile stage are the most severe bottlenecks to the development of aquaculture production of nontraditional species. To understand the unique challenges of developing a manufactured diet for larvae, digestive and sensory physiology from the initial phase of exogenous feeding through metamorphosis must be considered and explored.

This chapter is titled "Nutritional Physiology" and covers both digestive and sensory systems. Sensory systems are included because of the importance these systems have for feeding (see Chapter 13, by Lovell) and the challenges of diet acceptability when high levels of alternative feedstuffs (see Chapter 9, by Hardy and Barrows, and Chapter 10, by Hendricks) or therapeutants (see Chapter 12, by Gatlin) are used. The liver is also included because of its metabolic importance (see Chapter 6, by Dabrowski and Guderley). This chapter will serve as a starting point to the wider fish physiology literature.

To simplify the task of describing the remarkable diversity of structure and function of the nutritional physiology of fish as a group, this chapter focuses on species that are currently important to, or have the potential to be important to, intensive aquaculture. Thus, the focus is on teleosts, with more attention paid to carnivorous species than to omnivores or herbivores. The chapter deals with the anatomy and function of the major organs and

systems in the body that impact nutrition, especially as they relate to species being fed prepared feeds. The text and figures are designed to help the reader visualize and understand the function of each organ and the system as a whole. Changes in some of these systems during ontogeny and how these changes relate to larval fish culture are discussed in the second half of the chapter. Throughout this chapter, the focus is on the essential link between physiology and the feeding of prepared diets.

7.2 Gross Juvenile and Adult Anatomy

In a general sense, form and function go hand in hand; an understanding of anatomy enhances the understanding of physiology. Fish can be classified broadly by their feeding habits into the well-known classes of detritivores, herbivores, omnivores, and carnivores. Within each category, organisms can be thought of as either euryphagous (eating a great variety of foods), stenophagous (eating a limited variety of foods), or monophagous (eating only one type of food) (Moyle and Cech 1982). The majority of fish targeted for aquaculture are either euryphagous carnivores (such as salmon, basses, breams, halibut, turbot, flounders, and groupers), euryphagous omnivores (such as channel catfish and tilapia), or euryphagous herbivores (such as some carp and milkfish). While exceptions occur, the gross anatomy is often somewhat similar within each class but different between classes (Fig. 7.1; see also Section 7.7). Figures 7.2–7.8 illustrate the differences and similarities among the classes.

At first glance, carnivorous "flatfish" (Fig. 7.2) appear to be somewhat different from carnivorous "round" fish (Figs. 7.3–7.5); however, these distinctions are due largely to the different shape of the body cavities in each form. In reality, the digestive tracts of halibut (*Hippoglossus stenolepis;* Fig. 7.2) and sablefish (*Anoplopoma fimbria;* Fig. 7.3) are more similar than is the digestive tract of sablefish to that of either Atlantic salmon (*Salmo salar;* Fig. 7.4) or lingcod (*Ophiodon elongatus;* Fig. 7.5). Both sablefish and halibut have similar feeding habits and occur in the same areas deep in the north Pacific Ocean.

Pacific halibut (Fig. 7.2), sablefish (Fig. 7.3), Atlantic salmon (Fig. 7.4), and lingcod (Fig. 7.5) are all carnivores. Displayed (in Figs. 7.2–7.5) are the dentition (teeth and gill rakers), mouth, eyes, nasal pits, gills, tongue, esophagus, esophageal (also called cardiac) sphincter, stomach, pylorus, pyloric ceca, intestine (upper and lower), gall bladder, spleen, kidney, and liver. Figure 7.6 is of a channel catfish (*Ictalurus punctatus*), and Fig. 7.7 is a Nile tilapia (*Sarotherodon niloticus*), both omnivores. Catfish prefer animal sources of food, while tilapia typically eat plant material and detritus in the

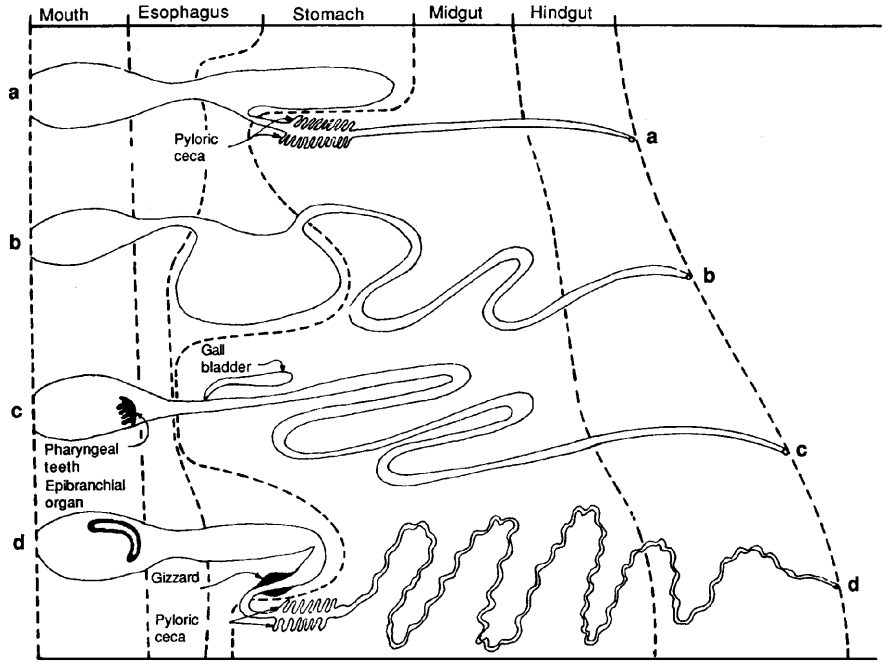

FIG. 7.1

Diagrammatic representation of typical digestive configurations. (a) Euryphagous carnivore with a y-shaped stomach (salmon, trout, lingcod, sablefish, and halibut). (b) Euryphagous omnivore emphasizing animal sources of food; pouched stomach or intestinal sac (catfish and tilapia). (c) Euryphagous omnivore emphasizing plant sources of food; stomach absent (carp and goldfish). (d) Stenophagous planktivore; tabular stomach with muscular gizzard (milkfish). From Smith (1989).

wild (though it will aggressively feed on pellets made with animal products, and its morbid tankmates in culture). Both have a pouch-shaped stomach, no pyloric ceca, and a long intestine; otherwise the gross anatomy is similar to that of carnivores. Tilapia consume a great deal of algae, which can be resistant to digestion unless an extremely low stomach pH (a pH of about 2–3) is available to rupture the cell walls (Smith 1989). Tilapia also have a very long intestine, which may compensate for the lack of ceca. Figure 7.8 shows the common carp, an herbivore. The carp (*Cyprinus carpio*) lacks a stomach and pyloric ceca; however, the length of its intestine is very long compared to that of carnivores.

In the following sections, each area of the digestive tract is examined in more detail. Refer to Figs. 7.1–7.8 as well as to the figures associated with each organ system. Many of the figures in the following sections contain histology

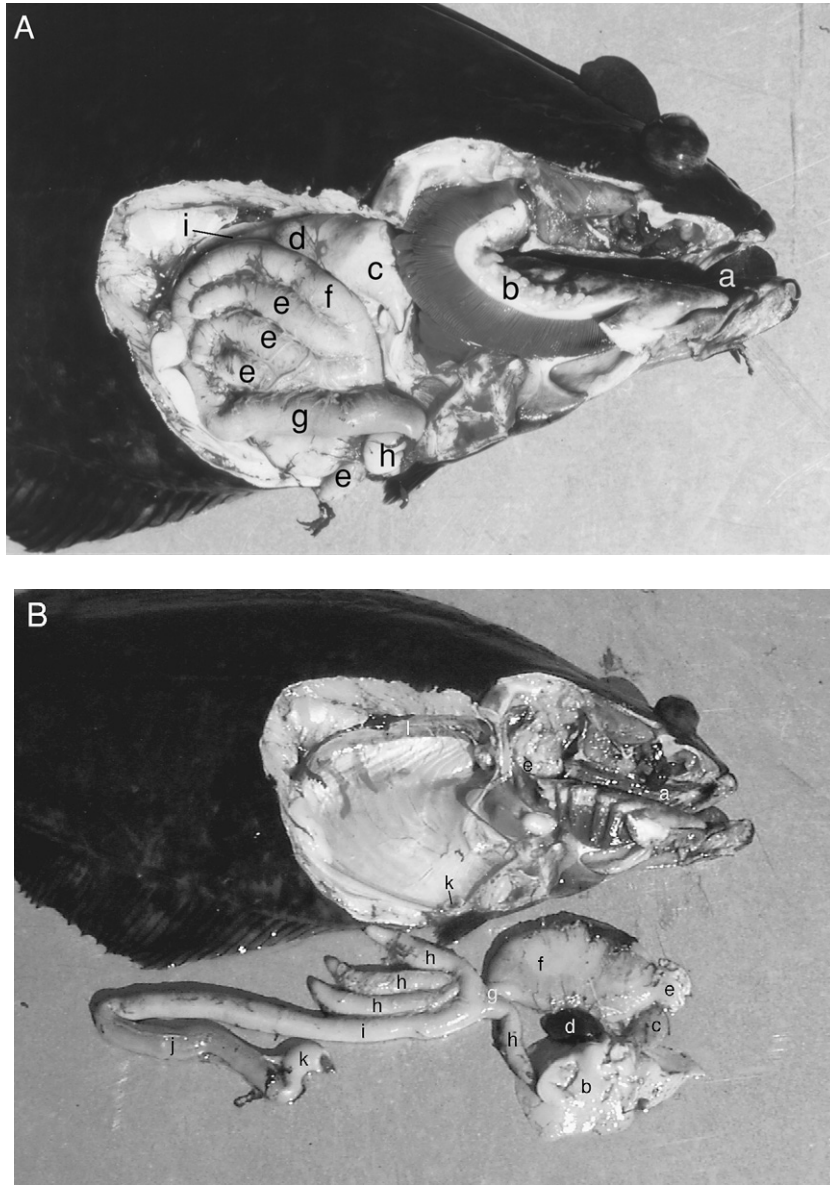

FIG. 7.2

(A) Dissection of a halibut showing the digestive tract. (a) Oral cavity, (b) gill arches with teeth-like rakers, (c) liver, (d) gallbladder, (e) pyloric ceca, (f) upper or small intestine, (g) lower or large intestine, (h) anus, and (i) kidney. (B) Digestive tract removed, showing (a) oral cavity, (b) liver, (c) gallbladder, (d) spleen, (e) esophagus, (f) stomach, (g) pylorus, (h) pyloric ceca, (i) upper or small intestine, (j) lower or large intestine, (k) anus, and (l) kidney. Photographs by Michael Rust.

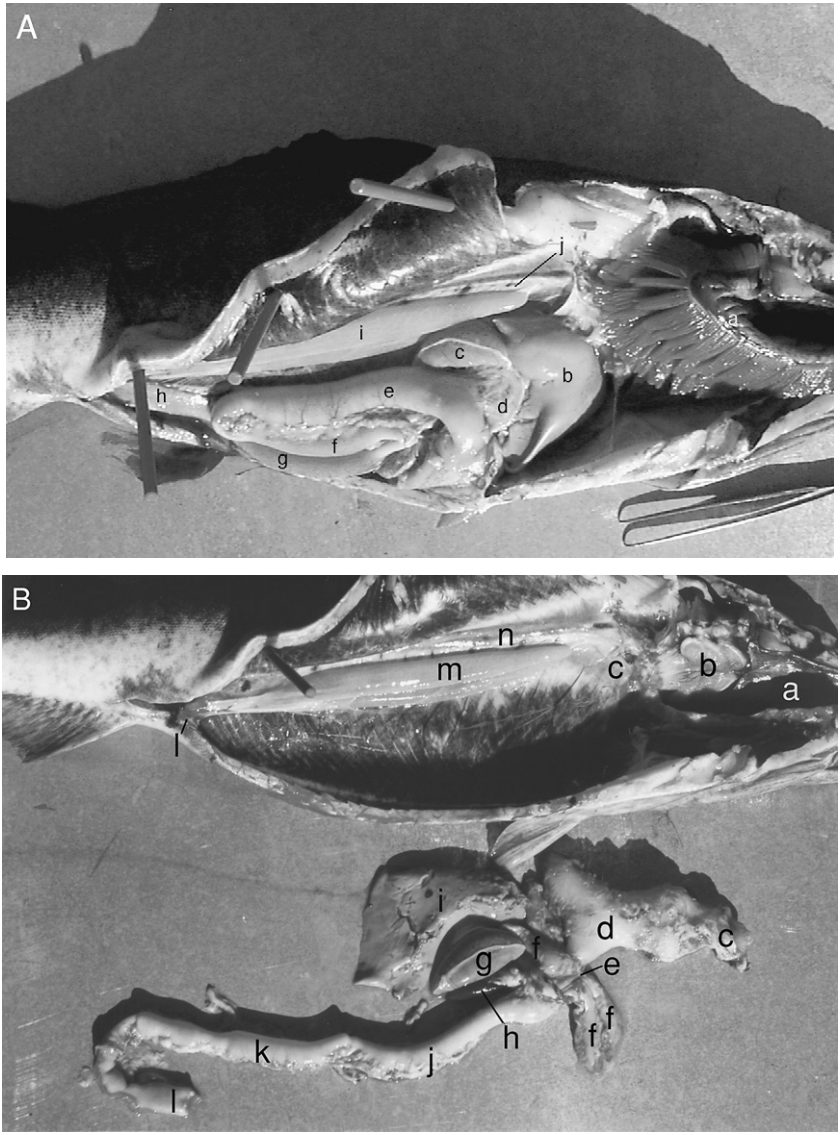

FIG. 7.3

(A) Dissection of a sablefish showing the digestive tract. (a) Gill arches, (b) liver, (c) gallbladder, (d) pyloric ceca, (e) upper or small intestine, (f, g) lower or large intestine, (h) anus, (i) ovaries, and (j) kidney. (B) Digestive tract removed. (a) Oral/pharyngeal cavity, (b) pharyngeal tooth plate, (c) esophagus, (d) stomach, (e) pylorus, (f) pyloric ceca, (g) gallbladder, (h) spleen (hard to see), (i) liver, (j) upper or small intestine, (k) lower or large intestine, (l) anus, (m) ovaries, and (n) kidney. Photographs by Michael Rust.

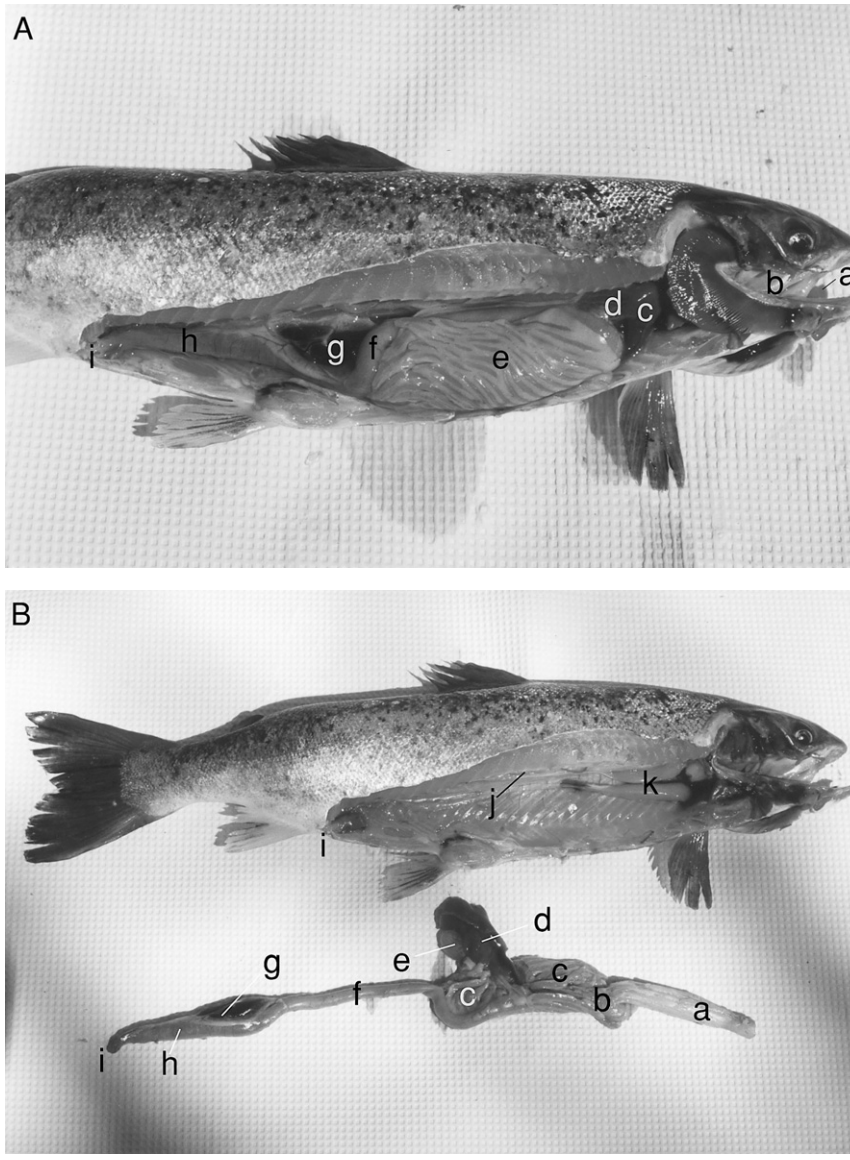

FIG. 7.4

(A) Dissection of an Atlantic salmon. (a) Oral cavity, (b) pharynx, (c) liver, (d) gallbladder, (e) pyloric ceca, (f) upper or small intestine, (g) spleen, (h) lower or large intestine, and (i) anus. (B) Digestive tract removed. (a) Esophagus, (b) stomach near the esophagal sphincter, (c) pyloric ceca, (d) liver, (e) gallbladder; (f) upper or small intestine, (g) spleen, (h) lower or large intestine, (i) anus, (j) kidney, and (k) ovaries. Photographs by Michael Rust.

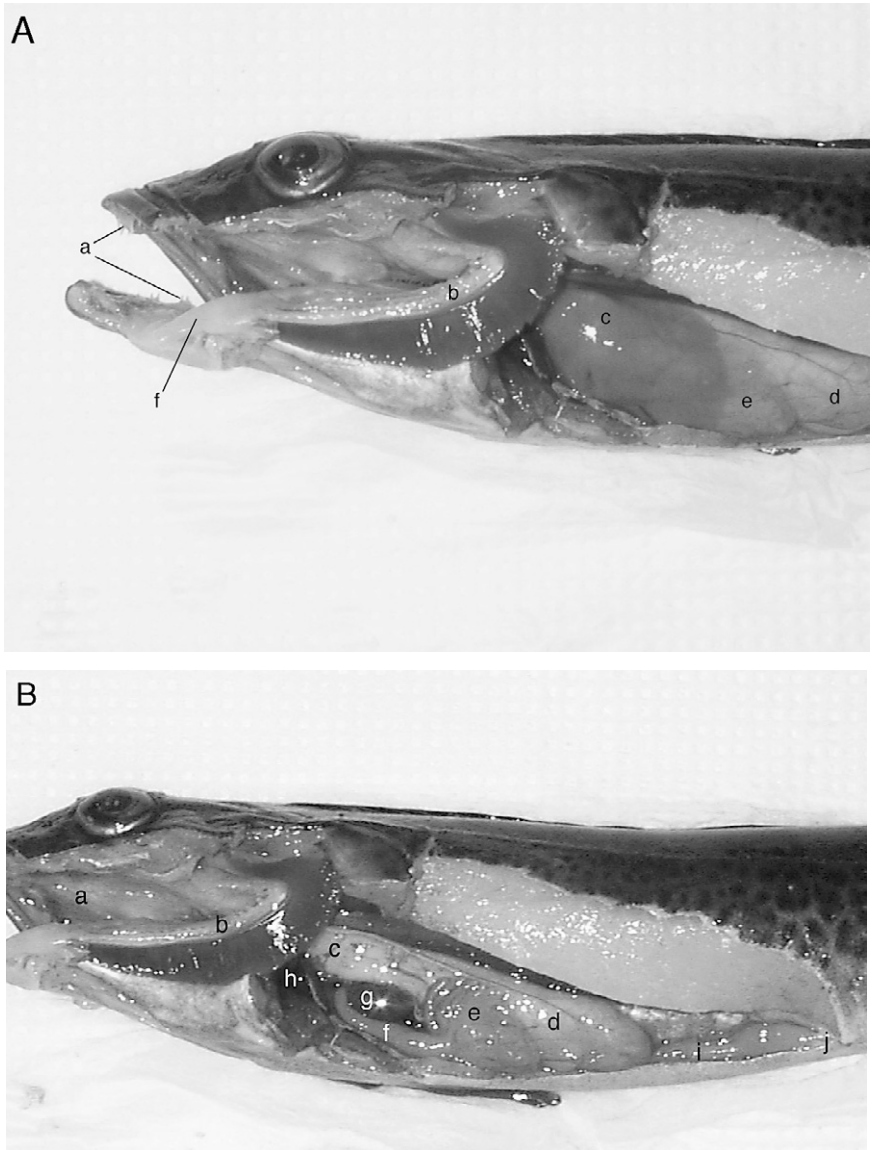

FIG. 7.5

(A) Dissection of the anterior portion of a juvenile lingcod. (a) Jaw teeth, (b) gill arches, (c) liver, (d) stomach, and (e) pyloric ceca (somewhat behind liver). (B) Same as A, with the liver removed. (a) Oral cavity, (b) gill arches, (c) cardiac stomach, (d) fundic stomach, (e) pyloric ceca, (f) upper or small intestine, (g) gallbladder, (h) spleen, (i) lower or large intestine, and (j) anus. Photographs by Michael Rust.

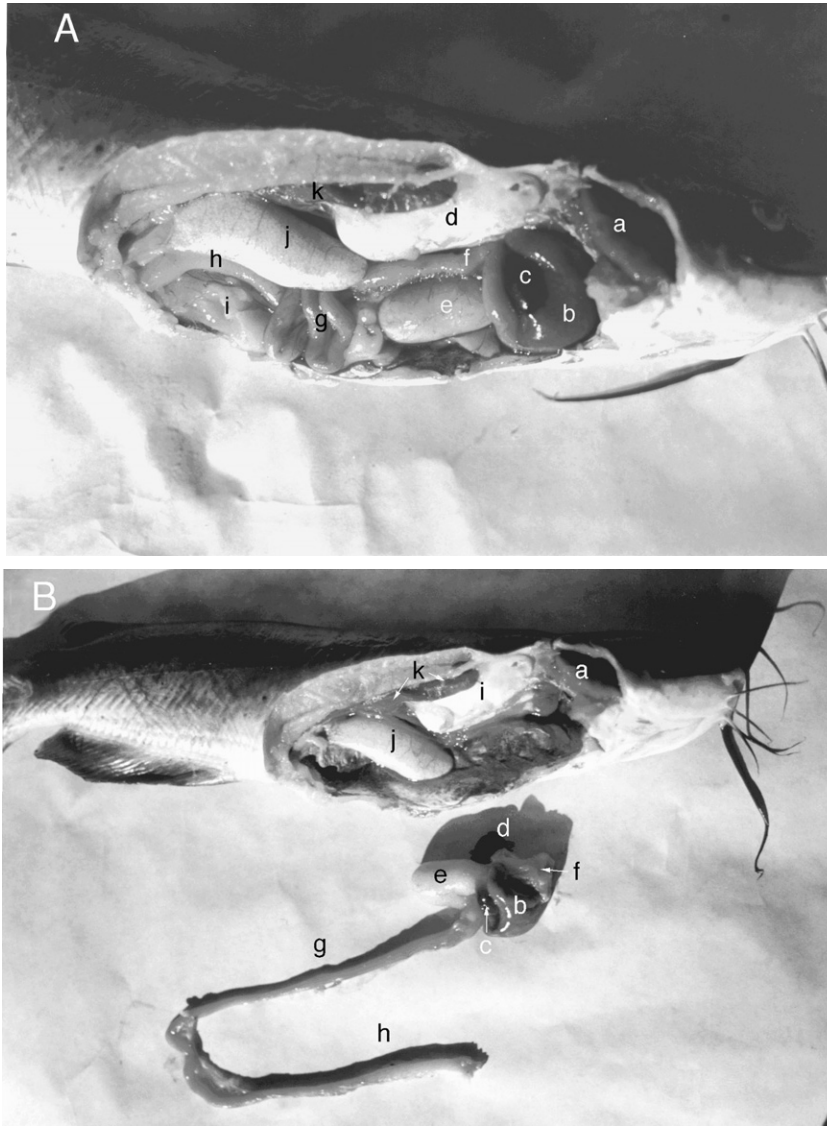

Fig. 7.6

(A) Dissection of a channel catfish showing the digestive tract. (a) Gills, (b) liver, (c) gallbladder, (d) swimbladder, (e) stomach, (f) esophagus, (g) upper or small intestine, (h) lower or large intestine, (i) adipose (fat) tissue, (j) ovary, and (k) kidney. (B) Channel catfish with digestive tract removed. (a) Gills, (b) liver, (c) gallbladder, (d) spleen, (e) stomach, (f) esophagus, (g) upper or small intestine, (h) lower or large intestine, (i) swimbladder, (j) ovary, and (k) kidney. Photographs by Michael Rust.

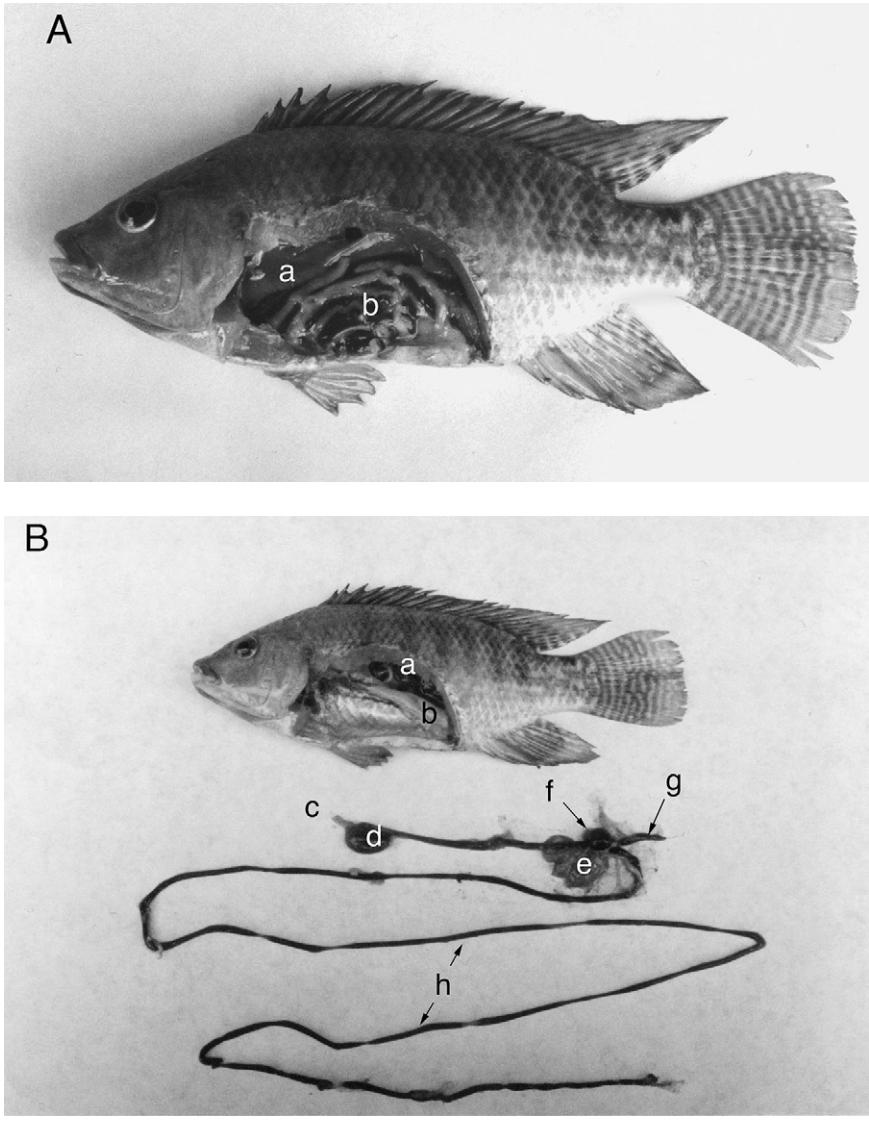

FIG. 7.7

(A) Dissection of a Nile tilapia with the intestinal tract in place. (a) Liver and (b) coiled gut. (B) Dissection of Nile tilapia with the intestinal tract removed. (a) Kidney, (b) ovary, (c) esophagus, (d) stomach, (e) liver, (f) gallbladder, (g) spleen, and (h) intestine. Photographs by Michael Rust.

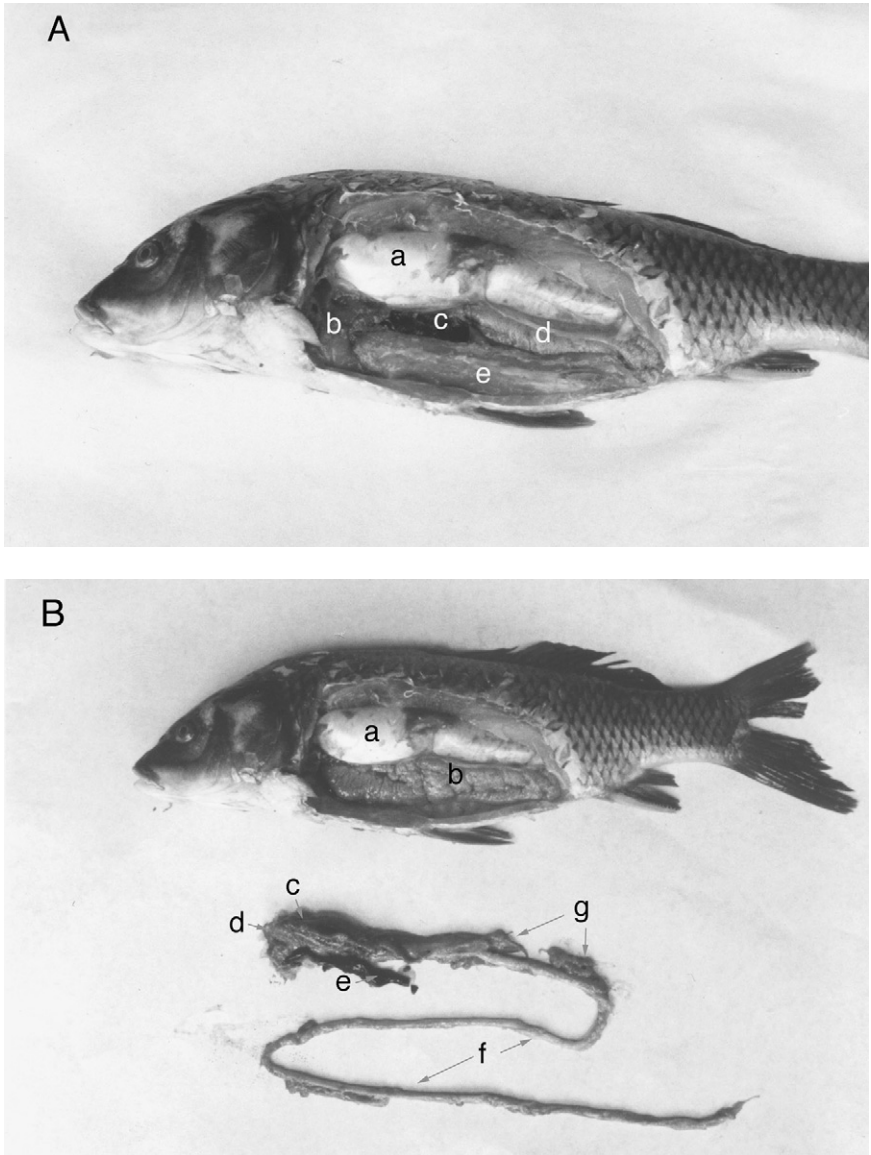

FIG. 7.8

(A) Dissection of the common carp. (a) Swimbladder, (b) liver, (c) spleen, (d) ovary, and (e) coiled gut. (B) Digestive tract removed. (a) Swimbladder, (b) ovary, (c) liver (under), (d) esophagus, (e) spleen, (f) intestine, and (g) pancreatic tissue (around the gut in several locations). Photographs by Michael Rust.

photographs chosen to show common structures in fine scale. The histology sections were produced by fixing the tissue or organ, then embedding it in a paraffin-based medium, and cutting thin (about 4- to 5-μm) sections (slices) of tissue once the medium hardened. These sections were mounted on a microscope slide, the paraffin-based medium was removed, and the section stained so that the cells and tissues could be seen. The stains used in the histological photographs in this chapter were hematoxylin and eosin, the most common stains used in animal histology. Hematoxylin stains acidic structures a purplish blue. Structures that contain acids (e.g., RNA or DNA), such as the endoplasmic reticulum and nuclei, turn blue, as do gastric cells and glands. Eosin stains basic structures various shades of red to pink. Most cytoplasmic proteins are basic and stain red or pink. Cells that contain huge amounts of lipid, such as adipose tissue, do not stain very well and show up as clear or white because the embedding process extracts the lipids.

Many of the tissues that are important in nutritional physiology contain an epithelium of columnar-shaped cells supported by a lamina propria and/or other subsurface cell layers. The epithelium cells interact with the environment or the lumen of an organ and include those that sense the environment (chemoreceptive cells), those that take up nutrients (enterocytes), and those that secrete mucus, enzymes, and/or other compounds.

For more detailed information on general histology and cellular physiology of fish, the reader is referred to Hibiya (1982). In addition, several species-specific histological atlases cover fish histology extensively. Examples include those by Anderson and Mitchum (1974a) for trout, Grizzle and Rogers (1976) for catfish, Gorman (1982) for striped bass, Yasutake and Wales (1983) for salmon, Bell (1986) for sablefish, and Morrison (1993) for larval cod. All are excellent sources of histological/anatomical information on fish.

7.3 Sensory Organs

The sensory organs discussed in this chapter include eyes, olfactory organs, and taste buds. Electromechanical senses are not discussed, although they may be important for feeding in some species. The sensory organs are important in fish nutrition, as these organs are used by the fish to locate and investigate the acceptability of any food or feed in the environment. This leads to the multistep decision to search for food, bite it, and then swallow it. The decision to ingest is based on the properties of the feed as determined by the fish's senses. Such things as the appearance of the feed (e.g., movement, size, shape, color), long-range attraction (olfaction and perhaps electrochemical properties), short-range attraction (texture, taste, and perhaps

electrochemical properties), environmental conditions (light, temperature, salinity, water quality, currents, tank color, outside disturbances, and other factors), and past feeding behavior (e.g., fixation, feeding habits, previous experience, endogenous rhythms) all affect how well fish will feed on a given diet. It is thus critical that the systems used by fish to gather the information needed to decide to feed or not to feed, and to choose among feeding strategies, are understood.

7.3.1. Vision

Vision in fish has been well studied over a long period. Two excellent texts on the subject, one by Nicol (1989) and another edited by Douglas and Djamgoz (1990), provide an excellent background and depth. A more recent review on UV vision in fish was written by Losey and colleagues (1999). UV vision has also been the subject of numerous international conferences, and selected papers from a recent meeting have been published (Losey and Cronin 1999).

As light enters the eye it first passes through the cornea (Fig. 7.9). The cornea is made of three clear layers: the dermal, scleral, and autochthonous layers. The dermal layer is continuous with the skin. The scleral layer is continuous with the sclera, a thick fibrous coat that covers the whole eye

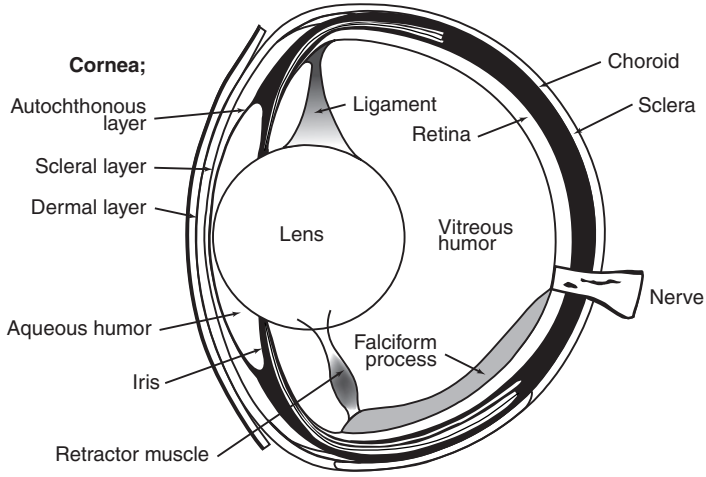

FIG. 7.9

Diagram of the cross section of a fish eye. Drawing by James Peacock based on Walls (1942).

but is transparent only where it is a part of the cornea. The cornea does not alter the incoming light but serves as a protective window for the eye.

Behind the cornea and in front of the lens is an anterior fluid-filled chamber containing the aqueous humor. This chamber is subdivided by the iris, a circular structure with an opening in the middle called the pupilar opening. A portion of the lens protrudes through the pupilar opening toward the cornea. The lens is a somewhat spherical structure made of structural and soluble proteins and water. The higher the concentration of soluble proteins in the lens, the higher the refractive index of the lens. Teleost eyes have very high refractive indices.

The lens is responsible for collecting and focusing the light coming into the eye onto the retina. Teleost eyes often bulge from the body, and the lens bulges through the pupilar opening, which has the practical effect of lifting the eye above the surrounding body (Fig. 7.9). This greatly increases the field of view forward and backward. Coupled with eye movement and the changing point of view induced from swimming, fish can see from almost directly behind to directly in front of themselves (Moyle and Cech 1982).

The retina is where images are discriminated from the light focused on it by the lens. Between the back of the lens and the retina is another fluid-filled space called the vitreal cavity. The vitreal cavity is filled with a viscous fluid called the vitreous humor or vitreous body. The distance between the retina and the lens determines focus. Focus is accomplished by movement of the lens by muscles in the eye. The muscles do not change the shape of the lens; they just move it toward or away from the retina to provide focus. The lens, with its associated muscles and ligaments, separates the vitreous humor from the aqueous humor.

Because the teleost lens is spherical and the retina is elliptical, the distance between the lens and the retina varies, resulting in comparatively better close-up vision directly in front of the fish and comparatively better far-away vision to the side. In addition, fish may have differing cell density, mosaic, and compositional patterns across the retina, which have strong effects on visual acuity across lines of sight. It appears that these patterns often reflect their feeding pattern and behavior.

Depth perception, which requires that both eyes see the same thing, is best to the front. Therefore, feed pellets offered to the side or rear of fish may not be recognized, especially if they are near the fish.

Light striking the retina first travels through a transparent layer of nerve cells and fibers before reaching the pigmented light sensitive layer. Below this layer is the choroid coat, which functions to supply nutrients and oxygen to the rest of the retina. Some fish also have a tapeta lucida (or tapetum

lucidum), which is a reflective layer between the light-sensitive layer and the choroid coat (Douglas and Djamgoz 1990). It is often the tapeta lucida that causes "eye shine" in fish—the reflection of light when a light is shined on the eyes, especially under darkened conditions. The tapeta lucida can increase the light absorption in the retina by a factor of about 1.5, a significant adaptation to dark conditions. The reflective nature of the tapeta lucida changes with the light intensity of the environment and can take 60–90 min to go from fully reflective to almost-unreflective, or vice versa (Nicol 1989). This could have implications for feeding fish in an aquaculture situation, especially when lights are suddenly turned on or off, and feeding is attempted soon after the lights are switched.

The light-sensitive layer of the retina is made up of two types of light-sensitive cells, the rods and cones, which are sometimes arranged in highly ordered mosaic patterns. These cells, as their names imply, are long and thin and arranged in columns, with the nonphotoreceptive end facing the lens and the photoreceptive end facing the choroid. Rods (with a peak absorbance of 490–510 nm) are more numerous and more closely packed together and often share one neural fiber among several rods, resulting in a high sensitivity under low-light conditions and a good sensitivity to lightness and darkness (similar to black and white). Cones are more widely spaced and can have between one and four pigments with differing peak absorbance values. The number and spectrum peaks for the cones vary from species to species; UV (340- to 380-nm)-, violet (380- to 420-nm)-, blue (420- to 480-nm)-, green (480- to 540-nm)-, yellow (540- to 600-nm)-, and red (600- to 650-nm)-sensitive cones are all possible (Nicol 1989; Douglas and Djamgoz 1990; Losey *et al.* 1999). It is the number and nature of rods and cones that, to a large extent, determine the ability of the fish to perceive color (extending in some species into the UV) and hues. The number and nature of rods largely define the eyes' ability to discern lightness and darkness (contrast). Both rods and cones interact to define the resolution of the eye; the rods dominate at low light intensities and the cones dominate at higher light intensities (Douglas and Hawryshyn 1990). Information from the retina is transferred to the brain via the optic nerve. The brain then takes the information and processes it into an image.

While the teleost eye is in many ways similar to the human eye, enabling us to relate to what the fish "sees," the visual environment underwater is very different from the terrestrial environment. In addition, some fish are able to "see" in several ways that are very different from the human experience. As mentioned above, some fish (and a great number of other organisms) have a UV-sensitive cone, enabling fish to see UV light (McFarland and Loew 1994; Losey *et al.* 1999). In addition, some fish can also discriminate among planes of polarized light (Douglas and Hawryshyn 1990). In fact, both appear to

be related to the types of cones found in the fish's eyes (Parkyn 1999). The function of UV vision is still not totally understood, but there appears to be some evidence pointing to its utilization in plankton feeding (Loew *et al.* 1993, 1996; Browman *et al.* 1994).

The implications for feeding fish in aquaculture may include the investigation of the use of full-spectrum (into the UV) bulbs and polarizing filters for indoor hatcheries (Mikheev *et al.* 1997). The provision of polarized light may also be useful for training fish to feeders or in orientation in a tank. This may be especially helpful for training larval stages to artificial feeds (Sandstroem 1999) (see Section 7.11.5).

7.3.2. Chemoreception

Like vision, chemoreception in fish has been studied for a long time. Two volumes on the subject are available (Hara 1982, 1992a); a third is nearing publication. Kleerekoper (1969) reviews work done on olfaction in fish back to 1744 and provide many excellent figures. A chapter by Kleerekoper (in Hara 1982) provides an interesting historical perspective from work done in the 18th, 19th, and early 20th centuries. Relatively little information is available on how knowledge of chemoreception physiology can be applied to practical diets for fish (Takeda and Takii 1992). This section reviews the physiology of chemoreception with special consideration of how it may relate to feeding practical diets to fish.

Chemoreception in land animals includes the related senses of taste and smell. Since fish are always in water, the distinction between the two may be less obvious than for terrestrial organisms. In the aquatic environment, compounds need to be soluble in water to be sensed by either system; in the terrestrial environment, compounds that are volatile (in air) are smelled, while compounds that are soluble (in solution) are tasted (Hara 1992b). As a result, highly soluble, low molecular weight compounds (such as amino acids, steroids, nucleotides, and sugars) are important in fish chemoreception (Takeda and Takii 1992; Jones 1992).

Unlike the stimuli for vision or hearing, chemical stimuli are highly specific with respect to which compounds are stimulatory, do not directly indicate the direction to the source, and persist in the environment long after they are released (Hara 1992b). An organism sees or hears everything over a wide range of frequencies, but chemical stimuli are specific to binding sites on receptor cells. Only compounds that fit receptors are stimulatory and can be sensed. Also, hearing and seeing allow for instant directional location of the stimulus, while it is necessary for the organism to move within a gradient of a chemical stimulus to determine the direction of the source. If the gradient has been disrupted, or has drifted, then it is possible

that no directional information or incorrect information will be conveyed. A chemical stimulus will persist in the environment for a long period of time, while visual and auditory stimuli persist only if they are continuously generated.

7.3.2.1. Olfaction

While there is a great diversity in olfactory organ morphology given the diversity of fish and the niches they occupy, a common motif can be generalized (Zeiske *et al.* 1992). Paired pits in the snout of the fish contain the olfactory organs (Fig. 7.10). Each pit contains a chamber that is

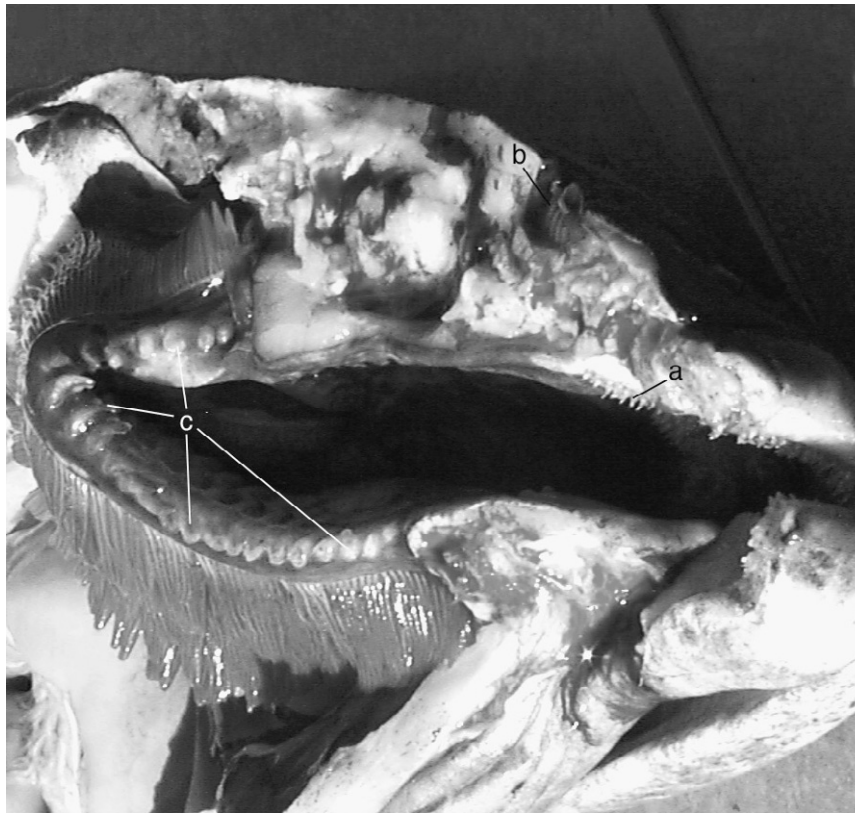

FIG. 7.10

A longitudinal dissection through the head and mouth of an adult sablefish. (a) Maxillary teeth, (b) olfactory pit containing the olfactory lamellae, and (c) teeth-like gill rakers. Photograph by Michael Rust.

connected to the outside by two openings (nostrils): one influent and one effluent. The nasal bridge separates the influent and effluent nostrils and provides a cover over the olfactory chamber. Within the olfactory chamber are the olfactory lamellae, often arranged in multiple rows. In some species, there is a side chamber called an olfactory ventilation sac that can expand and contract to create water movement. Some fish species have cilia or some other method to move water through the chamber (Zeiske *et al.* 1992).

The olfactory lamella is lined with epithelium containing both sensory and nonsensory columnar-shaped cells (Zeiske *et al.* 1992). The columnar epithelial cells are attached to a basal lamina and are covered with a mucous layer. Under the basal lamina is a supportive lamina propria. Four or more generic cell types make up the epithelium with the most important being the receptor (of at least two types: microvillous and ciliated), supporting, mucous (also called goblet cells), and basal (Yasutake and Wales 1983; Zeiske *et al.* 1992) cells. Each receptor cell is surrounded by supporting cells, with small basal cells scattered adjacent to the basal lamina. Supporting cells extend from the surface of the epithelium down into the lamina propria. Receptor cells are bipolar neurons, with a single dendrite extending to the surface of the epithelium and an axon extending down toward the lamina propria (Zeiske *et al.* 1992). Axons from many receptor cells come together in the basal lamina to form bundles that extend beyond the lamina propria. These bundles send their information to the olfactory bulb, which relays the information on to the brain for processing via the first cranial nerve (Hara 1992b; Satou 1992; Marui and Caprio 1992). Mucous cells are scattered irregularly near the surface of the epithelium (Yasutake and Wales 1983; Hibiya 1982).

Olfactory sensitivity is generally regarded to be greater than gustatory sensitivity, although this is not always the case (Kanwal and Finger 1992; Caprio *et al.* 1993). Compounds which are known to stimulate the olfactory receptor cells include amino acids, steroids, and prostaglandins (Hara 1992b). Of the three, amino acids are the most interesting for fish nutrition and the most widely studied. Electrophysiological studies have identified stimulatory thresholds for amino acids in the range of 10^{-7} to 10^{-9} M, with a response range that covers 6–7 log units of concentration (Hara 1992b).

7.3.2.2. Gustation

Gustation, or the sense of taste, is the province of the taste bud. Taste buds are located on the gills, barbels, and fins and all over the oral cavity and pharynx (Figs. 7.11 and 7.12), as well as all over the external surface of the body as solitary buds in some species (Hara 1992c; Whitear 1992). In contrast to humans, taste buds are not often located on the tongues of

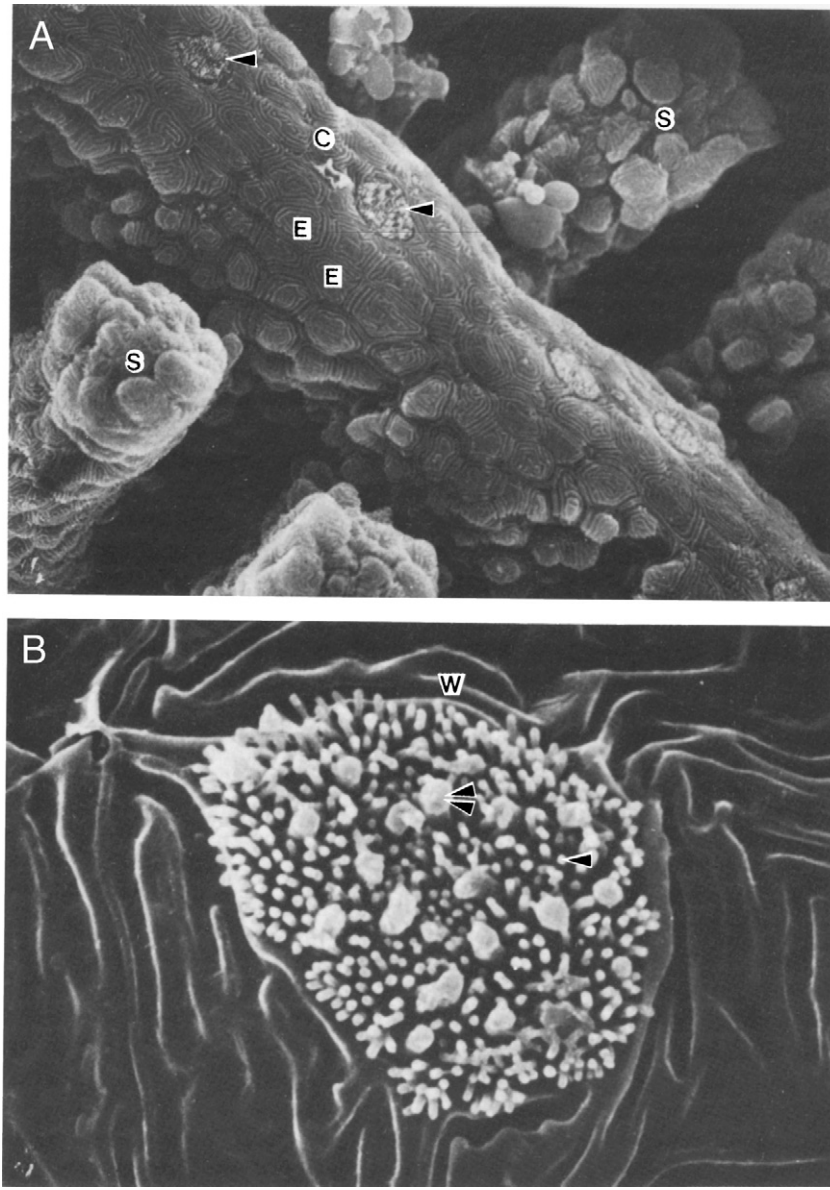

FIG. 7.11

(A) Taste buds on the gill rakers of a mullet (indicated by arrowheads) (magnification: ×3000); (B) thin (single arrowhead) and thick (double arrowhead) microvilli (magnification: ×27,000). SEM. From Hossler and Merchant (1983).

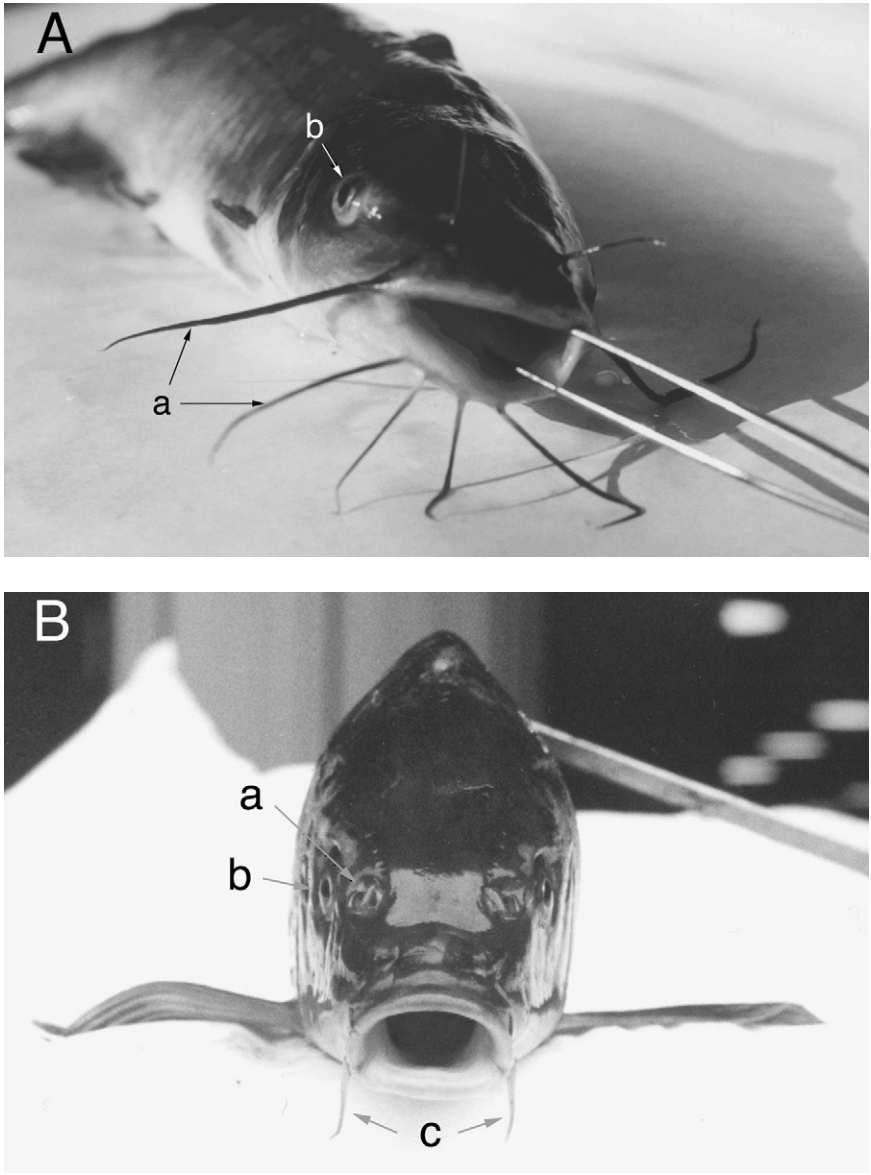

FIG. 7.12

(A) Mouth of a channel catfish showing (a) barbels and (b) eye. (B) Frontal view of a common carp showing (a) nasal pits, (b) eye, and (c) barbels. Photographs by Michael Rust.

fish (Hara 1992c). The individual or solitary taste buds are distinct from the gustatory system even though they are structurally related (Whitear 1992). These solitary cells are likely involved with a general chemical sensory system able to detect salinity or the presence of irritant chemicals, although their exact function is still unclear (Whitear 1992). Taste buds located within the oral cavity and pharynx are often located near teeth, presumably so that the juices of punctured food will be in close contact with the sensory epithelia of the taste bud (Kanwal and Finger 1992).

Information from the oral cavity and pharyngeal taste buds is communicated to the brain via the glossopharyngeal and vagal (cranial IX and X) nerves, while information from taste buds located on the barbels and fins (external to the oral cavity excluding the solitary cells) is communicated to the brain via the facial (cranial VII) nerve (Kanwal and Finger 1992). The anatomical segregation of the two gustatory systems implies a functional segregation as well. In the bullhead catfish (*Ictalurus nebulosus*) it appears that the external taste buds (those on the barbels, skin, and fins; Fig. 7.12) feed into the facial nerve and influence food search and pickup behavior, while the internal taste buds (in the oral cavity and pharynx) feed into the vagal nerve and influence selective ingestion behavior (Kanwal and Finger 1992). Depending on the species, one or the other behavior may be more important for successful feeding with prepared diets.

Taste buds contain several cell types that are similar in some respects to olfactory tissue including sensory, supporting, and basal cells. Unlike olfactory tissue, they are arranged in clusters elevated into a bud or "hill" above the surrounding epidermal layer (Fig. 7.11) and are surrounded by marginal cells that are similar to the surrounding stratified squamous epithelium (Reutter 1992). Taste bud sensory cells are much more dispersed than olfactory sensory cells and often contain mechanical receptors which are sensitive to touch (Marui and Caprio 1992). The mechanical receptors are likely very important for the fish to determine food texture (somatosensation), which may be just as important as taste in determining acceptance or rejection of a feed pellet (Marui and Caprio 1992).

Since taste buds are located on external structures of some fish as well as in the mouth, the gustatory system in fish can play a role in localizing food in the environment even before it is taken into the mouth. Taste receptors, like olfactory receptors, can respond to chemical stimuli originating from a distant source. In some species such as the channel catfish (Carprio *et al.* 1993), gustatory and olfactory receptors are similar in sensitivity to some amino acids (Kanwal and Finger 1992). However, gustatory tissue is generally regarded to be less sensitive than olfactory tissue, especially in species that lack external taste structures such as barbels (Fig. 7.12). Compounds which are known to stimulate gustatory receptor cells include amino acids and

nucleotides, as well as marine toxins, CO_2, and hydrogen ions (Hara 1992c). The amino acids and nucleotides are likely related to feeding, while the other compounds may be important for recognition of poisonous prey or to aid in the avoidance of water with low dissolved oxygen concentrations (Hara 1992c).

7.3.2.3. Chemoreception and Feeding

Fish exposed to food-related chemical stimuli initiate feeding behavior (Jones 1992). Feeding behavior is actually a sequence of behaviors that often includes specific activities related to arousal, searching, taking into the mouth, and swallowing (Hara 1992b; Jones 1992; Caprio *et al.* 1993). It is sometimes difficult to determine if the response is primarily one from the olfactory system or the gustatory system or a combination of both; however, there is great potential to apply what is known about chemosensory physiology to feeding in an aquaculture setting.

Feed additives which increase the acceptability or palatability of prepared diets (called gustatory stimulants or palatability enhancers) are of great interest to the aquaculture industry because they increase fish growth rates and farm efficiency by increasing feed consumption (Takeda and Takii 1992). Gustatory stimulants may also be beneficial by restoring the palatability of diets containing unpalatable feedstuffs or additives. Examples of important feed ingredients with a low palatability for some fish include soybean and other plant meals and several medications for fish (Takeda and Takii 1992). Stimulants may be effective in feeds for young fish which are being "trained" to eat prepared diets after being reared on natural or live diets (Lovshin and Rushing 1989; Takeda and Takii 1992; see also Chapter 9, by Hardy). Stimulants may also be useful in starter diets designed for first-feeding larvae (Kasumyan and Ponomarev 1985; Takeda and Takii 1992).

Several chemicals have been identified as feeding stimulants to fish. The most common are amino acids, betaine, quaternary ammonium bases, and nucleotides (inosine 5′-monophosphate, AMP, UMP, *etc.*) (Fuke *et al.* 1981; Murofushi and Ina 1981; Mackie and Mitchell 1982, 1985; Mitchell and Mackie 1983; Hidaka *et al.* 1985; Ikeda *et al.* 1988a,b; Jones 1989; Takeda and Takii 1992). The effectiveness of specific compounds or combination of compounds differs among species (Jones 1992) but, for a given species, is often related to the type of prey item (and content of these chemicals in those prey items) that the species consumes in nature (Mackie and Mitchell 1985). Betaine plus amino acids tends to be more effective in species consuming invertebrates (worms, mollusks, and crustaceans), and nucleotides plus amino acids more effective in species consuming vertebrates (Mackie and

Mitchell 1985). In most cases, these substances exhibit synergistic effects when used in combination. For a general review on the subject of feeding stimulants used in aquaculture see Takeda and Takii (1992).

In mammals, the cephalic reflex is stimulated by the smell and taste of highly palatable foods and results in salivation, increased mucous secretions, gastric juice secretions, and pancreatic (exocrine and endocrine) secretions which prepare the alimentary tract and related systems for feeding. This is a reflex that we have all experienced when we smell our favorite meal being prepared. Although high-quality studies are lacking for fish, in at least one trial, diets supplemented with amino acids and nucleotides not only stimulated feeding activity but also may have resulted in early induction of the cephalic reflex in Japanese eel (*Anguilla japonica*) (Takade and Takii 1992). Fish fed the stimulants had not only a higher feed intake, but also enhanced growth performance beyond that due to increased feed intake. The cephalic reflex is known to stimulate digestion, absorption, and metabolism in mammals; however, it is still unclear if there is a similar stimulation in fish.

Chemoreception and associated feeding behaviors and physiological responses need to be investigated further in fish and the results applied to practical diet formulation and feeding. Given the differences in chemosensory physiology (olfactory and gustatory), it may be practical to target each system and elicit each behavior differently. For example, an attractant targeting the olfactory organ might stimulate arousal and searching behavior simply by being added to the water prior to feeding, while one targeting the oral gustatory system to enhance ingestion might be coated on the outside of a pellet. In species that masticate (chew) their food, the stimulant might need to be mixed throughout the pellet, while a coating may be sufficient for species that swallow prey (and pellets) whole.

7.4 Food Capture Structures and Organs

Once food is sensed and located, a fish must capture it so it can be ingested. The oral or buccal cavity (less specifically, the mouth) is the area where food is first consumed by the fish. It extends from the jaw to the esophageal sphincter. The posterior portion of the mouth, near the esophagus and gills, is called the pharynx. The structures which aid in capture and retention of food are the taste buds (previously discussed), teeth, gill rakers, tongue, and esophagus (Fig. 7.13). They all work in concert to capture, taste, retain (or reject), and then swallow (or expel) food items.

Mouth structure is highly variable among species and invariably related to feeding habits. Because these structures are easy to observe, and relate to how and what organisms are consumed in the wild, they are a logical place to start when developing a diet for a new species or predicting the appropriateness of a pellet type (hard or soft, floating or sinking). For example, does an organism possess the appropriate structures to feed on shellfish (then it can crush hard pellets)? Does it normally feed off the bottom (use sinking pellets) or on the surface (use floating or slowly sinking pellets)? and so on. By looking at the mouth it is easy to tell if the fish is a carnivore or

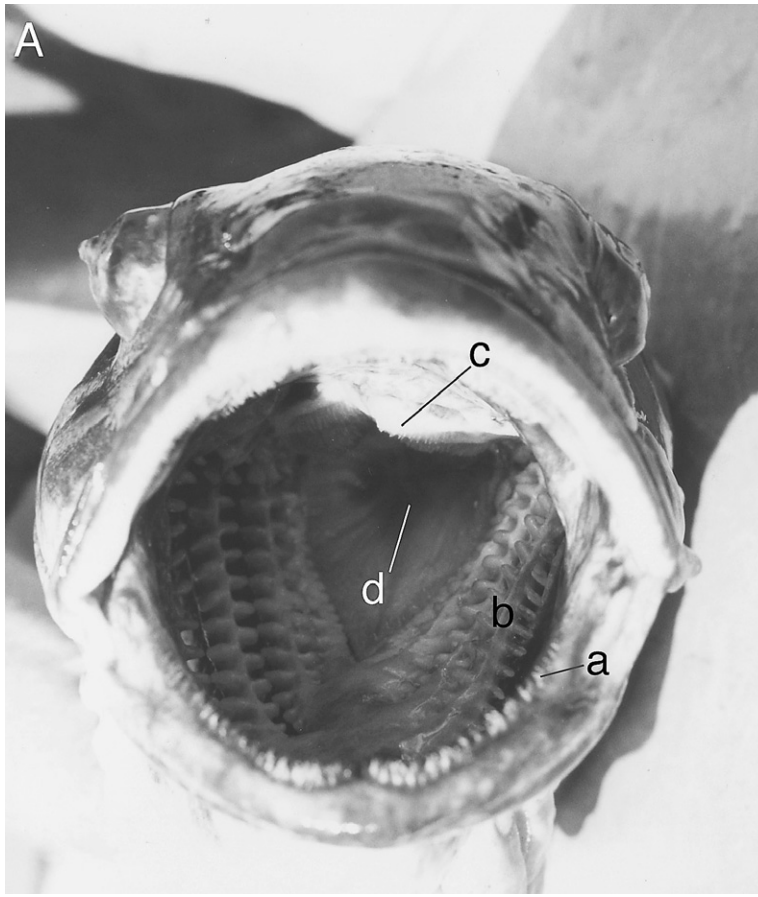

Fig. 7.13A

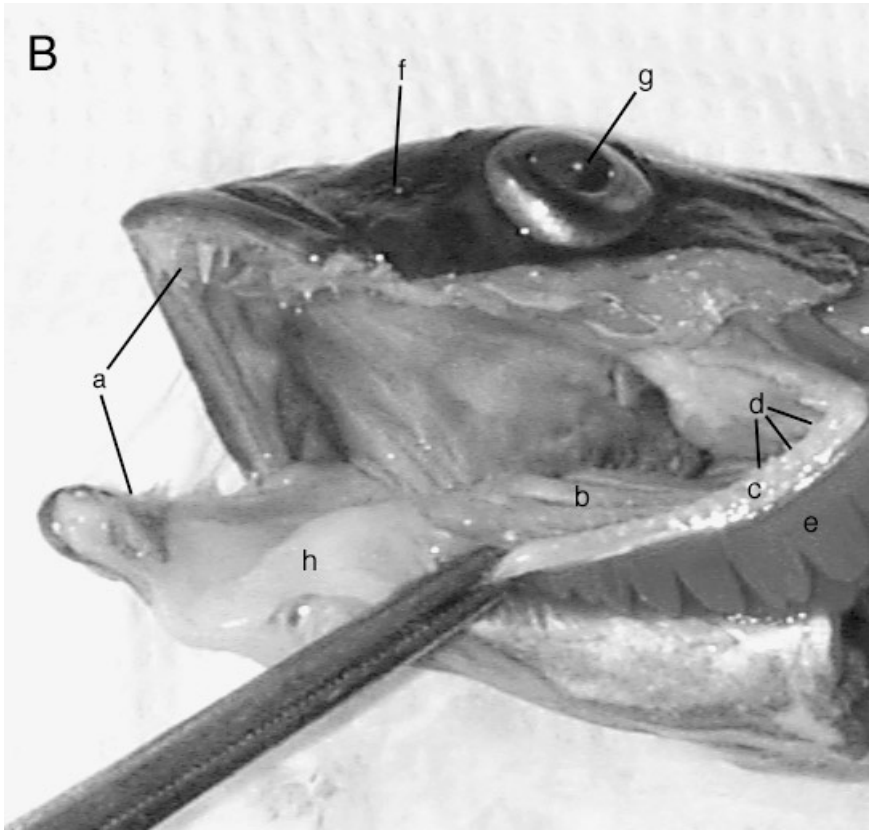

FIG. 7.13B (*continued*)

(A) Mouth of an adult sablefish. (a) Jaw teeth, (b) tooth-like gill rakers, (c) pharyngeal pad with multiple small pharyngeal teeth, and (d) esophageal sphincter. (B) Mouth of a juvenile lingcod, with the opercle removed. (a) Jaw teeth, (b) base of gill arches containing teeth-like rakers, (c) middle gill arch, (d) individual tooth-like gill rakers, (e) gill filaments, (f) olfactory pit, (g) eye, and (h) tongue. Photographs by Michael Rust.

omnivore, which may suggest what types of feedstuffs will be palatable, what potential feeding attractants might work, and what the general proximate composition of the diet is. The size of the mouth and the esophagus can also be used as a general guide for pellet size. While these traits can be used as a general guide, there are also many exceptions, and fish are often adaptable in a culture situation to foods that they would never eat in the wild.

7.4.1. Teeth

Teeth are located in several areas of the oral cavity: on the jaw (maxillary and premaxillary bones above and dentaries below), in the mouth (on the bones of the roof and sides of the oral cavity and, sometimes, on the tongue), and on plates in the pharynx (Figs. 7.3B and 7.13A). Tooth-like structures (gill rakers) also occur on the gill arches, especially in predatory fish.

The function of the teeth relates to their form (Lagler *et al.* 1977; Gosline 1985; Motto 1985; Herman *et al.* 1992; Peterson and McIntyre 1998). Sharp needle-like teeth (canine or cardiform) aid in grasping, puncturing, and holding prey, sharp-edged cutting teeth (incisors) help to slice prey into smaller bits, and broad, flat teeth (molariform) are used for crushing or grinding. The number and type of teeth relate to the natural feeding habits of the species. The dentition and oral anatomy of fish can also change over ontogeny, as larval, juvenile, and adult fish occupy different feeding niches during different life stages (Nakajima 1979, 1984; Luczkovich *et al.* 1995).

7.4.2. Gill Structures

The structure and function of gill rakers (Figs. 7.2, 7.5, 7.10, and 7.13) parallel or complement the structure and function of teeth in most respects. Like teeth, gill raker structure relates to feeding habit (Singh 1976; Wright *et al.* 1983; Livingston 1987; Guinea and Fernandez 1992; Chaudhuri and Datta 1994). Gill rakers in some species are fine and comb-like (or feather-like) and are used to strain small particles from the water in much the same way that whales use baleen to filter krill (Hampl *et al.* 1983). The gill rakers on predatory fish are sharp and pointed to hold and puncture prey. Other species have gill arches with molar-like teeth (rakers) used for grinding. While the gill rakers play a major role in feeding, they also serve to protect the delicate gill filaments located on the other side of the gill arches (see Figs. 7.2–7.8 and 7.13).

7.4.3. Esophagus

The esophagus serves as the transition from the mouth to the rest of the digestive system. Figure 7.13A (and Fig. 7.10) shows the anterior portion of the esophagus and the closed cardiac (or esophageal) sphincter of a sablefish. The portion of the esophagus (visible in Figs. 7.2–7.8), posterior to the sphincter, is continuous with the stomach in gastric fish or the intestine in stomachless fish and is similar histologically and functionally to those

organs (Hibiya 1982). In fact, some authors consider the cardiac sphincter to be the anterior of the stomach, and for all practical purposes this is the case (Smith 1989). The esophagus is highly elastic in predatory fish, expanding to hold the prey in the mouth while it is fed at a more controlled rate into the stomach. Mucus-secreting esophageal glands are located in the anterior portion of the esophagus to help lubricate the alimentary tract, while digestive juice-secreting glands (similar to chief cells or gastric glands in fish with stomachs) are located posterior of the sphincter. Evidence is lacking in fish whether cells anterior to the sphincter secrete any digestive enzymes or substances other than mucus (Smith 1989).

7.5 Digestive Organs

The digestive organs are where the nutrients are liberated from the food, broken down, and absorbed into the fish's system. The organs that are the sites of digestion include the posterior esophagus, stomach (in gastric species) or intestinal bulb (in agastric species), upper and lower intestine, and pyloric ceca. Supportive digestive organs are the pancreas, gallbladder, and liver.

Four distinct layers of tissue can be found in digestive organs of fish and other vertebrates (Hibiya 1982). These layers, starting from the lumen and working outward, are the mucosa, submucosa, muscularis (or muscular coat), and serosa (Fig. 7.14). The mucosa is composed of a great variety of columnar epithelium cells that form large undulated folds. The submucosa is a layer of connective tissue supporting the mucosa. The muscularis is composed of two layers of muscle (the outer longitudinal and the inner circular muscularis) that aids in movement of the contents of the lumen (digesta, bile, or other secretions). The serosa is a layer of mesothelial cells resting on loose connective tissue. All layers are not found in all tissues, and within a layer, there can be additional layered structures. Examples of the differences are pointed out in the sections that follow.

7.5.1. Stomach

The diversity of fish stomach configurations is very great. Harder (1975; cited by in Smith 1989) gives four general configurations: (1) a straight stomach with an enlarged lumen (Figs. 7.2 and 7.6; halibut and channel catfish), (2) a U- or J-shaped stomach with an enlarged lumen (Fig. 7.4; salmon), (3) a Y-shaped stomach with a cecum (Figs. 7.3, 7.5, and 7.7;

FIG. 7.14A

(A) Section of cardiac stomach near the esophageal junction from a juvenile lingcod. (a) Serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) bundles of skeletal or striated musculature (voluntary muscle), (e) submucosa, (f) submucosal veins, (g) attenuated stratum compactum, (h) gastric glands, (i) lamina propria, (j) columnar epithelium composing the mucosa, and (k) lumen. Hematoxylin and eosin (H&E) stain. Magnification: ×127. (B) Detail of a section of cardiac stomach near the esophageal junction from a juvenile lingcod. (a) Serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) bundles of skeletal or striated musculature (voluntary muscle), (e) submucosa, (f) submucosal veins, (g) attenuated stratum compactum, (h) gastric glands, (i) lamina propria, (j) columnar epithelium composing the mucosa, and (k) lumen. H&E stain. Magnification: ×254. (C) Sagittal section of the fundic stomach of a juvenile lingcod, demonstrating (a) serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) submucosa, (e) submucosal veins, (f) gastric glands, (g) columnar epithelium composing the mucosa, and (h) lumen. Also present external to the serosa are components of a mesentery, including (h) mesenteric vasculature and (i) adipose tissue. H&E stain. Magnification: ×51. (D) Detail of the fundic stomach of a juvenile lingcod, demonstrating (a) submucosa, (b) submucosal veins, (c) gastric glands, (d) submucosal lamina propria, (e) columnar epithelium composing the mucosa, and (f) lumen. H&E stain. Magnification: ×254. Photographs by Mark S. Myers.

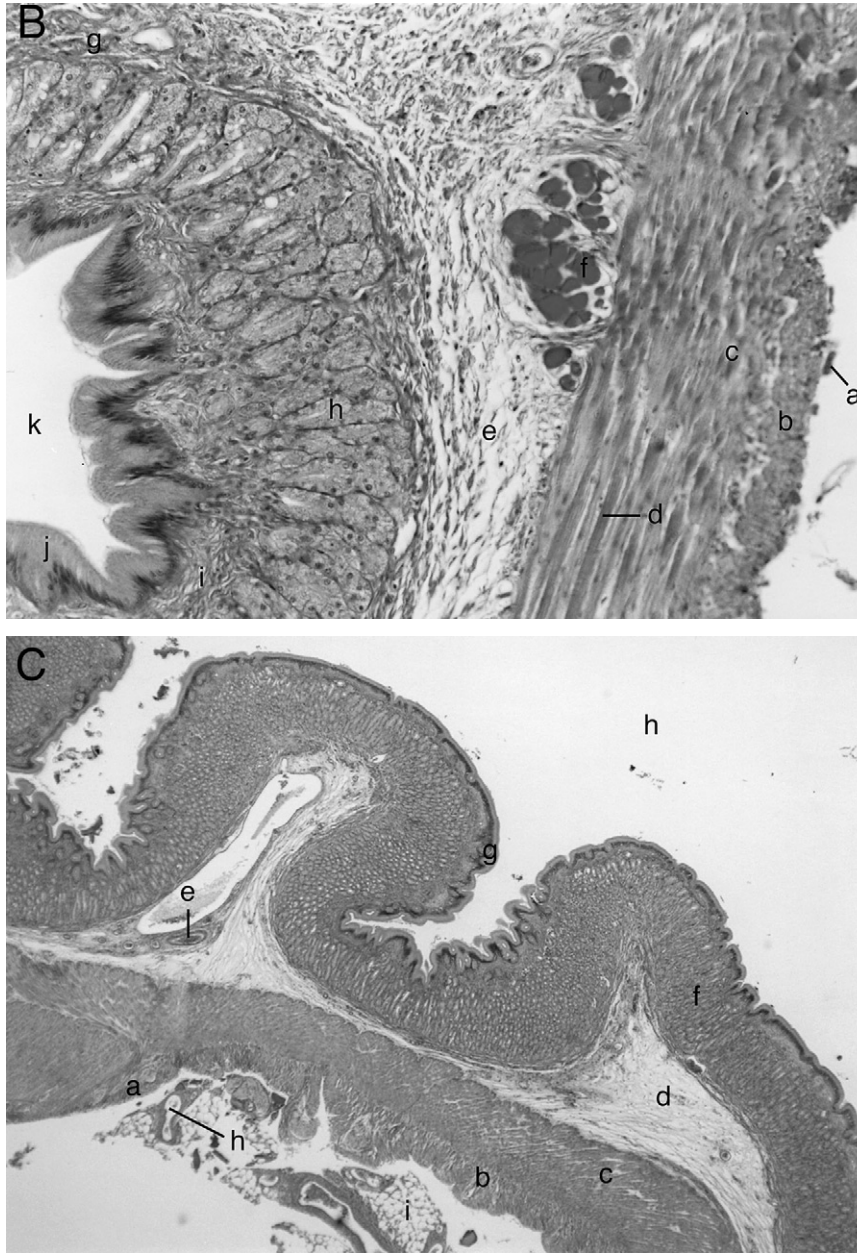

Fig. 7.14B–C (*continued*)

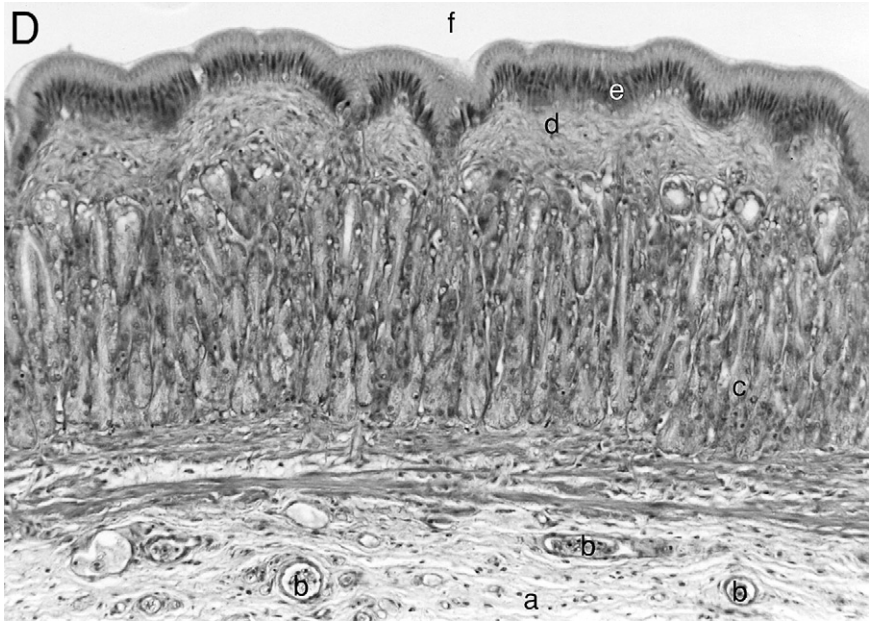

Fig. 7.14D (*continued*)

sablefish, lingcod, and tilapia), and (4) no stomach (Fig. 7.8; carp). Three of these stomach types are depicted graphically in Fig. 7.1 (from Smith 1989).

The stomach can be divided into the cardiac (anterior) portion and the fundic or pyloric (posterior) portion, separated by a transitional region. All four tissue layers, the mucosa, submucosa, muscularis, and serosa, are found in the stomach (Fig. 7.14). The mucosa can be further defined with layers of epithelium, gastric glands (also called serous cardiac glands or chief cells), lamina propria, stratum granulosum, stratum compactum, and sometimes a muscularis mucosa (Hibiya 1982). The gastric glands form pits (gastric pits) in the cardiac stomach that can extend deep into the mucosa and are the sites for acid and enzyme secretions from oxyntic cells or oxynticopeptic cells. Mammalian gastric glands have two cell types: oxyntic cells (also called parietal cells), which produce only acid; and chief cells, which produce only gastric enzymes. In most fish (and other lower vertebrates) there is only one cell type, oxynticopeptic cells, that produces both acid and enzymes (Shafi 1980; Michelangeli *et al.* 1988; Wang and Want 1989; Ostos Garrido 1993; Einarsson and Davies 1996). Gastric glands are less numerous in the fundic stomach (Smith 1989). As in other tissues, the lamina propria is a

layer of connective and vascular tissue. The stratum granulosum is made up of glandular tissue and held together by the collagen-rich stratum compactum. Often these two layers are somewhat intertwined. The muscle fibers of the muscularis are of two types: skeletal muscle (or striated) and smooth muscle (forming the circularis and longintudinalis) (Hibiya 1982). Skeletal muscle is found primarily in the cardiac stomach near the esophagus and is under the voluntary control of the fish (Figs. 7.14A and B). The possible use of this voluntary muscle is in grasping and holding prey and regurgitating or rejecting food. The muscularis circularis and longitudinalis are composed of smooth muscle and found in many digestive organs. Smooth muscles are under involuntary control. The circularis muscle bundles form rings that circle around the stomach so that they constrict the lumen when they contract. Longitudinal muscle bundles run along the long axis of the stomach and tend to shorten the stomach when they contract. Together these two involuntary muscle layers are responsible for the peristaltic movements that mix and move the digesta.

In addition to the oxynticopeptic cells that secrete pepsin and HCl, the mucosa has two other types of cells that have a major role in digestion: endocrine cells and mucous cells (Hibiya 1982; Smith 1989). The endocrine cells may be of several types, depending on the hormone they secrete (Wendelaar Bonga 1993). Examples are gastrin, somatostatin and pancreatic polypeptide. These hormones are discussed further in Section 7.10. Mucous cells can also be of several types, depending on whether they secrete sialomucins, sulfomucins, or neutral mucosubstances (Smith 1989).

The function of the stomach is to begin the process of breaking down the food into digesta. Gastric juice is secreted from the gastric glands to break down structural nutrients and release soluble nutrients. Gastric juice typically has a pH of 2.5 and contains a host of acidic digestive enzymes and hormones (see Table 7.1, Section 7.10, and Table 7.3). The stomach muscles mix and churn the contents to facilitate contact between the bolus (digesta) and the gastric juices.

7.5.2. Pylorus/Pyloric Sphincter

The pyloric sphincter (or pylorus) defines the posterior limits of the stomach in the same way that the cardiac (esophageal) sphincter defines the anterior limits of the stomach. It functions to feed the highly acidic digesta into the upper intestine at a controlled rate. The opening and closing of the pylorus therefore determine the gastric emptying time. This is important not only to aid in controlling the contact time between the food item and the gastric juices in the stomach, but also to control the amount

Table 7.1

General Characteristics of Some Digestive Enzymes in Fish[a]

Enzyme	Synthesis location	Action location	pH optimum	Substrate/action	Product(s)	References
Pepsin	Oxynticopeptic cells in the stomach mucosa	Stomach lumen	Acid	Nonspecific endoprotease activated by exposureto acid	Peptides	Lentner (1981), Glass *et al.* (1987, 1989), Smith (1989), Sabapathy and Teo (1993), Einarsson *et al.* (1996, 1997), Einarsson and Davies (1996, 1997), Douglas *et al.* (1999)
Trypsin	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Endoprotease activated by enterokinase. Catalyzes hydrolysis of peptide bonds next to amino acids with basic side chains	Peptides	Glass *et al.* (1985), Raae and Walther (1989), Smith (1989), Bjarnason *et al.* (1993), Sabapathy and Teo (1993), Slalaas *et al.* (1994), Einarsson *et al.* (1996), Einarsson and Davies (1996), Kuz'mina and Gelman (1997)
Chymotrypsin	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Endoprotease activated by trypsin. Catalyzes hydrolysis of peptide bonds next to amino acids with hydrophobic side chains	Peptides	Glass *et al.* (1985), Raae and Walther (1989), Smith (1989), Bjarnason *et al.* (1993), Sabapathy and Teo (1993), Einarsson *et al.* (1996, 1997), Einarsson and Davies (1996, 1997), Kuz'mina and Gelman (1997)
Elastase	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Endoprotease activated by trypsin that catalyzes hydrolysis of peptide bonds next to glycine or alanine	Peptides	Yoshinaka *et al.* (1982, 1983, 1984, 1985), Clark *et al.* (1985a,b), Glass *et al.* (1985), Raae and Walther (1989), Smine *et al.* (1993), Bassompierre *et al.* (1993), Sabapathy and Teo (1993), Bjarnason *et al.* (1993), Smine and Le Gal (1995), Kuz'mina and Gelman (1997), Aittaleb *et al.* (1997)

Collagenase	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Endoprotease activated by trypsin that primarily cuts collagen	Peptides	Yoshinaka *et al.* (1978a,b), Clark *et al.* (1985c), Divakaran and Ostrowski (1990), Bjarnason *et al.* (1993), Teruel and Simpson (1995), Divakaran *et al.* (1999)
Aminopeptidase (several types, acidic and neutral)	Stomach, exocrine pancreas, and enterocytes	Stomach lumen; lumen and brush border of intestine and pyloric ceca	Acid or neutral	Exopeptidase cleaves amino acid from the amino end of peptide chain	Small peptides and free amino acids	Lentner (1981), Smith (1989), Kuz'mina and Gelman (1997)
Leucine amino-peptidase (example of neutral amino-peptidase)	Enterocyte	Membrane linked in brush border	Neutral	Exopeptidase cleaves leucine from the amino end of peptide chain	Smaller peptides and free leucine	Smith (1989), Sabapathy and Teo (1993), Kuz'mina and Gelman (1997)
Carboxypeptidase	Exocrine pancreas and enterocytes	Lumen and brush border of intestine and pyloric ceca	Neutral	Exopeptidase cleaves amino acid from the carboxyl end of peptide chain	Smaller peptides and free amino acids	Lentner (1981), Smith (1989), Yoshinaka *et al.* (1984, 1985a,b), Kuz'mina and Gelman (1997)
Nonpancreatic lipase or gastric lipase	Gastric glands of stomach mucosa	Lumen of stomach	Acid	Primarily triacylglycerol lipase of gastric origin	See triacylglycerol lipase and phospholipase	Smith (1989), Gisbert *et al.* (1999)
Pancreatic lipase	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Includes multiple lipases of pancreatic origin, often bile salt activated	See triacylglycerol lipase and phospholipase	Smith (1989), Gjellesvik *et al.* (1994), Izquierdo and Henderson (1998), Iijima *et al.* (1998), Gisbert *et al.* (1999)
Triacylglycerol lipase	Stomach, exocrine pancreas, and enterocytes	Stomach lumen; lumen and brush border of intestine and pyloric ceca	Acid or neutral	Cleaves fatty acids from the 1 and 3 positions of triacylglycerol, sometimes with a colipase	2-Monoglycerides and free fatty acids	Lentner (1981), Smith (1989), Kuz'mina and Gelman (1997), Higgs and Dong (2000)

(*continues*)

Table 7.1 (*Continued*)

Enzyme	Synthesis location	Action location	pH optimum	Substrate/action	Product(s)	References
Monoglyceride lipase	Enterocyte	Brush border of intestine and pyloric ceca	Neutral	Cleaves fatty acid from 2-monoglycerides	Fatty acids and glycerol	Kuz'mina and Gelman (1997)
Phospholipase	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Cleaves fatty acids from phospholipids	Fatty acids and lysophospholipids	Izquierdo and Henderson (1998), Higgs and Dong (2000)
Wax ester hydrolase	Exocrine pancreas	Lumen of intestine and pyloric ceca	Neutral	Hydrolyzes wax esters	Fatty alcohols	Mankura *et al.* (1984), Smith (1989)
Amylase	Enterocyte, gut microflora	Membrane linked in brush border or associated with gut flora	Neutral	Hydrolyzes starch	Polysaccharides and monosaccharides (sugars)	Kuz'mina and Gelman (1997), Hidalgo *et al.* (1999)
Cellulase	Gut microflora; endogenous production has not been confirmed	Lumen of intestine	Neutral	Hydrolyzes cellulose	Glucose	Lindsay and Harris (1980), Gerking (1984), Das and Tripathi (1991), Saha and Ray (1998)
Chitonase	Stomach and exocrine pancreas	Lumen of stomach, intestine, and pyloric ceca	Acid or neutral	Hydrolyzes *N*-acetylglucosamine (chitin)	Polysaccharides and monosaccharides (sugars)	Danulat (1987), Smith (1989), Sabapathy and Teo (1993), Divakaran *et al.* (1999), Moe and Place (1999)

[a] Typically there will be several forms of each enzyme, with slightly different activities, pH optima, or substrates. See the references for more specific details on each enzyme.

of acidic material entering the intestine and pyloric ceca. Secretions from the pancreas and gallbladder enter the digestive tract via the common bile duct posterior to the pylorus and contain sodium bicarbonate and neutral digestive enzymes (Table 7.1). Thus the pH on the stomach side (anterior) of the pylorus is of the order of 2–5, while the pH on the intestinal (posterior) side of the pylorus is 7–8. The complement of enzymes secreted into the digesta also changes from those with peak activities in the acidic region to those with peak activities near neutral.

7.5.3. Pyloric Ceca

Located just posterior to the pylorus and stomach and branching from the intestine are the pyloric ceca (in some species). Ceca are individual sac-shaped structures with the open end filled from and emptying back into the upper intestine. Figures 7.2–7.8 show the diversity of size and number of ceca among species. For example, Pacific halibut (Fig. 7.2) have four large ceca similar in diameter to the intestine, while Atlantic salmon (Fig. 7.4) have numerous thin ceca much smaller in diameter than the intestine. Sablefish (Fig. 7.3) and lingcod (Fig. 7.5) are somewhat intermediate in both respects. The ceca are typically absent or much reduced in omnivorous and herbivorous species (Figs. 7.6–7.8). Ceca are often surrounded by pancreatic tissue and by ducts that deliver pancreatic juice and bile (Table 7.1).

Pyloric ceca are basically extensions of the upper intestine and are histologically similar to the upper intestine (Fig. 7.15). The one notable difference is that the ceca may have a narrower outer muscular wall than the intestine. The wall of pyloric ceca and intestine differs from the wall of the stomach in several ways. The pyloric ceca and intestine lack a prominent submucosa. Also, the stratum compactum and stratum granulosum are larger, while the muscularis is smaller than in the stomach (Hibiya 1982).

The function of the ceca and intestine is to continue the digestive process begun in the stomach and to absorb nutrients that are already liberated from the digesta. In the columnar epithelium of the mucosa of both the ceca and the intestine are mucous (goblet) cells and endocrine cells scattered among a great number of enterocytes (Hibiya 1982). Mucous cells secrete mucus and certain digestive enzymes. Endocrine cells secrete hormones that help to regulate digestion. These functions are discussed in more detail in Sections 7.8 and 7.10. Enterocytes have both digestive and absorptive functions and are key cells to the function of the digestive system. The enterocyte cellular membrane that borders the lumen is highly folded into thousands of microvilli. This region is termed the brush border due to its appearance under light microscopy (Hibiya 1982). The highly folded nature of the brush

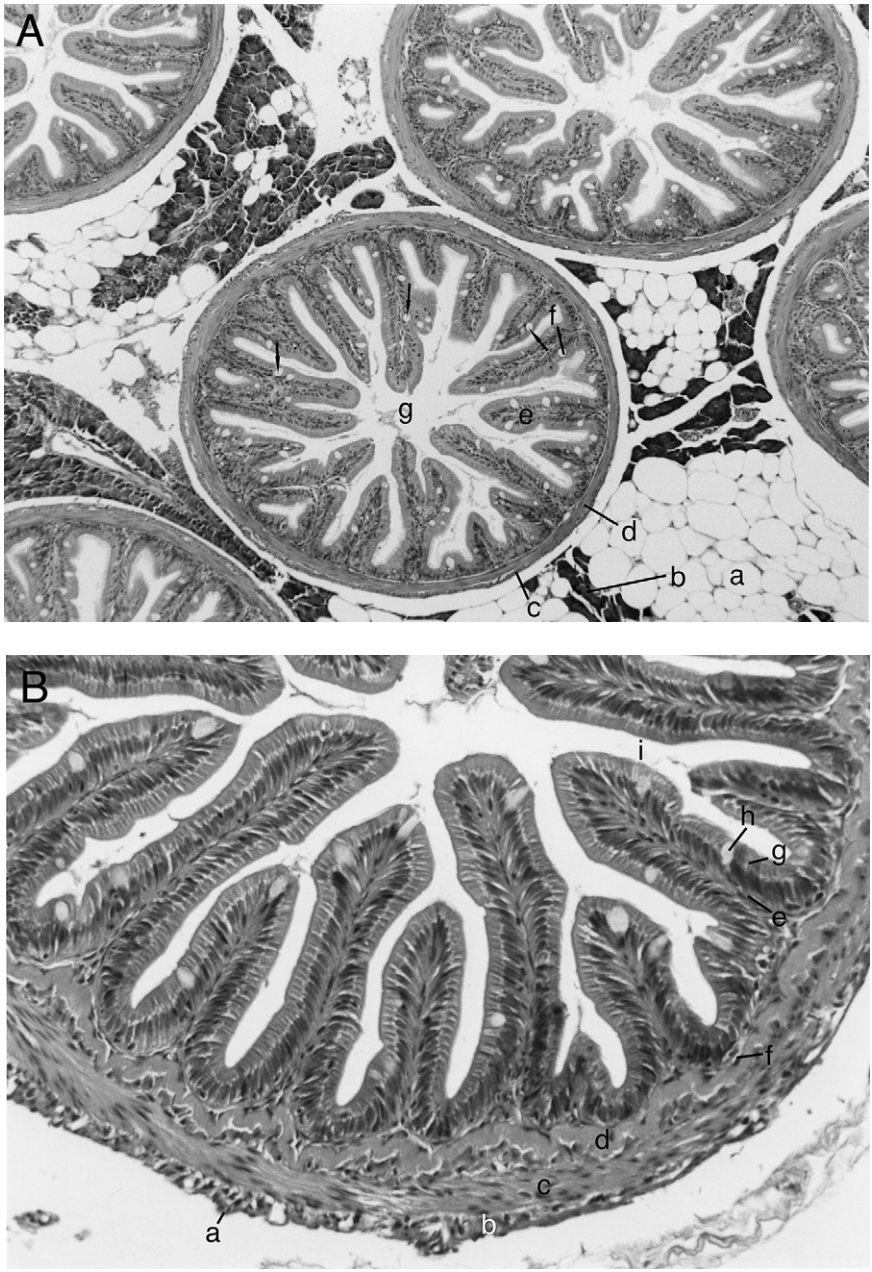

FIG. 7.15

(A) Sagittal section of multiple pyloric ceca in a juvenile lingcod. (a) Adipocytes composing the mesentric adipose tissue and (b) acini of exocrine pancreas

border increases many times over the surface area exposed to the lumen relative to an unfolded membrane. The practical result of this folding is to increase the absorptive capacity of the cell. Linked with the cell membranes of the microvilli are numerous enzymes responsible for both nutrient digestion and absorption (Kuzmina and Gleman 1997). The enzymes and processes involved are discussed in greater detail in Section 7.8.

7.5.4. Intestine

The intestine is commonly considered to have two parts: the upper (also called the small or ascending intestine, ileum, or midgut) anterior portion (Figs. 7.2, 7.3, and 7.16) and the lower (also called the large or descending intestine, rectum, or hindgut) posterior portion (Figs. 7.2, 7.3, and 7.17). These parts are divided by an ileocecal valve in some species and differ in gross anatomy, with the upper intestine typically being more slender than the lower intestine. Histologically, the upper intestine differs from the lower intestine in that goblet cells become fewer and the muscularis becomes thinner in the lower intestine. There may be a change from a columnar epithelium of secretory and absorptive cells in the upper intestine to a cuboidal to squamous epithelium that secretes mostly mucus in the lower intestine (Smith 1989). The lower intestine may also have an annulospiral septa that extends into the lumen. The function of the intestine is similar to that described for the pyloric ceca, with a decreasing secretory and nutrient absorptive function as the intestine progresses posteriorly.

7.5.5. Intestinal Bulbs and Gizzards

Some fish have an intestinal bulb which looks somewhat like a stomach, however, it is functionally and histologically more like a large single pyloric cecum. Differences that can be used to distinguish between a true stomach and an intestinal bulb are that (1) there is no pylorus at the posterior end of

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surrounding the pylorica ceca, (c) outer serosa, (d) muscularis longitudinalis and circularis, (e) lamina propria, (f) musosal epithelium with (arrows) scattered mucous cells, and (g) lumen. Hematoxylin and eosin (H&E) stain. Magnification: ×127. (B) Detail of pyloric cecum, juvenile chinook salmon showing the detailed histology of a pyloric cecum, including (a) outer serosa, (b) muscularis longitudinalis (outer smooth muscle layer), (c) muscularis circularis (inner smooth muscle layer), (d) stratum compactum, (e) lamina propria, (f) basement membrane, and (g) musosal epithelium, including (h) mucous cells, and (i) lumen. H&E stain. Magnification: ×254. Photographs by Mark S. Myers.

an intestinal bulb; (2) the bile duct always enters posteriorly to the pylorus in a fish with a true stomach, while it may enter anteriorly or directly into an intestinal bulb; and (3) the wall of an intestinal bulb is usually thinner and does not produce acidic secretions (Smith 1989).

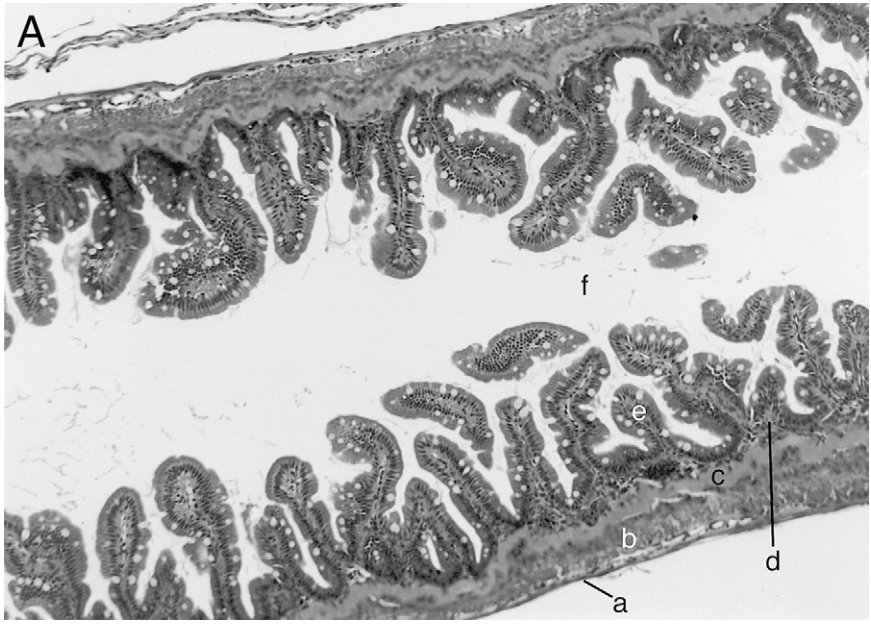

FIG. 7.16A

(A) Longitudinal section of the upper or small intestine of juvenile chinook salmon. (a) Outer serosa, (b) outer and inner muscularis layers, (c) stratum compactum, (d) submucosa and lamina propria, (e) mucosal epithelium with goblet cells, and (f) lumen. Hematoxylin and eosin (H&E) stain. Magnification: ×127. (B) Longitudinal section of the upper or small intestine in a juvenile lingcod. (a) Serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) submucosa, (e) lamina propria, (f) mucosal epithelium with goblet cells, and (g) lumen. H&E stain. Magnification: ×127. (C) Detail of a sagittal section of the upper or small intestine of a juvenile lingcod. (a) Serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) submucosa, and intestinal villi composed of (e) lamina propria, (f) mucosal epithelium with numerous goblet cells (indicated by arrows), and (g) lumen. H&E stain. Magnification: ×254. (D) Detail of the upper or small intestine of an adult English sole. (a) Serosa, (b) outer muscularis longitudinals, (c) inner muscularis longitudinalis, (d) submucosa and (e) lamina propria, (f) mucosal epithelium with goblet cells (indicated by arrows), and (g) lumen. H&E stain. Magnification: ×254. Photographs by Mark S. Mayers.

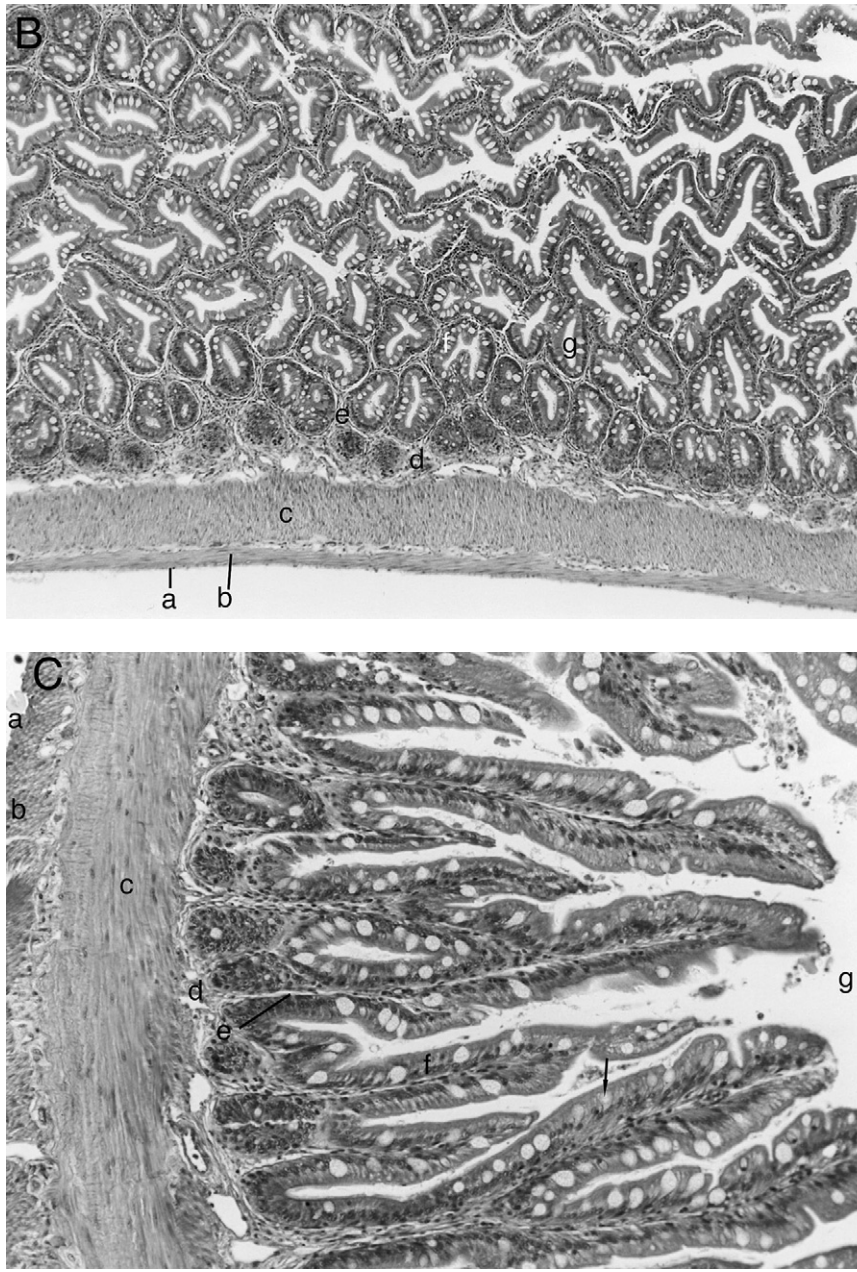

Fig. 7.16B–C (*continued*)

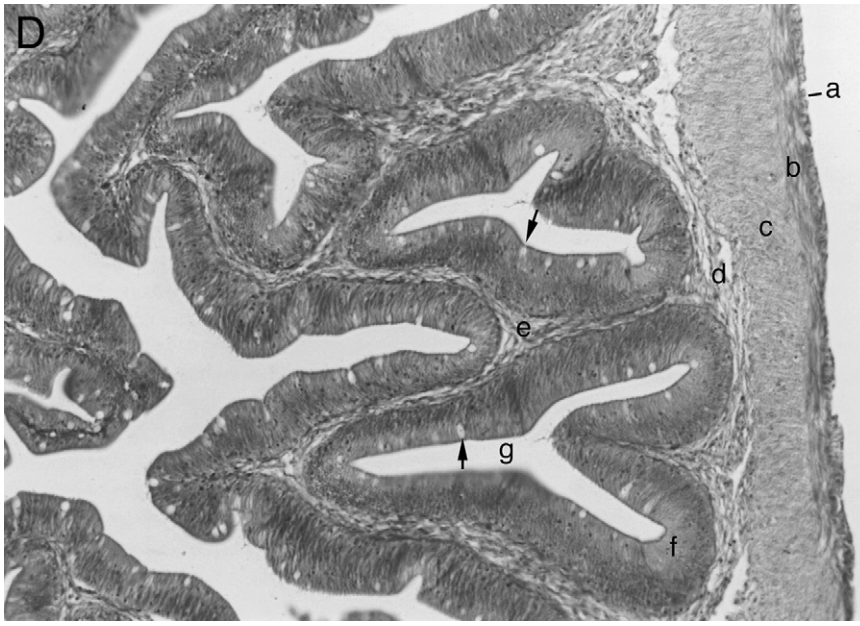

Fig. 7.16D (*continued*)

Some fish may also have a gizzard similar in function to the gizzard of birds. Unlike the intestinal bulb in fish and the gizzard of birds, the gizzard in fish can be posterior to, or not associated with, a true stomach (Smith 1989). Just as in birds, the gizzard in fish is used to grind the digesta into small food particles, thus increasing the surface area open to enzymatic action. If the fish is a planktivore, the gizzard can help to break the exoskeletons of zooplankton and the cell walls of phytoplankton.

7.5.6. Pancreas

The pancreas in fish can be either discretely located (such as in the Northern pike, *Esox lucius,* sharks, sturgeon, and anguillid eels) into one organ or diffuse (the more common case), with pancreatic nodules spread in the adipose, mesentery, and liver, around the bile duct, gallbladder, pyloric ceca, and intestine, and at other sites (Yasutake and Wales 1983; Smith 1989). When the pancreas is discrete it may form either one or two organs located near the spleen, along the portal vein, anterior to the liver, along the intestine, or in other areas near the intestine (Yasutake and Wales 1983; Smith 1989).

Pancreatic tissue is of two types: exocrine and endocrine. Secretions from exocrine pancreatic tissue (acinar cells) (Figs. 7.15, 7.18, and 7.19) include digestive enzymes and bicarbonate, and enter ducts connected to the intestinal or cecal lumen. Pancreatic ducts may be common with the bile duct from the gallbladder (which is then called the common bile duct) and/or be separate ducts (Figs. 7.18 and 7.19). There may be many small ducts associated with individual islands of exocrine pancreatic tissue leading to the lumen along the whole length of the intestine (Einarsson and Davies 1997). Exocrine pancreatic enzymes and their action and control are discussed in more detail in Sections 7.8 and 7.10. Differing from the exocrine tissue, endocrine pancreatic tissue does not secrete into ducts and is often arranged into clusters of glandular cells called Brockmann bodies or islets

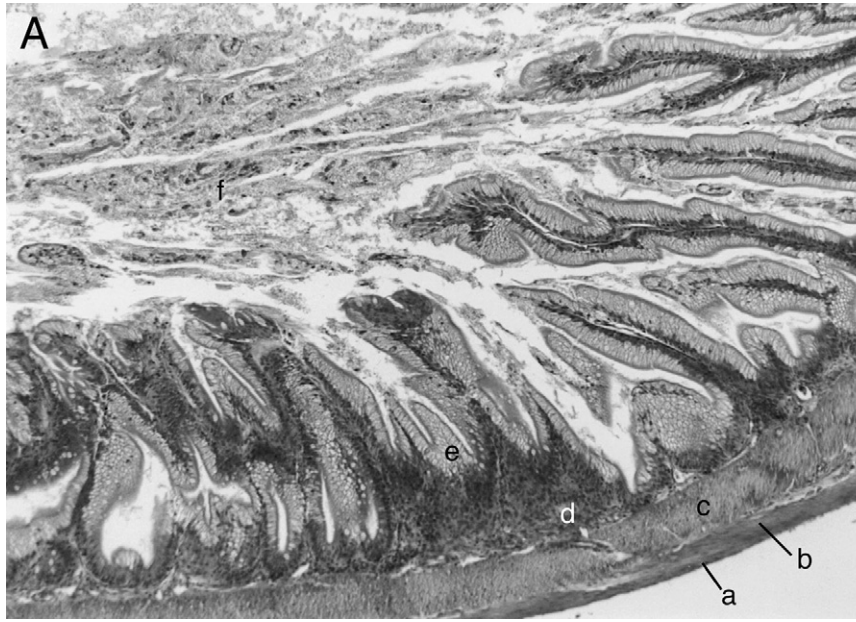

FIG. 7.17A

(A) Section of the lower or large intestine in a juvenile lingcod. (a) Serosa, (b) outer muscularis longitudinalis, (c) inner muscularis circularis, (d) submucosa and lamina propria, (e) mucosal epithelium with numerous goblet cells, and (f) lumen filled with digesta. Hematoxylin and eosin (H&E) stain. Magnification: ×127. (B) Longitudinal section of the lower or descending intestine of a juvenile chinook salmon. (a) Outer muscularis longitudinalis, (b) inner muscularis circularis, (c) stratum compactum, (d) lamina propria, (e) mucosal epithelium with abundant mucous cells, and (f) lumen. H&E stain. Magnification: ×127. Photographs by Mark S. Myers.

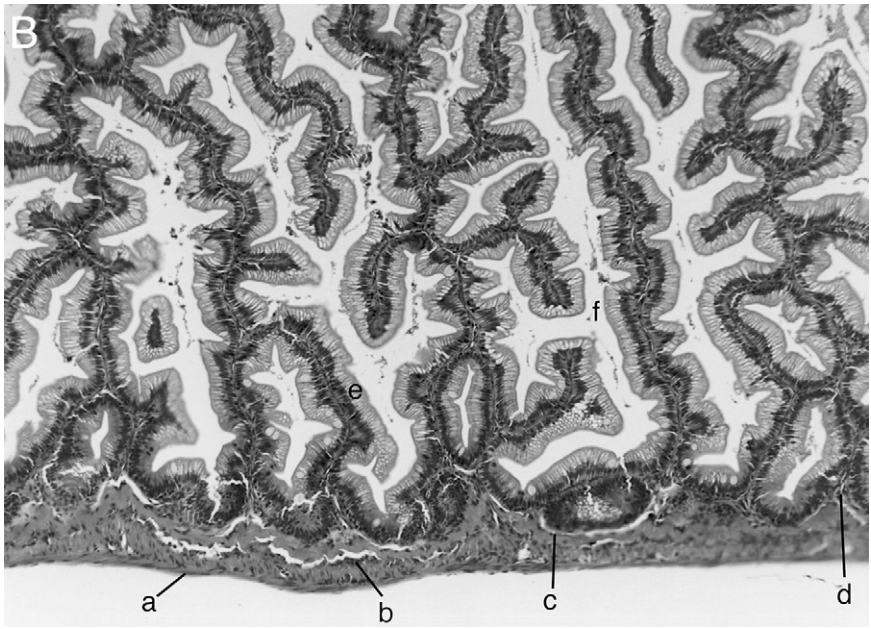

FIG. 7.17B (*continued*)

of Langerhans (Fig. 7.19) (Hibiya 1982). Endocrine pancreatic tissue is associated with blood vessels and/or the lumen and secretes hormones that regulate metabolism and digestion directly into the vasculature (Wendelaar Bonga 1993). Metabolism is discussed in more detail in Chapter 6, and regulation of digestion in Section 7.10.

Figures 7.18 and 7.19 show the fine structure of pancreatic tissue. Fish pancreatic exocrine cells resemble the typical vertebrate pancreatic acinar

FIG. 7.18

(A) Section of liver and exocrine pancreas (hepatopancreas) in an adult English sole. (a) Hepatic parenchyma composed of hepatic tubules, (b) vascular sinusoids, (c) macrophage aggregates, and (d) exocrine pancreatic tissue composed of pancreatic acini surrounding a centrally located hepatic portal vein (e) Hematoxylin and eosin (H&E) stain. Magnification: ×254. (B) Detail of exocrine pancreas in the liver of an adult English sole. Shown in the pancreas are (a) acini composed of exocrine pancreatic cells containing centrally located, eosinophilic zymogen granules (small arrows) and prominent nucleoli, (b) centrally located hepatic portal vein with erythrocytes, and (c) arteriole. Hepatic parenchyma (d) surrounds the pancreatic tissue. H&E stain. Magnification: ×508. Photographs by Mark S. Myers.

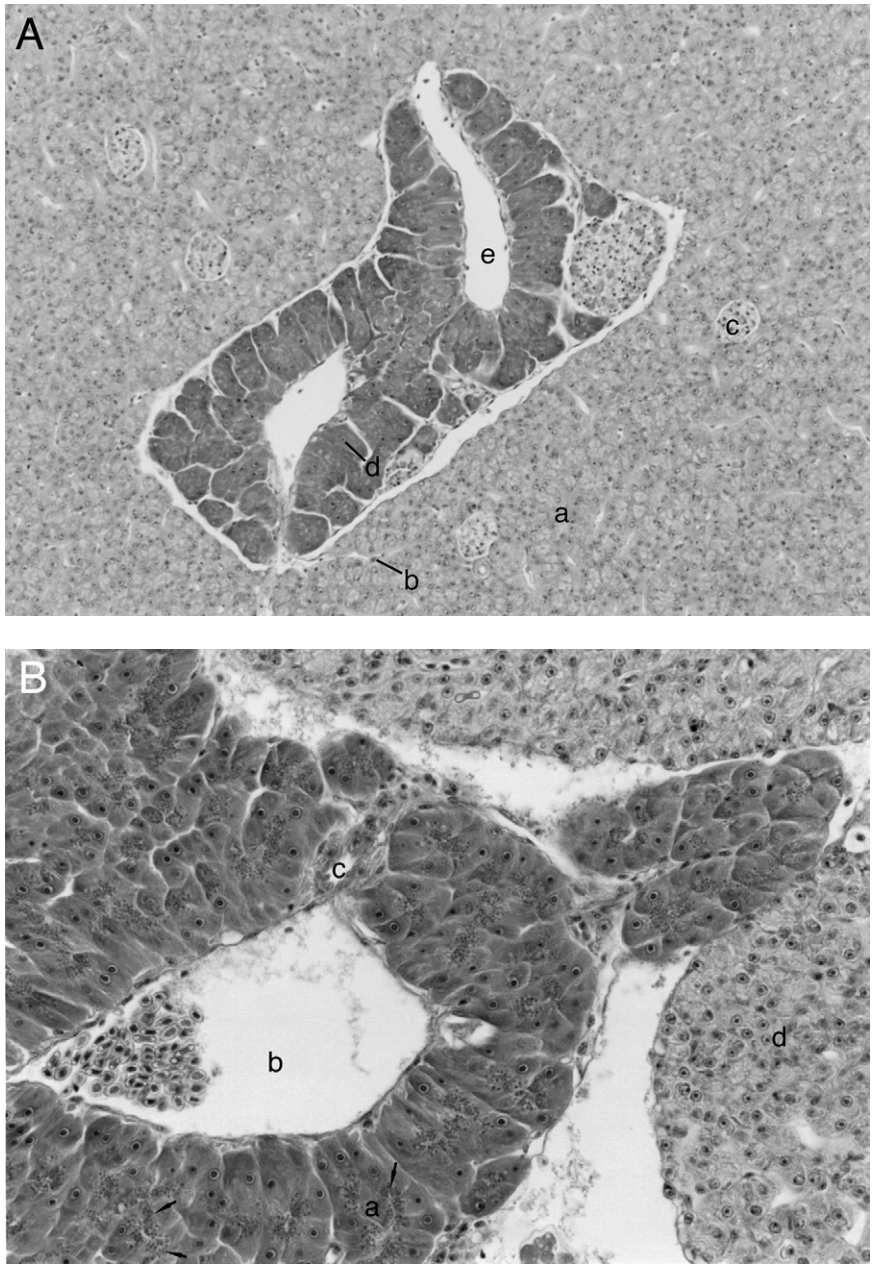

Fig. 7.18

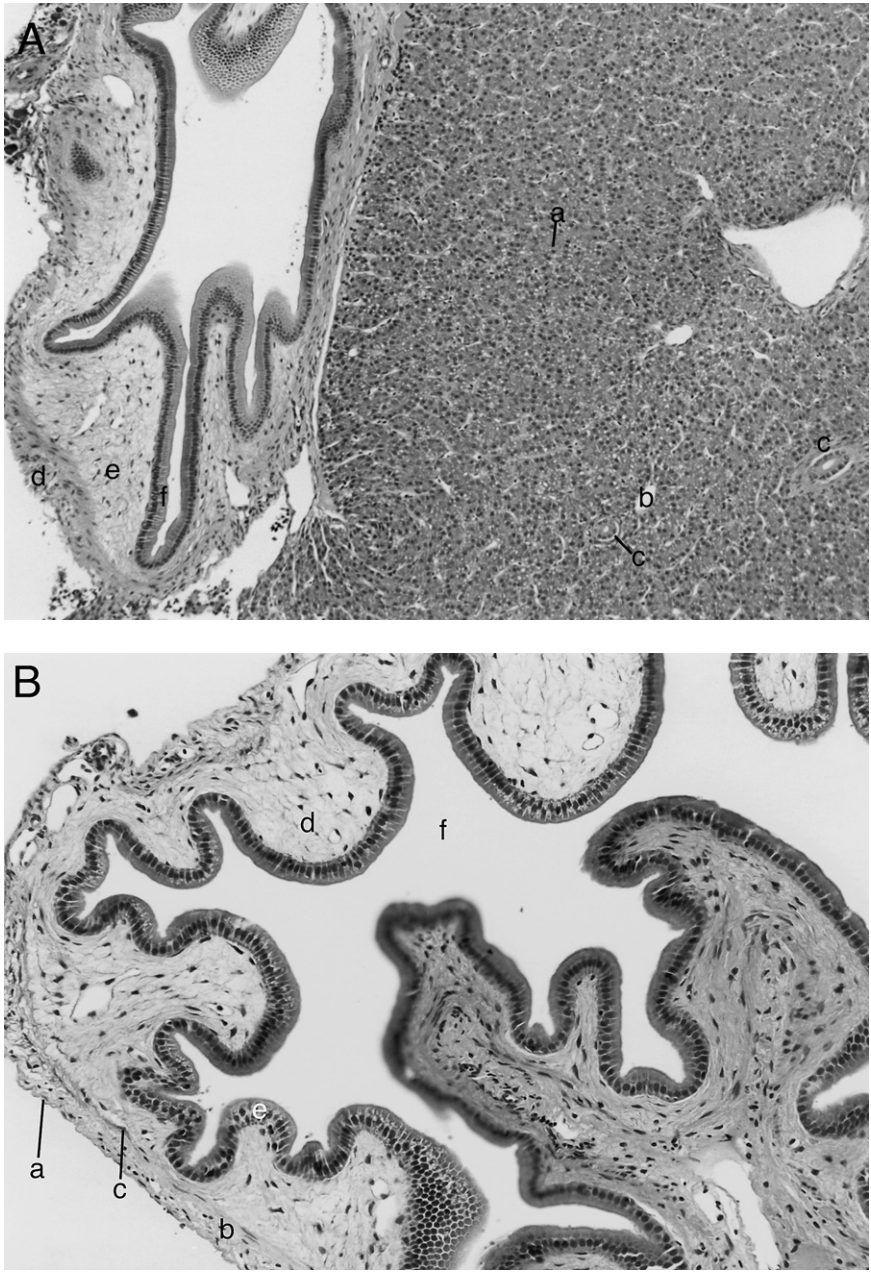

FIG. 7.19A–B

(A) Section of liver with adjacent gallbladder in a juvenile chinook salmon. Within the liver are (a) hepatic tubules composed of hepatocytes, b) hepatic veins, and (c) small bile ducts. In the gallbladder are (d) outer muscularis, (e) lamina propria, and

Fig. 7.19C (*continued*)

(f) mucosa composed of columnar epithelium. Hematoxylin and eosin (H&E) stain. Magnification: ×127. (B) Detail of sagittal section of a gallbladder from a juvenile chinook salmon. Demonstrated are (a) serosa, (b) muscularis, (c) stratum compactum and granulosum, (d) lamina propria, (e) lamina epithelialis (mucosal epithelium), and (f) lumen. H&E stain. Magnification: ×254. (C) Detail of gallbladder and adjacent exocrine and endocrine pancreatic tissue in a juvenile chinook salmon. Shown in the gallbladder are (a) the outer muscularis, (b) lamina propria, and (c) mucosa. Note also the large bile duct (d) adjoining the gallbladder. The endocrine pancreas represented by islets of Langerhans (e) are shown within surrounding exocrine pancreatic tissue. H&E stain. Magnification: ×254. Photographs by Mark S. Myers.

cells and are located in spherical clusters (Hibiya 1982). Fish endocrine pancreatic tissue has α, β, δ, and sometimes other cells similar in form and function to that of mammals and other animals.

7.5.7. Gallbladder

Most, but not all, fish have a gallbladder (Lagler *et al.* 1977). The gallbladder is a hollow spherical organ located adjacent to the liver and near the anterior portion of the intestine (Figs. 7.2–7.8) and is connected to the

intestine by the bile duct (or common bile duct if the exocrine pancreatic tissue also uses the duct). The gallbladder is histologically similar to other organs that have a lumen (Fig. 7.19). The gallbladder wall is made up of serosa, muscularis, stratum compactum, stratum granulosum, a lamina propria, and an epithelium (Figs. 7.19b and c) (Hibiya 1982). This basic structural motif is also seen in stomach, intestine, ceca, olfactory tissue, and other organs (Hibiya 1982).

The gallbladder stores bile and then excretes it into the intestinal lumen when food enters the intestine from the stomach (or the esophagus in stomachless fish). Contraction of the gallbladder is under endocrine and nervous control (Lagler *et al.* 1977; Smith 1989; Takeda and Takii 1992; Wendelaar Bonga 1993; Aldman and Holmgren 1995) in response to nutrients in the digestive tract and perhaps other stimuli. Control of digestion is discussed in greater detail in Section 7.10. Bile is made and recycled in the liver and is composed of a mixture of breakdown products, immunoglobulins, and xenobiotics that concentrate in the liver and gallbladder (Lentner 1981; Smith 1989). Two hepatic ducts, one from each lobe, flow into the cystic duct which terminates in the gallbladder (Lagler *et al.* 1977). There are two classes of breakdown products that have important digestive functions: bile salts and biliverdin/bilirubin (Smith 1989; Baldisserotto *et al.* 1990). Therefore the gallbladder plays a role in both digestion and waste removal (excretion).

Bile salts are the breakdown products of complex cyclic lipids such as cholesterol and steroids. While there are many specific bile salts and these may change over time and species, common bile salts include taurocholate, taurochenodeoxycholate, cholic acid, and allocholic acids (Smith 1989; Baldisserotto *et al.* 1990). Bile salts are produced in the liver and accumulate in the gallbladder via small bile ducts (Fig. 7.19). Bile salts in the digestive tract help to break up lipid into chylomicra (small droplets) so they can be absorbed by the enterocytes (discussed in more detail in Section 7.8). Of the order of 60–95% of bile salts are reabsorbed in the intestine and recycled to the liver (Smith 1989; Teshima *et al.* 1999).

The breakdown products of heme catabolism are bilirubin (red bile) and biliverdin (green bile). These compounds are what gives bile and the gallbladder a blue–green color. Dysfunction of the liver or gallbladder can result in a yellow–green or a blue–green coloration in serum, skin, and other tissues, although this might also occur naturally in some species (Smith 1989). The fullness (Gwak *et al.* 1999) and color of the gallbladder can also be used to determine how long it has been since it was contracted and emptied, with a full, dark-green gallbladder indicating that a long time has passed since the last meal. Bilirubin and biliverdin also function to emulsify lipids.

Bile also contains waste products, immunoglobulins, and metabolites of xenobiotic chemicals, such as toxins, pollutants, and a wide variety of other breakdown compounds (Lentner 1981). These compounds come from polluted water, feed, or absorption from the benthos and may be naturally occurring or man-made (Smith 1989).

7.6 Liver

The liver in fish may be arranged into two or more distinct lobes (Fig. 7.2) or as one single organ (Fig. 7.4). The majority of the mass of the liver is offset to one side. Note the different view of the liver in Fig. 7.5a, where the left side body wall of the lingcod was removed, compared to the liver covered by the digestive tract in Figs. 7.2–7.4, where the right side body wall was removed. In Fig. 7.5a, where the fish is facing left, the liver is on top of the digestive tract, while in the other figures, where the fish faces right, the liver is mostly covered by the digestive tract. In species with multiple lobes, one lobe will generally be on either side of the digestive tract; however, the lobes will be unequal in size. In general, the liver is located in the anterior portion of the peritoneal cavity in contact with the transverse septum. Anterior to the transverse septum is the pericardial cavity, where the heart is located.

The tissue morphology of fish livers is similar to that of other vertebrate livers with the exception that the microscopic anatomy of fish liver is not partitioned into well-organized, discrete lobules or acinar units (Yasutake and Wales 1983; Hibiya 1982). The liver is a highly branched maze of ducts, tubules, and sinusoids composed mainly of hepatocytes, with the solid portion organized into branching tubular units with various pancreatic and bile ducts, veins (or venules—small veins), and arteries (or arterioles—small arteries) interspersed (Fig. 7.20). At the gross level two lobes are distinct, even in species with a single liver. However, the lobes are not defined as they are in mammals. A more detailed description of the morphology of teleost liver is provided and reviewed by Hinton (1994).

The liver is supplied with nutrients and body metabolites by the hepatic portal vein coming from the alimentary tract and oxygen from the hepatic artery. This provides ample contact between the circulatory system and the liver tissue. The hepatic portal vein provides a direct conduit of nutrients absorbed in the digestive tract to the liver, where they can be further processed and sent to other body tissues. Liver hepatocytes (parenchymal cells) make up most of the liver volume and can contain

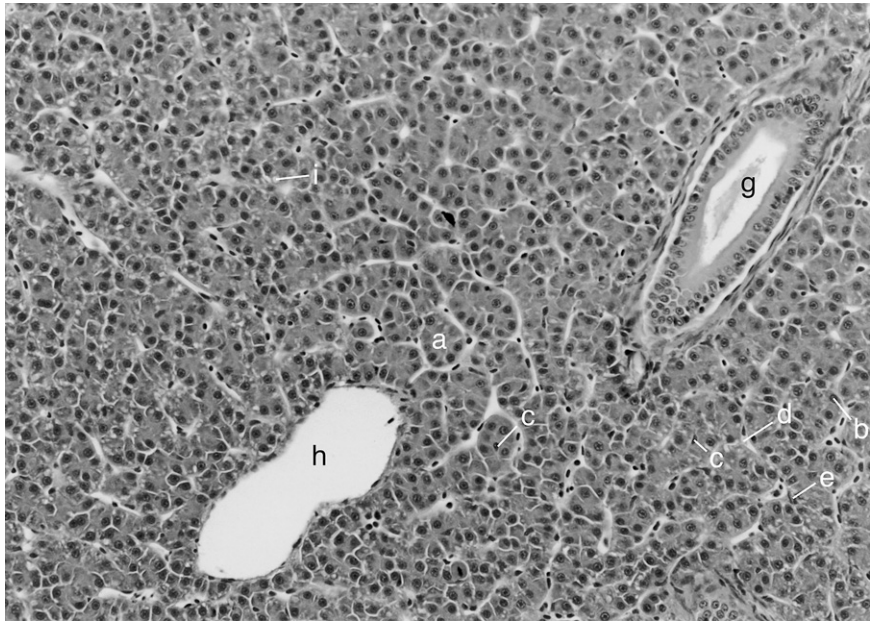

FIG. 7.20

Detail of the liver in a juvenile chinook salmon. (a) Hepatic tubules composed of (b) hepatocytes and (c) centrally located preductular biliary epithlial cells. (d) vascular sinusoids lined by (e) endothelial cells, (f) bile duct, (g) and (h) heptic vein. Clear zones within hepatocyte cytoplasm represent (i) glycogen vacuoles. Hematoxylin and eosin (H&E) stain. Magnification: ×254. Photograph by Mark S. Myers.

variable amounts of glycogen and lipid. Differences in the amount of glycogen vacuoles are clearly evident on comparing Figs. 7.18, 7.19, and 7.20. Changes in the gross and histological morphology as well as the amount of glycogen and lipid found in the liver are often related to the diet, health, toxin load, and energy status of the fish (Gingerich and Weber 1979; Hilton *et al.* 1981; Hilton and Dixon 1982; Smith 1989; Fynn-Aikins *et al.* 1993; Woods *et al.* 1995; Hemre *et al.* 1996, 2000; Tucker *et al.* 1997; Craig *et al.* 1999).

The liver also serves as the primary lipid storage organ in some species (Laglar 1977; Smith 1989). In fact, the extraction of oil from the livers of cod and sharks added significantly to the economic value of the fisheries for these species. The size of the livers in these species can be quite large, accounting for as much as 25% of the total weight of the fish. Other species store lipid in muscle and/or adipose tissue.

7.7 Anatomy and Diet

Smith (1989) correctly states that " fish maintain an intimate relationship between the form and function of their gut and their food resource." However, further generalization of this relationship needs to be made with care. The generalization that carnivores have short guts, omnivores have medium-length guts, and herbivores have long guts (relative to body length) has numerous exceptions (Smith 1989). Basing measurements on gut absorptive surface area to fish volume may show differences to be less pronounced, as this would take into account the ceca, the degree of epithelial folding, and specialized structures such as the presence of a spiral valve into the calculation of the digestive system size. In addition, it appears that fish, especially when raised in aquaculture, are more flexible in their diet than would be suggested by physiology or food preferences in the wild (Chan and Horn 1999).

Fish which feed on algae, coral, mollusks, detritus, or plankton represent special cases which do not often follow the general digestive anatomy of the carnivore, omnivore, and herbivore classifications outlined in Section 7.2. For example, both zooplanktivores and herbivores may have a gizzard. Presumably this helps to grind the plankton or plants, breaking down the chitinous exoskeleton or cell walls and improving digestion in the intestine. Some species have both a stomach and a gizzard or just one or the other (Smith 1989). Fish feeding on invertebrates containing a great deal of calcium carbonate (such as coral and mollusks) may lack acid (gastric) digestion even though they are carnivores. In addition, some tilapias which in the wild consume detritus, algae, and bacteria have extremely low pH stomach secretions (<1.5), which may be an adaptation to the need to lyse algal cell walls (Smith 1989; Chakrabarti *et al.* 1994). The existence of low-pH stomachs in herbivores has been demonstrated in a number of other species (Zemke-White 1999).

The role of microbes in digestion has been well studied in land animals and there may also be a significant role of gut microflora in fish. Microbes were responsible for a significant portion (perhaps all?) of the cellulase activity in a wood-eating catfish [*Panaque* sp. (Nelson *et al.* 1999)] and rohu carp [*Labeo rohita* (Saha and Ray 1998)], however, gut microflora played little role in the digestion of chitin in anchovy (Seiderer *et al.* 1987). The marine herbivore *Sarpa salpa* digested antibiotic-treated green algae (*Ulva* sp.) just as well as untreated algae, indicating that endogenous fish enzymes were responsible. During development, buffalo bream (*Kyphosus cornelii*) juveniles change from being strictly carnivorous to being strictly herbivorous (Rimmer 1986). Concurrently with this change, a previously nonexistent gut

microflora develops that is capable of fermentative digestion, allowing the switch to the strictly herbivorous diet (Rimmer 1986).

Understanding and manipulating the gut microflora in aquaculture species may be an important area of nutritional research in the future. One approach to this has been in the use of probiotic bacteria. Probiotic bacteria are commonly used in diets for terrestrial animals and are beginning to be used in aquaculture. Positive results with dietary supplementation of probiotics have been reported for turbot [*Scophthalmus maximus* (Gatesoupe 1997; Schrijver and Ollevier 2000)], Atlantic salmon [*Salmo salar* (Robertson *et al.* 2000)], rainbow trout [*Oncorhynchus mykiss* (Robertson *et al.* 2000)], rohu [*Labeo rohita* (Sahoo *et al.* 1999)], flounder [*Paralichthys olivaceus* (Cai *et al.* 1998)], and cod [*Gadus morhua* (Gildberg *et al.* 1996)]. However, Metaillier and Hollocou (1991) found no improvement from feeding three probiotics to sea bass (*Dicentrarchus labrax*). For a review of the use of probiotics in aquaculture see Gatesoupe (1999). For a review of the use of probiotics with larval fish see Skjermo and Vadstein (1999), Ringo and Birkbeck (1999), and Gatesoupe and Lesel (1998).

Much has been written about the relative environmental merits of culturing herbivores versus carnivores for food production. The main argument is that the culture of herbivores is environmentally preferred because herbivores feed lower on the food chain. Herbivores are usually cultured in fertilized ponds, where the plant, plankton, and bacterial material that serves as feed are produced concurrently in the same pond. Increasingly, even in highly fertilized ponds, supplemental feeds are being used to improve yields (see Chapter 13, by Ghittino). Carnivores are usually produced in intensive systems using high-protein complete feeds, which are produced externally to the aquaculture system. Traditionally, complete feeds have been based on fish meal, seafood by-products, and other animal proteins; however more and more plant-based proteins are now being used in diets for "carnivorous" fish (see Chapter 9, by Hardy).

From the perspective of the digestive system of agastric (stomachless—mostly herbivores) and gastric (with a stomach—mostly carnivores) species, the most important difference in diet is nutrient density rather than nutrient source. For the most part, all fish, regardless of feeding mode and physiology, require the same 40 or so nutrients, regardless of whether they come from plants, animals, industry, or by-products. Complete feeds have a much higher nutrient density than feed organisms produced in a fertilized pond. Smith (1989) indicates that the protein assimilation efficiency of stomachless herbivores (i.e., agastric fish) is typically about 50%, while carnivores (i.e., gastric fish) are approximately 80%. However, it is unclear if this difference is due to the differing nutrient density of the diets or different digestive efficiencies between gastric and agastric species. A definitive study on this

subject has yet to be done. The low assimilation efficiency of agastric fish eating pond-produced food organisms is not much of a disadvantage, because the undigested (excreted) protein stimulates the production of more feed organisms. However, in an intensive aquaculture system, this could be a significant disadvantage as the protein will be lost, decreasing total system efficiency and potentially causing nutrient pollution.

Nutrient-dense prepared feeds made from plant feedstuffs challenge traditional definitions when these diets are fed to carnivores. Likewise, supplemental feeds containing animal feedstuffs fed to improve the production of herbivores blur the distinction between the classical ecology-based herbivore and carnivore classifications. From an environmental standpoint it may be advantageous to reserve nutrient dense plant- and animal-based feedstuffs for the species that can most efficiently utilize them, be they carnivore or herbivore. The ability of a species to utilize efficiently by-products of seafood and other animal production industries should also not be overlooked when determining the environmental merits of culturing carnivores. Since the dress-out percentage (round weight to fillets) from wild-caught fish is typically of the order of 50% (Crapo *et al.* 1988), there is a considerable amount of processing waste that could be utilized as feedstuffs in aquaculture (Rathbone *et al.* 2000). While there is no doubt that fertilized ponds are an environmentally advantageous method of producing fish in aquaculture, there is also benefit to having a diversity of species with different physiological adaptations available for culture in different circumstances. Species which can recycle waste products into human foods provide environmental advantages.

7.8 Digestive Processes

The process of digestion is a coordinated combination of physical, chemical, and enzymatic activities that begins as soon as food is taken into the mouth and ends when feces are excreted from the anus. Included in the physical processes are puncturing, crushing, and mixing. The secretion of acid in the stomach is a chemical process that helps to hydrolyze and break down compounds. Enzymatic processes are more specific for the molecular breakdown of proteins, lipids, and carbohydrates and involve a large number of enzymes. Enzymes and cellular processes also actively and passively transfer all types of nutrients from the lumen across the brush border into enterocytes. These nutrients include proteins, polypeptides, amino acids, lipids of all types, carbohydrates, vitamins, carotenoid pigments, and minerals.

7.8.1. Physical Processes

Physical processes start in the mouth where food is punctured or crushed by the teeth, buccal pads, and gill rakers. Straining may also occur to concentrate small particles for filter feeders. Once feed is transferred to the stomach and intestine, muscles contract to mix the digesta with acid and other digestive juices. The strong muscles of the gizzard helps to break up cell walls, chitin, and other hard particles. Peristalsis transfers the digesta down the digestive tract toward the anus while it also mixes the digesta. This puts nutrients in close contact with the enzymes located in the brush border and digestive juices.

7.8.2. Chemical Processes

In species with true stomachs, hydrochloric acid is secreted by oxynticopeptic cells (both acid and pepsinogen is produced in these cells) into ducts connecting to the lumen when food is present. This secretion is under both endocrine and nervous control (Wendelaar Bonga 1993). The acid denatures proteins and carbohydrates, breaks down cellular structures, emulsifies lipids, and generally make the nutrients in the digesta more available to enzymatic break down and nutrient release (Smith 1989; Chakrabarti *et al.* 1994). The activity of acidic digestive enzymes such as pepsin, gastric lipase, and chitinase is higher at a low pH.

Once digesta is passed out of the stomach, bicarbonate is secreted. Bicarbonate is produced by the acinar cells in the exocrine pancreatic tissue (Figs. 7.15, 7.18, and 7.19). These secretions enter ducts connected to the gallbladder and/or directly into the intestinal/cecal lumen. Bicarbonate raises the pH of the digesta to a range where the digestive enzymes secreted into the intestine and pyloric ceca have high activity and protects the brush borders of the enterocytes from the hydrochloric acid of the stomach.

7.8.3. Enzymatic Processes

Enzymes function to break down nutrients in foods into compounds that can be absorbed across the brush border membrane of the enterocyte. These enzymes can be excreted into the lumen, bound to membranes, or contained in supranuclear vacuoles within the enterocyte. Therefore, digestion can occur in fish using one or more digestive motifs: extracellular (or lumenal) digestion, membrane-linked digestion, and intracellular digestion. There has been a great deal of focus on extracellular digestion in the lumen and, to a lesser extent, membrane-linked digestion (reviewed by

Kuz'mina and Gelman 1997), but very little work has been done with intracellular digestion. The functional importance of these modes of digestion appears to change during ontogeny (discussed in Section 7.11) and perhaps phylogeny; however, in the more generalized case, all likely work in concert.

Classes of enzymes are often named for the function they perform or the type of compound on which they act. Often the compound name followed by the suffix"-ase" is the name of the enzyme. For example, proteases act on proteins, lipases on lipids, and so on. Enzymes are stored in cells in an inactive form (proenzymes or zymogens) and become active only after they are secreted, when there is digesta or acid present, or when they are acted on by another enzyme. The inactive proenzyme form is often noted by the addition of the "-ogen" suffix. For example, pepsinogen is the inactive storage form of the active enzyme pepsin. In addition, there are often two or more enzymes with slightly different activities, affinities for substrates, pH optima, or tissue locations, but with the same function. These are often denoted by a letter or number after the enzyme name. For example, carboxypeptidase A and carboxypeptidase B are both enzymes that cleave amino acids from the carboxy end of a peptide chain. There are also isozymes of most, if not all, enzymes. Table 7.1 provides a list of some of the digestive enzymes in fish along with a description of their function, the location where they are active in the digestive tract, and the tissue producing the enzyme.

7.8.3.1. Proteases and Peptidases

The general digestive scheme for protein in monogastric animals occurs in the lumen of the digestive organs in a linear fashion, where protein is broken down to polypeptides by proteases, polypeptides are broken down into free amino acids by peptidases (both extracellular and membrane linked), and free amino acids are absorbed (Fig. 7.21). However, it has also been shown that protein assimilation in larval and adult fish can occur by pinocytosis and intracellular digestion in the posterior intestine (Gauthier and Landis 1972; Noaillac-Depeyre and Gas 1978; Stroband and Van der Veen 1981; Watanabe 1981, 1982; Gabaudan 1984). In addition, peptides can be absorbed directly (Baumrucker *et al.* 1989; Gaertner *et al.* 1989; Sarwar and Paquét 1989), and in developing rats, small peptides are assimilated at a higher rate than free amino acids (Gardner 1984).

Since protein has numerous levels of structure, there are a great number of proteases and peptidases, each having a specific function relating to the protein structure (Table 7.1). The amino acid (polypeptide) chain makes up the primary (1°) structure of protein. The coiled or helical shape of the chain formed by local hydrogen bonding of the amino acids with

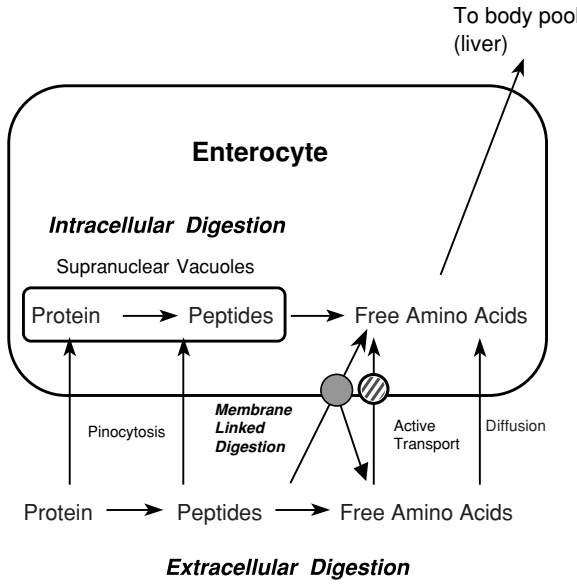

FIG. 7.21

Protein digestion in fish. Digestion can occur inside the enterocyte (intracellular digestion), in association with enterocyte membranes (membrane-linked digestion), and outside (extracellular or lumenal digestion) of the enterocyte.

other nearby amino acids represents the secondary (2°) structure. More distant bonding (hydrogen, electrostatic, and other types of bonding) within a chain and/or bonding of two or more peptide chains creates tertiary (3°) structure. Sometimes, several 3° protein subunits come together to give a protein quaternary (4°) structure. Acid and proteases (such as pepsin in the stomach and trypsin in the intestine and ceca) break down the 4°–1° structure. Peptidases cut peptide bonds between amino acids, which breaks down the 1° and 2° structure.

Proteases can be endoproteases, which cut in the middle of a peptide chain, or exoproteases, which work on the free ends of peptide chains. For example, aminopeptidase is an exopeptidase that cuts amino acids from the amino end of a peptide chain one at a time. Proteases and other enzymes can also be classified based on their optimal pH (acid or neutral), the tissue of production (gastric, pancreatic, mucosal, or even bacterial for enzymes produced by gut microflora), and the mode of digestion (intercellular,

membrane linked, extracellular). The ultimate function of the extracellular and membrane-linked suite of proteases is to break down the ingested protein into amino acids and peptides so they can be absorbed across the brush border of the enterocytes. Often, the membrane-linked enzymes function both to cleave the peptide chains and to transport the products across the brush border (Kuz'mina and Gelman 1997).

Proteases are important to activate the released zymogens of a great number of digestive enzymes into the active form. The classical example of this is the cascade of activation that occurs with the pancreatic enzymes. Trypsinogen is activated by limited proteolysis by enterokinase, a protease specific for this function. The active trypsin then activates other digestive zymogens into the active enzymes. Examples of enzymes activated by trypsin include chymotrypsin, elastase, collagenase, and lipase.

7.8.3.2. Lipases

The general digestive scheme for lipids (Fig. 7.22) is extracellular hydrolysis of lipids (Higgs and Dong 2000) in the stomach, intestinal, and cecal lumen by a variety of lipases and colipases (Sargent *et al.* 1989; Smith 1989) (Table 7.1). It does not appear that membrane-linked digestion plays a significant role in lipid digestion (Kuz'mina and Gelman 1997), although this needs to be investigated further. In some species there is hydrolysis in the stomach by gastric lipase (Gisbert *et al.* 1999), but the primary site of lipid hydrolysis for most species appears to be in the pyloric ceca and anterior intestine. It is generally understood that short chain fatty acids (2–10 carbons) and glycerol are absorbed directly through the brush border of the enterocytes. Long-chain fatty acids (12 and more carbons) are cleaved by lipase and emulsified by bile salts to form negatively charged aggregates called micelles. Micelles are transported from the lumen to the brush border, where they dissociate and the fatty acids diffuse across the epithelial membrane. Once inside the enterocyte, the fatty acids are reesterified and grouped with proteins to form aggregates called chylomicra (singular is chylomicron).

Lipases, like proteases, are named to reflect the substrates on which they work; triacylglycerol lipase cleaves the fatty acids from triglycerides; phospholipases cleave fatty acids from phospholipids, and so on (Table 7.1). The most important lipids found in fish diets are triacylglycerols, wax esters, cholesterol/cholesterol esters, and phospholipids (Sargent *et al.* 1989; Higgs and Dong 2000) (see Chapter 4, by Sargent *et al.*). These substrates are hydrolyzed to free fatty acids (from all dietary lipids), glycerol, 2-monoacylglycerol (from triacylglycerol and phospholipids), fatty alcohols (from wax esters), sterols (from cholesterol), and lysophospholipids (from phospholipids). These compounds are absorbed across the brush border

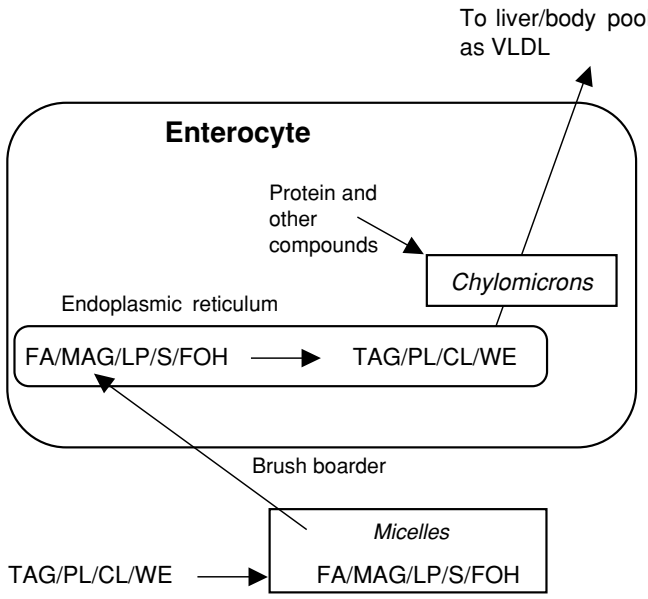

FIG. 7.22

Lipid digestion in fish. Digestion is extracellular with the formation of micelles that transport lipids across the brush border. TAG, triacylglycerol; PL, phospholipid; CL, cholesterol; WE, wax esters; FA, fatty acids; MAG, monoacylglycerol; LP, lysophospholipids; S, sterol; FOH, fatty alcohol; VLDL, very low density lipoprotein.

primarily in the anterior and midintestine and cecum (Sargent *et al.* 1989; Higgs and Dong 2000). Lipids are also absorbed (by pinocytosis) as small droplets (Smith 1989). It appears that the bioavailability of fatty acids can differ among species and due to the fatty acid chain length and degree of saturation (Sargent *et al.* 1989; Higgs and Dong 2000). This is likely due to differences in the specificity for substrates among lipases.

7.8.3.3. Carbohydrases

The general digestive scheme for carbohydrates is hydrolysis of complex carbohydrates extracellularly in the stomach, intestine, and ceca (Danulat 1987; Sabapathy and Teo 1993; Moe and Place 1999; Divakaran *et al.* 1999) and in association with membrane-linked hydrolysis (Kuz'mina and Gelman

1997) in the anterior intestine (Kuz'mina 1985; Olatunde *et al.* 1991; Nevalyonny *et al.* 1991; Kuz'mina 1988) and ceca (Glass *et al.* 1987, 1989) by a variety of carbohydrases (Smith 1989) (Table 7.1). The products of this hydrolysis are polysaccharides and monosaccharides (sugars), which are more easily assimilated. In mammals, monosaccharides are transported from the lumen of the small intestine by an active transport mechanism in the brush border (Lentner 1981). It is unclear if this is also true for fish.

Starches, polysaccharides, and cellulose are the primary carbohydrates in plants, whereas chitin is the primary carbohydrate in invertebrates. Animals also store a limited amount of glucose as glycogen; however, it is unlikely that much glycogen remains by the time the organism reaches the gut (Kuz'mina 1996).

The ability to digest and utilize carbohydrates differs greatly among species (Buddington and Christofferson 1985; Olatunde *et al.* 1991; Kuz'mina *et al.* 1996; Kuz'mina 1996; Chan and Horn 1999; Mwachireya *et al.* 1999; Divakaran *et al.* 1999). Differences in digestibility are likely due to differences in the amount and types of carbohydrases found in different species (Clark *et al.* 1984; Olatunde *et al.* 1991; Sabapathy and Teo 1993; Chan and Horn 1999). Uncooked (raw) starch is poorly utilized by many species (Cowey and Walton 1989; Hemre *et al.* 2000). In contrast, gelatinized (cooked) starch (Takeuchi *et al.* 1990; Hemre *et al.* 1996) and simple polysaccharides such as dextrin (Ufodike 1988) appear to be better digested in fish. Several studies have found a positive correlation with the degree of gelatinization and the digestibility of starch (Jeong *et al.* 1991, 1992a,b; Podoskina *et al.* 1997).

Once digested, the carbohydrate may or may not be well utilized by the fish. The rainbow trout (*Oncorhynchus mykiss*) is known to poorly utilize high levels of dietary carbohydrates for energy. No protein-sparing or enhanced growth effect was noted when diets containing elevated levels of digestible carbohydrate (gelatinized potato starch) were fed to trout (Bureau 1998). In this same study, glucose was shown to be lost in the urine, indicating that even though the starch was digested and assimilated, it was not well utilized.

Chitin, which is an unbranched polymer of *N*-acetylglucosamine, is a primary component of invertebrate exoskeletons. This is likely to be the primary carbohydrate experienced by most carnivorous species in the wild. Moe and Place (1999) found a correlation of chitonase activity with diet type for eight marine species caught from the wild. However, Lindsay (cited by Smith 1989) did not find a correlation among 29 species. Activity was found in the stomach at gastric pH and in the intestine and ceca at neutral pH in a number of species (Danulat 1987; Sabapathy and Teo 1993; Moe and Place 1999; Divakaran *et al.* 1999). This indicates that there are at least two

Table 7.2

Important Selective Nutrient Transport Systems[a]

Transport system	Substrates, cofactors, and inhibitors	Location(s)	Reference(s)
Amino acids			
Alanine (A)	Most amino acids; sodium dependent	Brush border	Baumrucker *et al.* (1989)
Leucine (l)	Large branched chain or aromatic side chains	Brush border	Baumrucker *et al.* (1989)
Large neutral brush border (LNBB)	Most neutral amino acids; sodium dependent	Brush border	Mercer *et al.* (1989)
Phenylalanine (PHY)	Phe and Met; sodium dependent	Brush border	Baumrucker *et al.* (1989)
Cationic (y)	Cationic amino acids (Arg, Lys, Orn)	Brush border	Baumrucker *et al.* (1989)
Anionic (X or x)	Anionic amino acids (Asp, Glu); may be sodium dependent	Brush border	Baumrucker *et al.* (1989)
Imino (IMINO)	Imino acids (Pro); sodium dependent	Brush border	Baumrucker *et al.* (1989)
Minerals			
Calcium	Vitamin D may play a role in gut absorption. Absorption reduced by dietary fiber, zinc, magnesium, and phytate	Gills and oral epithelia; gut may be secondary	Lall (1989), Chap. 5
Phosphorus	As soluble phosphate; uptake inhibited by iron and magnesium	Gut; gills and skin may be secondary due to low phosphorus concentrations in water	Lall (1989), Chap. 5

Magnesium	Vitamin D dependent; may be same as for calcium; environment is significant source. Absorption reduced by dietary fiber, calcium, phosphorus, and phytate	Gut	Lall (1989), Chap. 5
Sodium/potassium/ chlorine	Energy-dependent "pumps" transfer ions across membranes	Gills, skin, brush border	Lall (1989), Chap. 5
Iodine	Branchial pumps (in gills) and as a part of other molecules (e.g., iodinated tyrosine)	Gut and gills	Lall (1989), Chap. 5
Iron	Complex process where iron is absorbed via protein system (ferritin) and transferred in blood via another protein (transferritin)	Gut; gills may play minor role	Lall (1989), Chap. 5
Zinc	Gut absorption reduced by dietary fiber, calcium, and phytate	Gut and gills	Lall (1989), Chap. 5
Selenium	Efficient uptake across gills	Gills and gut	Lall (1989), Chap. 5

[a] Most require energy to transport the substrate actively.

chitonases, one gastric and one pancreatic (Smith 1989). Chitonase may also have nondigestive roles related to defense against microorganisms and parasites in other tissues (Lundblad *et al.* 1979; Smith 1989).

7.8.4. Absorptive Processes

Transport of nutrients from the intestinal lumen into the enterocyte can occur by pinocytosis, simple diffusion, ion exchange (in the case of some minerals), or active transport (Smith 1989). Pinocytosis can transport large complex compounds that are later digested intracellularly or used for other purposes such as priming the immune system or recycling components of digestive secretions (enzymes, bile salts, etc). Diffusion or "nonsaturable" transport is driven by concentration gradients of the nutrient between the lumen and the enterocyte. Ion exchange is selective and functions to maintain the electrical potential of the tissue. For example, the absorption of monovalent ions in the intestine is through a Na^+/H^+ and Cl^-/HCO_3^- ion-exchange system. Na^+ is absorbed in exchange for H^+ and Cl^- is absorbed in exchange for HCO_3^-. Active transport is selective and often requires a sodium gradient to pump the nutrient (e.g., essential amino acids) across the brush border (Storelli and Verri 1993). Active transport requires energy and is governed by Michaelis–Menten saturation kinetics. Selective transport mechanisms where they occur are thought to be quantitatively more important than diffusion or pinocytosis, especially for essential amino acids (Bakumrucker *et al.* 1989; Storelli and Verri 1993).

At least two important enzymes in fish and other vertebrates are involved with active transport of nutrients: alkaline phosphatase and γ-glutamyltransferase. Both enzymes are membrane linked and often associated with transport mechanisms in the brush border and are used as a proxy for the development of enhanced nutrient uptake in fish larvae (Ribeiro *et al.* 1999; Martinex *et al.* 1999; Tengjaroenkul *et al.* 2000; Gawlicka *et al.* 2000). Phosphatase dephosphorylates nutrients and plays a role in lipid and protein absorption by being a catalyst for transphosphorylation reactions (Kuz'mina and Gelman 1997; Villanueva *et al.* 1997). γ-Glutamyltransferase catalyzes the hydrolysis of γ-glutamyl peptide bonds and likely provides membrane transport of amino acids at the same time (Baumrucker *et al.* 1989).

In mammals, seven active transport systems have been identified for amino acids in the brush border of the intestinal mucosa (Table 7.2; see pages 424–425). The alanine system (A) has a broad specificity for most amino acids (Bakumrucker *et al.* 1989). The leucine system (l) has a wide scope of amino acids but prefers amino acids with large branched and aromatic side chains. The large neutral brush border (LNBB) system is specific

for methionine, histidine, leucine, isoleucine, valine, tyrosine, phenylalanine, tryptophan, and threonine (Mercer *et al.* 1989). The phenylalanine system (PHY) is specific for methionine and phenylalanine (Baumrucker *et al.* 1989). The cationic system (y) transfers cationic amino acids such as arginine and lysine. The anionic system (X) transfers anionic amino acids such as asparagine and glutamine. Finally, the IMINO system transports imino acids (proline). The A, LNBB, PHY, X, and IMINO systems are all sodium dependent and are denoted by capital letters, while the l and y systems are not sodium dependent and are denoted by lowercase letters (Baumrucker *et al.* 1989).

It is unclear the extent to which these systems function in fish; however, classical evidence suggests that fish systems are similar (Smith 1989). The use of molecular probe techniques is ideally suited to sort out the various enzyme and transport systems and to quantify the degree of similarity with higher vertebrates. These techniques are only just beginning to be used in fish nutritional physiology studies (Douglas *et al.* 1999; Gawlicka *et al.* 2000).

Minerals represent a special case in fish nutrition because many of them can be absorbed at the gills and skin as well as in the gut. They may enter the gut either in the food or from swallowed water. There are a number of selective transport mechanisms for minerals summarized in Table 7.2. Since several minerals can compete for the same uptake site, a careful balance must be achieved in the diet so that an excess of one mineral does not saturate the site and reduce uptake of another mineral. For example, an excess of calcium in the diet can lead to the need for greater levels of magnesium. The bioavailability of minerals differs depending on the chemical form, whether it is chelated, the presence of antagonistic interactions, mineral binding compounds (such as phytate), water chemistry, and other factors. See Chapter 5 for more details.

7.9 Postabsorptive Transport and Processing

Nutrients absorbed by the enterocytes are transported to the liver via the circulatory and lymphatic systems (see Section 7.6). The liver is the central metabolic organ of the body. It has the primary role in intermediary metabolism (see Chapter 6, by Dabrowski) and an important role in digestion, detoxification (see Chapter 8, by Roberts and Chapter 10, by Hendricks), and waste removal. Its role in bile production and as a site for pancreatic tissue has already been discussed. However, the most important function of the liver is as the primary regulating organ for circulating levels

of amino acids, lipids, and glucose, making it central to whole-body energy and nutrient balance (see Chapter 1, by Bureau *et al.,* and Chapter 6, by Dabrowski and Guderley).

From the enterocyte, proteins are transported to the liver as free amino acids and lipoproteins. Lipids are re-esterified to phospholipids and triacylglycerols in the enterocytes and transported to the liver as lipoprotein complexes called chylomicrons, or very low density lipoproteins (VLDL). Carbohydrates are transported as glucose in the blood.

7.10 Control and Regulation of Digestion

The control and regulation of such a complex system so that all the various processes occur in a coordinated and timely way are complex. The secretion of digestive juices, coordination of muscle contractions to mix and move the bolus, and activation of enzymes, as well as communication to the brain to stimulate or suppress appetite and influence feeding behavior, all must occur in an appropriate and measured process. This is accomplished through a combination of hormonal and nervous control. Few studies have been done with fish in this area, therefore some of the material in this section has been extrapolated from what is known for other animals (mostly mammals). This should be kept in mind, as future studies may find differences between the mammalian model and fish.

The overall metabolic state of the organism influences feeding and digestion. Many of the regulators of metabolism (insulin, insulin-like growth factor, glucagon, glucagon-like peptide, pancreastatin, somatostatin, growth hormone, and so on) also affect the digestive tract; however, these effects tend to be coordinated at the organismal level. This includes control of appetite, transport of nutrients within the body, uptake of nutrients from the blood by tissues, control of blood nutrient level, and cell and tissue growth. There is also a system of gastroenteropancreatic (GEP) hormones/neurotransmitters (Wendelaar Bonga 1993) and specific autonomic nerves (Smith 1989) that exert control over the specific processes of the gastrointestinal tract. It is the latter set of controls that is the subject of this section. The understanding of the GEP endocrine and nervous system is likely to have practical application for the improvement of prepared diets for fish being raised in aquaculture, because this is the system that regulates and controls the efficiency of digestion on a single meal basis. For information on control and regulation of metabolism see Chapter 6, by Dabrowski and Guderley.

Nerves from the cranial, spinal, and enteric sections of the autonomic system innervate various parts of the gastrointestinal tract. The vagus nerve (cranial X) runs to the pharynx, esophagus, and stomach. The (spinal) celiac ganglion innervates all of the visceral organs and also connects to the vagus nerve. The splanchnic nerve (also spinal) connects to the urogenital organs and rectum. The enteric nerves connect to the circular and longitudinal muscle layers of the gut wall and gland cells and receive inputs from extrinsic cranial and spinal nerves (Smith 1989). The roles of these nerves, in concert with GEP hormones, include the detection of fullness, control of peristalsis, vasoconstriction/dilation, control of smooth muscle (for regurgitation or relaxation of the stomach to allow distension), and secretion of digestive fluids (Smith 1989). For example, in humans, the strongest stimulation of acid and pepsin secretion is produced via the vagus nerve (Lentner 1981), although a number of GEP hormones also result in these secretions.

Endocrine cells of the GEP system are located in the pancreas (as discussed in Section 7.5.6.) and in the gut (stomach, intestine, and ceca). Within the gut, diffuse endocrine cells are of two types, open and closed (Wendelaar Bonga 1993). Both cell types are in contact with the basal lamina, but only the open type extends all the way through to the lumen. The open cell has microvilli-like processes that are likely to contain chemoreceptors for compounds in the gut (Wendelaar Bonga 1993). This gives the open cell the opportunity to communicate to both the lumen, on one side, and capillaries adjacent to the basal lamina, on the other side (Smith 1989). Both cells secrete into the lateral and basal cell membranes (Wendelaar Bonga 1993). The gut endocrine cells can produce the same major hormones as the pancreatic islets (glucagon, glucagon-like peptide, somatostatin, pancreatic polypeptide, pancreastatin, and, in some species, insulin) as well as several others (gastrin, cholecystokinin, bombesin, enkephalin, vasointestinal peptide, tachykinins, neuropeptide Y-like peptides, neurotensin, secretin, gastric inhibitory peptide, and serotonin) (Wendelaar Bonga 1993).

Some of the known actions of the various neuropeptide and nerves of the GEP system are listed in Table 7.3. The study of digestive endocrinology in fish is lagging compared to other aspects of endocrinology such as reproduction, development, growth, and metabolism, so much of the information in Table 7.3 and this section has been based on what is known in higher vertebrates. There is a tremendous opportunity to utilize modern molecular methods to better understand the workings of the GEP system (Schmitz *et al.* 1996; Johnson *et al.* 1997; Peyon *et al.* 1998, 1999; Suzuki *et al.* 1999). This understanding may provide the insight needed to control and improve digestion in farmed fish in the future.

Table 7.3

Selected Hormones and Neurotransmitters of Gastroenteropancreatic (GEP) System[a]

Hormone	Synthesis location	Activity location	Function(s)	References
Gastrin	Released by stomach (pyloric) and intestinal endocrine cells in response to food in lumen	Stomach oxynticopeptic cells	Release of HCl and pepsinogen	Elbal *et al.* (1988), Cimini *et al.* (1989), Holmgren (1993), Wendelaar Bonga (1993), Barrenechea *et al.* (1994), Pan *et al.* (1995), Nielsen *et al.* (1998)
Cholecystokinin (CCK)	Released by anterior intestinal endocrine cells in response to food in lumen and vagal stimulation.	Gallbladder, pancreas, and brain	Stimulates contraction of the gallbladder and secretion of pancreatic digestive enzymes and hormones; decreases feeding behavior and gastric emptying	Cimini *et al.* (1989), Holmgren (1993), Wendelaar Bonga (1993), Barrenechea *et al.* (1994), Peng and Peter (1997), Nielsen *et al.* (1998)
Bombesin and gastrin releasing peptide	Released by stomach and/or anterior intestinal endocrine cells	Systemic	Stimulates endocrine cells to release gastrin/CCK, growth hormone, and other stimulatory compounds; decreases feeding behavior	Cimini *et al.* (1989), Rajjo *et al.* (1989), Jensen and Conlon (1992), Holmgren (1993), Wendelaar Bonga (1993), Barrenechea *et al.* (1994), Peng and Peter (1997), Phale (1998)

Vasoactive intestinal peptide (VIP)	Released by stomach and/or intestinal endocrine cells in response to gut distention	Smooth muscles of gut wall, pancreas, intestinal endocrine cells, and blood vessels	Causes contraction of gut wall and pancreatic exocrine and endocrine secretion; increases blood flow to gut; activates salt secretion in rectum and inhibits gastrin secretion	Rajjo *et al.* (1989), Holmgren (1993), Wendelaar Bonga (1993), Barrenechea *et al.* (1994), Plesch *et al.* (1999)
Somatostatin	Released by endocrine cells in stomach and pancreas	Gut and brain	Inhibits gastrointestinal motility, rectal salt secretion, and gastrin and growth hormone release	Abad *et al.* (1987), Elbal *et al.* (1988), Cimini *et al.* (1989), Holmgren (1990), Chan and Hale (1992), Barrenechea *et al.* (1994), Plesch *et al.* (1999)
Neuropeptide Y-like peptides	Released by stomach and/or intestinal endocrine cells	Gut and brain	Enhances ion transport and stimulates feeding behavior	Wendelaar Bonga (1993), Peter (1997), Gomez-Visus *et al.* (1998)
Secretin	Released by anterior intestine endocrine cells in response to gastric acids	Pancreas	Releases sodium bicarbonate; inhibits gastric acid secretion; may increase insulin and other pancreatic hormone levels	Ince (1983), Wendelaar Bonga (1993)

[a] All are peptides, and many have functions and tissue locations in addition to those listed here.

7.11 Nutritional Physiology in Larval Fish

The development of an off-the-shelf microparticulate diet for small larval fish is one of the most important nutritional technologies needed by aquaculture that has not yet been developed. Replacing live feed production and/or fertilized ponds for hatcheries with a storage room of high-quality microparticulate diets will greatly increase seed production, improve the consistency of hatchery production, and lower costs for many species. What stands in the way? A major hurdle to overcome is improving the acceptability and bioavailability (or digestibility) of the diets. A key part of the problem for most species is a mismatch between the current technology being used to make microparticles and the functional development of sensory and digestive systems of larval fish.

Most larval fish start feeding before the digestive system has developed into its adult form (Gabaudan 1984; Boulhic and Gabaudan 1992; Kjoersvik and Reiersen 1992; Lazo *et al.* 2000). The gut is often just a simple transparent tube lacking in differentiation (Fig. 7.23). Instead of a stomach, an upper intestine, and a lower intestine, larvae have a foregut, midgut, and hindgut. Pyloric ceca have often not yet formed. Gastric glands often do not exist and support organs such as the pancreas, gallbladder, and liver are also immature. Often, it is possible to see *Artemia* still moving inside the guts of first-feeding larvae such as is the case with the halibut larvae shown in Fig. 7.24. It is not unheard of to observe live *Artemia* nauplii swimming away after being excreted from the digestive tract of first-feeding larvae. Clearly, there is not much digestion going on.

7.11.1. Changes in Diet Assimilation During Ontogeny

To understand the functional changes of larval fish guts as they mature, it is instructive to take a look at the feces over this important developmental interval. Figure 7.25a shows a larval halibut near the time of first feeding (approximately 250 C° days postspawn). Note that the gut is full of *Artemia* nauplii. Note that in Fig. 7.25b, nauplii are still clearly visible in the feces of the young halibut larvae. Figure 7.26 was taken 2 weeks later [approximately 550 C° days (temperature in °C × days since fertilization; also called degree days or temperature units) postspawn] of larvae taken from the same tank as those shown in Fig. 7.25. Note the changes in both the anatomy of the gut and the degree of homogenization of the feces. Clearly, more digestion is occurring than at first feeding. Figure 7.26 was taken prior to metamorphosis, 39 days after first feeding (approximately 700 C° days postspawn). Note that the eye is just starting to migrate and the gut has developed further. The

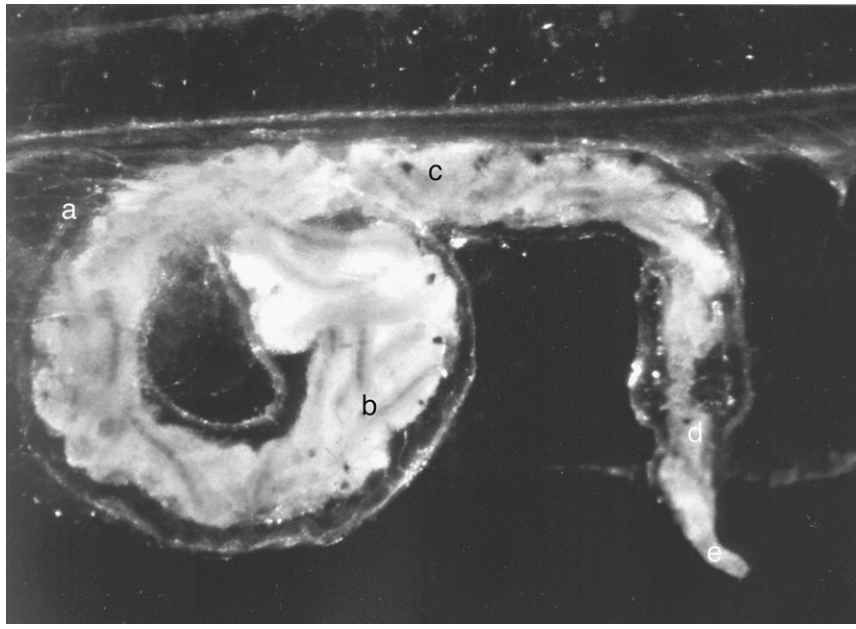

FIG. 7.23

The looped digestive tract of a first feeding Atlantic halibut larva. (a) Esophagus (above), (b) foregut, (c) midgut (below), (d) hindgut, and (e) anus. Anterior is to the left. Photograph by Michael Rust.

feces are well homogenized, with most of the nauplii broken down. Concentrated droplets of oil and the nearly empty exoskeletons of the *Artemia* are visible. Looking at the series it is easy to see that early in the larval period, very few of the structural (mostly protein?) components of the feed are digested, while later they are quite well digested.

7.11.1.1. Development of the Digestive System in Fish

Direct absorption of complex compounds from the yolk to the syncytium followed by intrasyncytial digestion is considered to be the primary mode of endogenous digestion during embryonic development (Heming and Buddington 1988). It is likely that both extracellular hydrolysis of complex compounds followed by assimilation of smaller by-products of hydrolysis (extracellular model including membrane linked digestion) and direct assimilation of complex compounds followed by intracellular hydrolysis (intracellular model) occur during the larval stage in fish (Fig. 7.21). It may

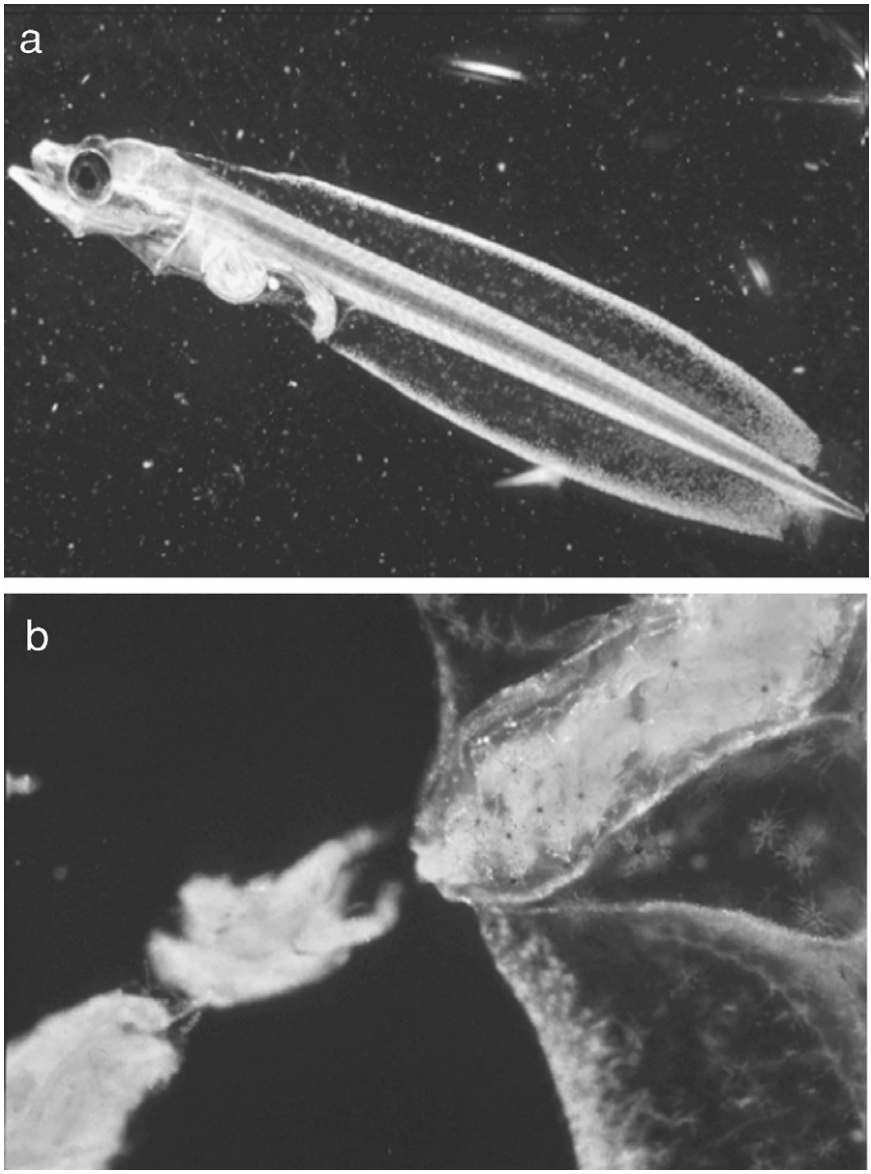

FIG. 7.24

Atlantic halibut larvae 2 days after first feeding (250 C° days postspawn). (a) Whole larvae. (B) magnified view of the anus and feces. Photographs by Michael Rust.

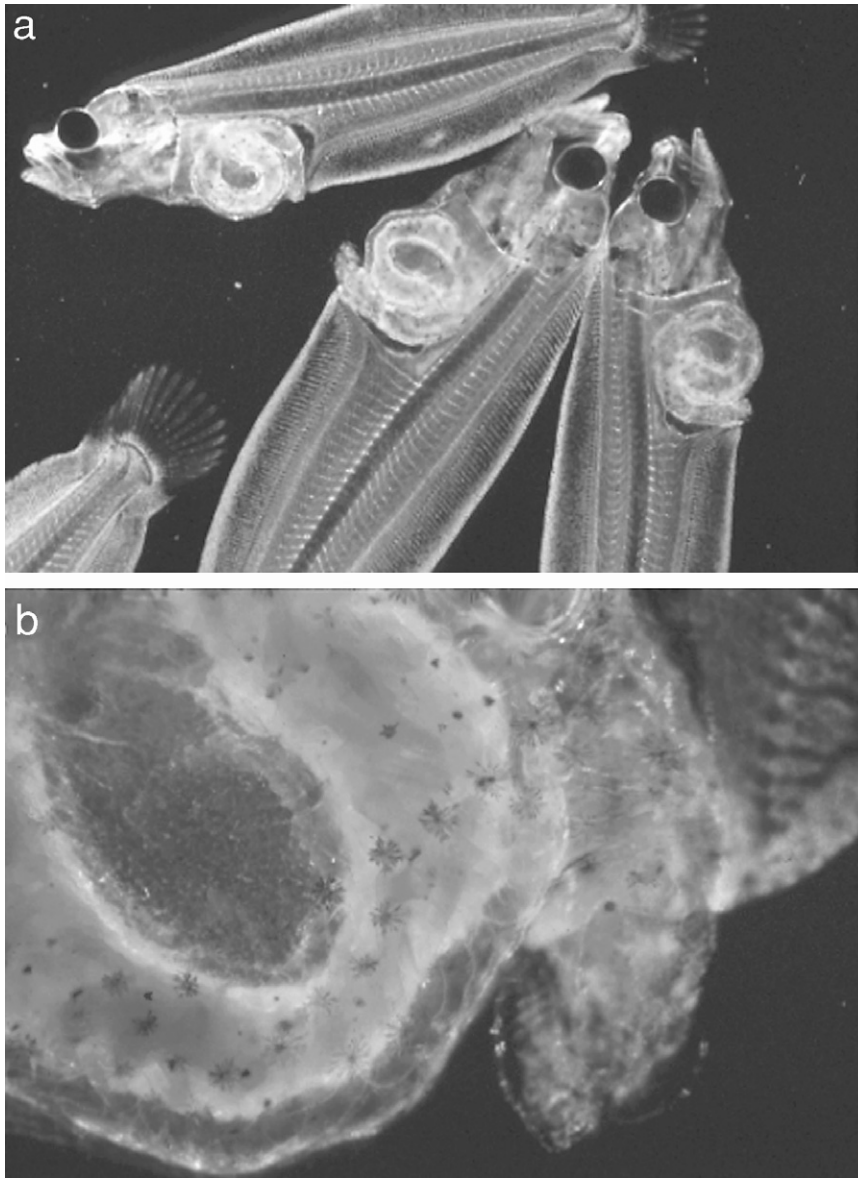

FIG. 7.25

Atlantic halibut larvae from the same tank, 2 weeks later (550 C° days postspawn). (a) Whole larvae; (B) magnified view of the anus and feces. Photographs by Michael Rust.

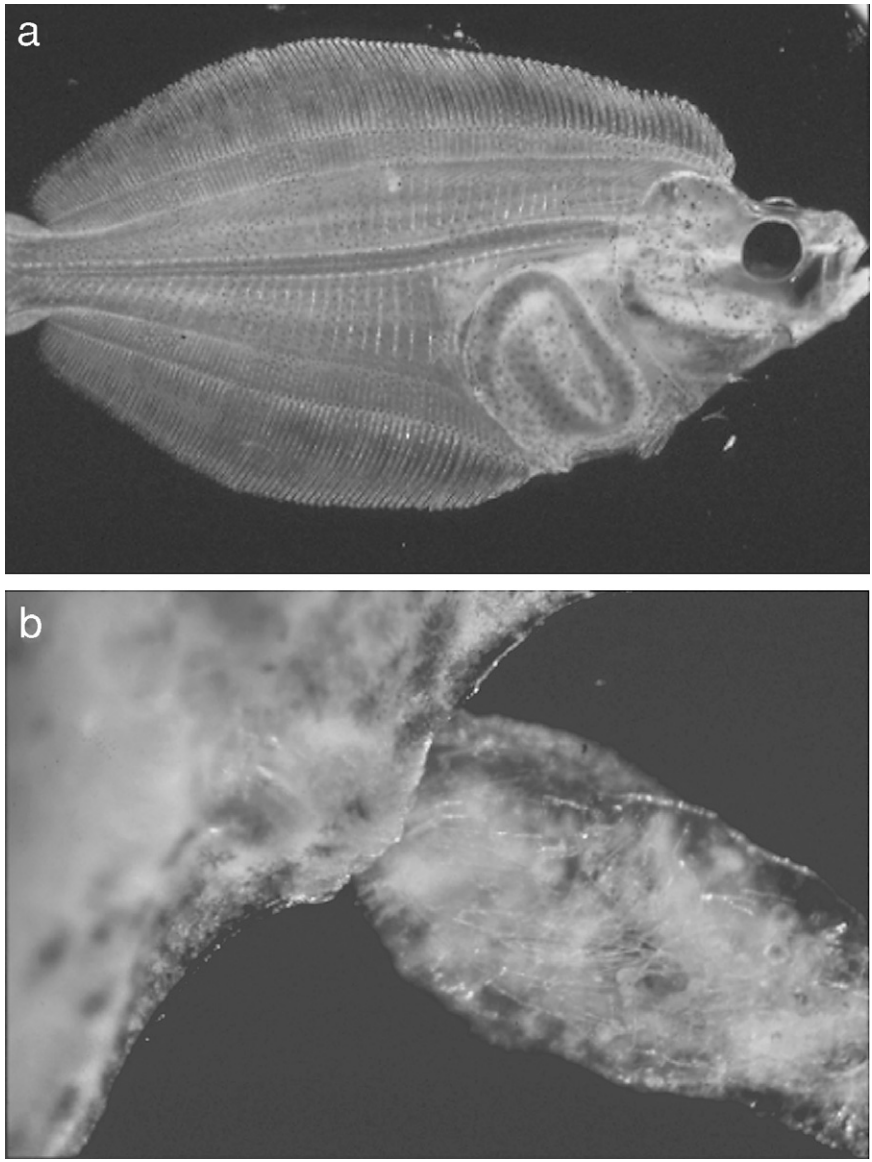

FIG. 7.26

Atlantic halibut larvae from the same tank near the start of eye migration 39 days after first feeding (700 C° days postspawn). (A) Whole larva; (B) magnified view of the anus and feces. Photographs by Michael Rust.

be that the relative contribution of each mode of digestion differs among species and changes during larval development. The quantitative importance of each model for larval fish digestion has not yet been determined.

An example of ontogenetic changes in the physiology and assimilation efficiency for protein in the striped bass may be illustrative of changes taking place in a large number of monogastric altricial fish species (Roo *et al.* 1999). Gabaudan (1984) studied the histological and histochemical development of the digestive system of striped bass larvae. He divided the larval stage into two periods after the start of exogenous feeding of what he termed "physiological relevance." The first period was from first feeding until about 15 to 18 days after first feeding [at 21°C; 0 to 340–370 C° days after first feeding]. During the first period, the stomach is not developed and "the digestive processes of the larvae are probably comparable to those of stomachless fishes such as cyprinids" (Gabaudan 1984). The second period begins about 17 days after first feeding and extends to about day 50 (at 26°C; 350 to 1200 C° days after first feeding). This second period is a state of transition where "the digestive processes presumably become similar to those of the adult" (Gabaudan 1984).

According to Gabaudan (1984), the stomach anlage appears about 15 days after first feeding (300 C° days after first feeding), along with differentiation of intestinal goblet cells. Before that time, the exocrine pancreas is formed and contains zymogen granules; however, the density (e.g., amount of zymogen) of the zymogen granules as well as the amount and distribution (after 25 days; 574 C° days) of exocrine pancreatic acinar cells continues to increase as the larva ages. Intestinal ceca and gastric glands (oxynticopeptic cells) begin to differentiate about 19 days after first feeding (419 C° days) but not all of the presumptive oxynticopeptic cells contain pepsinogen granules. Not until 27 days after first feeding (626 C° days) is zymogen seen. Although Gabaudan did not measure changes in acid proteolytic activity in striped bass, Vu (1983) reported that measurable acid proteolytic activity began to increase 5 days after gastric gland differentiation began in sea bass larvae. The development of these glands and structures is required for extracellular (luminal) digestion. In a different study, where a pH indicator solution was intubated into the stomach anlage of live larval striped bass, no color change (indicative of acid production) was evident until 24 days after first feeding [at 19°C; 456 C° days (Rust *et al.* 1993)]. Even then, the extent of color change due to the acid was limited to the region next to the epithelium and did not extend into the middle of the lumen (Fig. 7.27).

In addition to the histological evidence of developing extracellular digestive capacity, Gabaudan (1984) found evidence of pinocytosis and intracellular digestion from first feeding until the end of the study, after metamorphosis. Around first feeding, the enterocytes of the anterior intestine

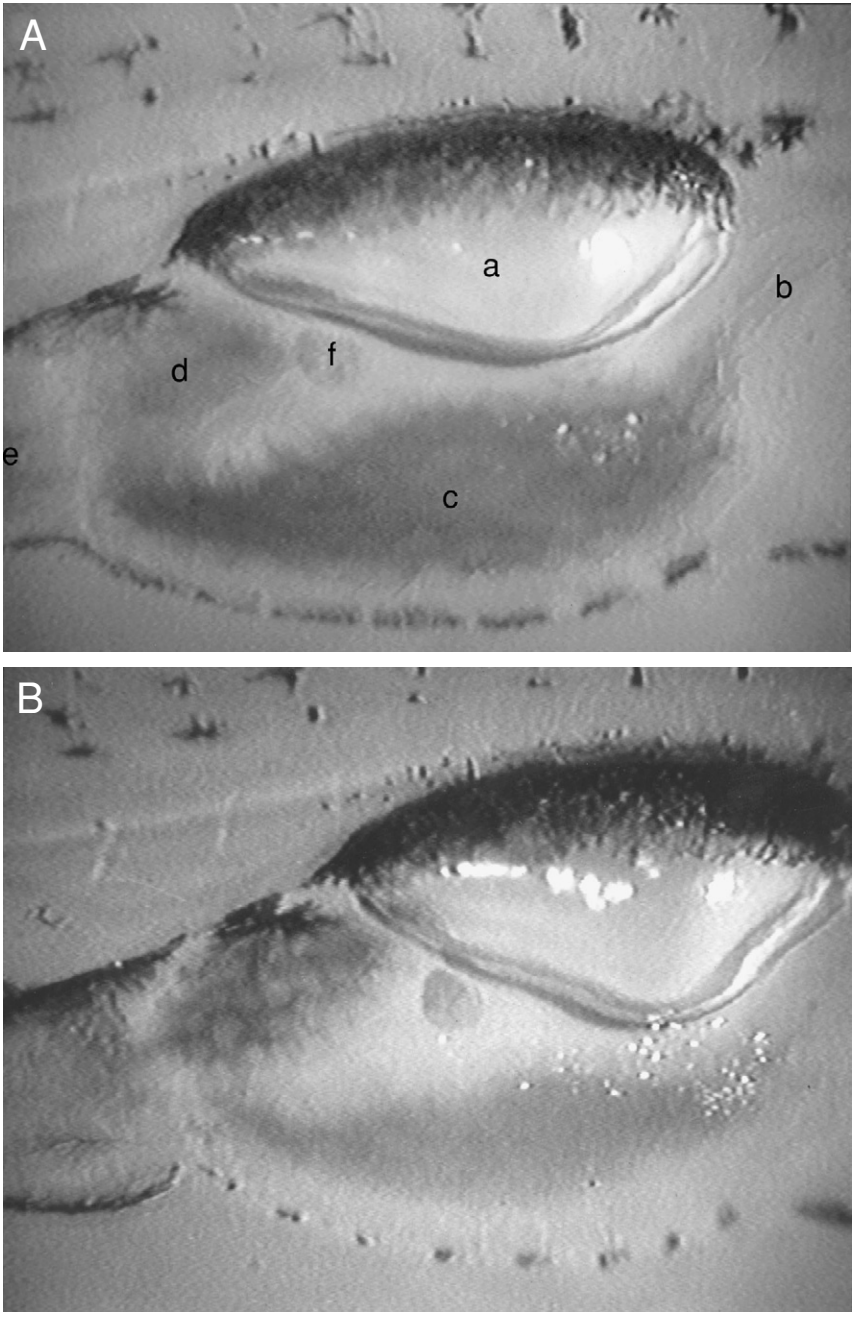

Fig. 7.27

contained lipid vacuoles and stained positive for alkaline phosphatase in the microvilli. Gabaudan (1984) postulated that the anterior intestine may be actively involved in the active transport of lipid, amino acids, and carbohydrates. The enterocytes of the posterior intestine contained large eosinophilic supranuclear bodies and had the ability to take up intact dietary horseradish peroxidase (HRP). HRP activity was not extinguished until it was contained in supranuclear bodies, indicating that the protein was assimilated intact, and then digested intracellularly in the supranuclear body. Similar histochemical studies have been conducted with goldfish (Gauthier and Landis 1972), common carp [*Cyprinus carpio* (Noaillac-Depeyre and Gas 1974)], grass carp [*Ctenopharyngodon idella* (Stroband 1977)], walking catfish [*Clarias lazera* (Stroband and Kroon 1981)], and other species, with similar results (Watanabe 1982).

In another study (Rust 1995), striped bass were fed radiolabeled amino acid-containing compounds between 19 days after first feeding (at 19°C; 361 C° days) to 47 days after first feeding (at 19°C; 893 C° days), which corresponds to Gabaudan's second period. The assimilation efficiency during that period for protein-bound methionine increased from about 30 to 60%. These results agree well with the histological changes discussed previously.

If an increase in assimilation of intact protein for intracellular digestion is the only process responsible for the observed increase in assimilation efficiency, then the transport of proteins from the lumen into the enterocyte by pinocytosis must also increase. Increasing protein assimilation rates would likely be accompanied by increases in absorptive surface area and/or increases in pinocytotic activity within existing enterocytes. Stomachless fish typically have long, highly folded guts and have high absorptive surface areas. The alimentary canal length in striped bass (which is correlated with the gut surface area) increases only from 60 to 75% of the total fish length during the larval period (Gabaudan 1984). In contrast, the alimentary canal length in goldfish increases from 80 to more than 300% of the total fish length over a similar period (Smith 1989). If assimilation of nutrients were due to surface area-limited processes alone (e.g., pinocytosis), then we would expect to see a relatively constant assimilation rate for larval striped bass. The results of the radiolabeled protein study are in conflict with this expectation (Rust 1995). Therefore, it is likely that the

FIG. 7.27

The development of acid secretion in larval striped bass (*Morone saxatilis*). Yellow–green indicates a pH below 6.0; blue, pH 7.4 and above. Time is 0 min (A) and 4 min (B) after intubation with indicator solution. From Rust (1993). (a) Swimbladder, (b) esophagus, (c) foregut stomach anlage), (d) midgut, (e) hindgut, and (f) presumptive spleen. Photographs by Michael Rust. (Reprinted from *Aquaculture,* **116,** Rust, M. B., Hardy, R. W., and Stickney, R. R. *A new method for force-feeding larval fish.* pp. 341–352. Copyright ©1993, with permission from Elsevier Science.)

increase in assimilation is due to an increasing ability of the larvae to utilize extracellular and/or membrane-bound digestive processes.

Extracellular digestion is enhanced as larvae produce increasing quantities of acid, develop nutrient transport mechanisms, and secrete digestive enzymes (pancreatic and gastric) and bile into the lumen. As more hydrolytic substances are secreted into the lumen, the effective volume of material that can be digested increases regardless of increases in the gut surface area. Increasing active transport sites and/or other membrane-bound enzymes can also increase the digestion and assimilation of nutrients without the need for an increased gut surface area.

Extracellular digestion is considered to be the more quantitatively important digestive pathway in postmetamorphic monogastric fish (Smith 1989; Pederson 1993). Uptake of intact proteins followed by intracellular digestion also occurs in postmetamorphic monogastric fish in the posterior intestine; however, the relative contribution to amino acid nutrition is questionable (Stroband and Van der Veen 1981; Gardner 1985; Smith 1989). Pinocytosis may function primarily as a mechanism to provide antigens for the immune system (Smith 1989) or to recover digestive enzymes (Hofer 1982).

It may be that the second period in larval striped bass development, described by Gabaudan (1984), is a change from a quantitative reliance on intracellular digestion to one on extracellular and membrane-bound digestion (Fig. 7.28). A model for digestive system functional development in altricial gastric larvae based upon this interpretation explains the assimilation data found for striped bass and other similar species. The model assumes a reliance on intracellular digestion from first feeding in those species until some time after the gastric glands begin to develop (corresponding to Gabaudan's first period), followed by an increasingly efficient extracellular/membrane digestion period (corresponding to Gabaudan's second period). The activity of intracellular digestion may not necessarily decrease in absolute terms. As more protein is processed by extracellular digestion, less is available in the posterior intestine, where intracellular digestion occurs. At some point, extracellular digestion reaches maximum efficiency and total assimilation efficiency reaches a plateau.

7.11.2. Development and Timing of First Feeding

The point at which embryos from different species start feeding is not entirely fixed developmentally, but may be dependent on (1) the developmental competence, (2) the yolk utilization efficiency, and (3) the quantity of endogenous nutrient reserves (Fig. 7.29). In contrast, the timing of first feeding for a single species is fixed within a window of feeding opportunity. If larvae do not begin feeding, then they pass a point of no return, after which feeding will not prevent starvation.

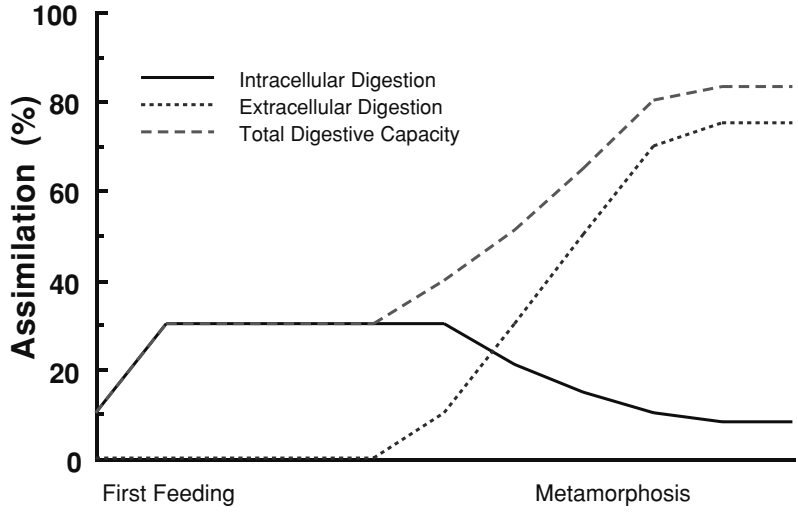

FIG. 7.28

A model for the development of protein digestion in gastric fish larvae. Early reliance on intracellular digestion increases as the gut surface area increases. Later, extracellular digestion and membrane-linked digestion become more important, reducing the substrate available for intracellular digestion in the hindgut. For postlarvae, intracellular digestion may be nutritionally less important but may provide a function for the immune system. From Rust (1995).

Digestive competence may proceed as outlined in the model (Fig. 7.28), with a critical minimum level of development necessary for successful assimilation of exogenous nutrients. If yolk utilization efficiency is high, and/or nutrient reserves are large, then the embryo can develop well beyond the critical minimum level before it requires exogenous nutrients. Salmon embryos, for example, have very large nutrient reserves in the eggs and incubate at low temperatures (resulting in a high yolk utilization efficiency) for long periods of time. Because of this, salmon start feeding at a developmentally advanced stage. Protein assimilation efficiencies for first-feeding precocial species such as salmon are relatively high [<80% (Yasutake and Wales 1983; Rust 1995; Rønnestad *et al.* 1999)]. This is in contrast to altricial species (such as halibut, striped bass, and most others), which start feeding at an early stage of development.

7.11.3. Ontogenetic Changes in Life History Strategies

Developmental patterns of nutrient assimilation relate to changes in the natural diet of each species. Almost all fish feed on zooplankton or other

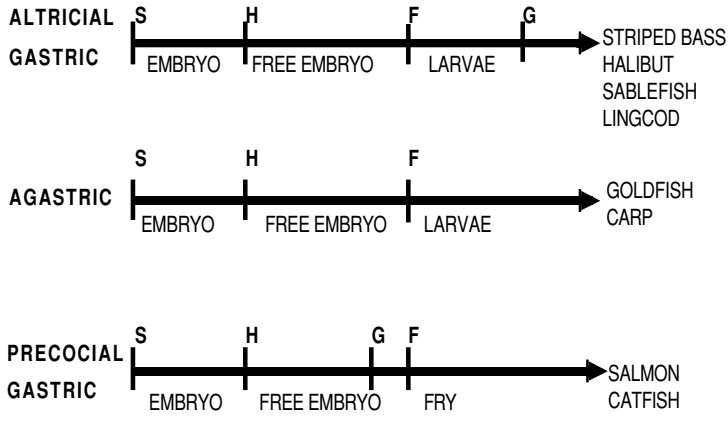

FIG. 7.29

Generalized early life histories of fish, with examples as they relate to digestive system development. S, Spawning; H, hatching; F, first feeding; G, gastric digestion is developed. Adapted from Rust (1995).

fish larvae during the larval stage. A typical agastric herbivore/detritivore changes to a foraging feeding behavior, where they ingest large quantities of relatively easy-to-seize plant material, sediments, detritus, and other nutrient-poor material. The metabolic cost of maintaining a high extracellular digestive efficiency may be higher than the potential return from such a diet. After developing acid digestion, carnivorous gastric fish change to a diet of insect larvae, fish, and other animals. As adults, they ingest a small quantity of material that is relatively difficult to seize but has a high nutritional value. The metabolic cost of developing a highly efficient digestive capacity is probably low compared to the increased net nutrient yield for those species. The functional development of the digestive systems for species with each life history is likely to be suited to the behavioral and ecological requirements for survival in the different niches they occupy, and will change accordingly during ontogeny.

7.11.4. Larval Fish Diets and Digestive Physiology

Zooplankton, the prey of larval fish, varies greatly in nutrient composition and therefore in its potential nutritional value for fish larvae. Zooplankton typically contain 50 to 70% protein, 10 to 20% lipids, and 4 to 20% ash on a dry weight basis (Creswell 1993). However, cladocerans can contain 28% carbohydrate (Creswell 1993). Copepods can have 70% of their lipids

in the form of wax esters (Smith 1989), which may be more difficult to digest than other lipids (Sargent *et al.* 1989). The amino acid and fatty acid composition of zooplankton may change due to dietary and environmental conditions. Therefore, the total nutrient composition of zooplankton may not be nutritionally optimal for first-feeding larval fish.

The nutritional value of live zooplankton to first-feeding altricial gastric larval fish is likely determined more by the bioavailability (form) of nutrients contained in the zooplankton than the nutrient composition of the zooplankton per se (Fyhn 1989, 1993). A high percentage of the total amino acids in zooplankton is in the form of free amino acids (Dabrowski and Rusiecki 1983; Fyhn 1989; Næss *et al.* 1995). For example, the amount of arginine in the free form contained in several zooplankton species ranged from 4.2 to 47.9% of the total arginine (free + bound) and free methionine accounted from 1.1 to 5.4% of the total methionine (Dabrowski and Rusiecki 1983) in several zooplankton species. Differences in levels of free amino acids among species of zooplankton may partly explain differences in the value of these species as live feeds for fish larvae (Fyhn 1989, 1993). For example, the level of free methionine in wild zooplankton (mostly copepodid stages of calanoid copepods) was about twice as high as the level of free methionine in *Artemia* sp. [4.7 vs 2.2 μmol/g wet weight (Næss *et al.* 1995)]. Higher assimilation efficiencies for free amino acids relative to bound amino acids may have the effect of shifting the relative contribution of different amino acids in zooplankton to larval nutrition (Fyhn 1989, 1993; Rust 1995; Rønnestad 1999). The overall bioavailability of a given nutrient is dependent not only on its concentration in zooplankton but also on its form and the competence of the larval gut for assimilation of that nutrient. Therefore, diets prepared to resemble the nutrient composition of zooplankton (or larval fish) will not necessarily be nutritionally balanced.

Bengtson *et al.* (1993) noted that live and microencapsulated homogenized *Artemia* sp. were digested differently by larval silversides (*Menidia beryllina*). The live brine shrimp were partially broken down in all areas of the intestine, with significant absorption (based on observations of the numbers and sizes of supranuclear vacuoles in intestinal epithelial cells) occurring in the posterior intestine, while the encapsulated *Artemia* sp. were broken down in the anteriomedian intestine, with much less total absorption by the posterior intestine. More total assimilation was histologically evident with the live *Artemia* sp., despite the fact that the nauplii were never fully broken down (Bengtson *et al.* 1993). Even though the homogenized *Artemia* sp. were fully released from microcapsules, they were apparently assimilated at a much lower level. Based upon the results of leaching trials (Lopez-Alvarado *et al.* 1994), it is likely that most microparticulate diets used for fish larvae lose most of their free amino acids before being ingested. Although no details

of diet manufacture are given, this was also likely to have been the case with respect to the microencapsulated diet used by Bengtson *et al.* (1993). The difference between the live *Artemia* sp. and the encapsulated *Artemia* sp. diets which would account for the observed histological differences found by Bengtson *et al.* (1993) may be the amount of low molecular weight nutrients found in the two diets. This possibility is consistent with both the results obtained by Bengtson *et al.* (1993) and the model presented above (Fig. 7.28).

Several authors have noted that the anterior intestine (foregut) of various species of late larval fish becomes slightly acidic (Mahr *et al.* 1983; Buddington 1985; Bengtson *et al.* 1993; Rust *et al.* 1993) as the larva develops. As shown in Fig. 7.27, in larval striped bass (24 days after first feeding), an area of low pH formed a microregion near the epithelium and was associated with localized mucus secretion. When zooplankton are being consumed by a fish larva of this age, areas of low pH in the anterior intestine would be in contact with the integument of the zooplankton. If hydrolytic activity were concentrated on the surface of the prey item, lysis and death of the zooplankter would result. As the zooplankter dies, it loses its ability to regulate nutrient pools, resulting in the release of low molecular weight nutrients. These nutrients may be more easily assimilated by the larval gut and may be critical for growth and survival.

The short digestive tract and short retention times that are characteristic of larval fish suggest a low food utilization rate (Hofer and Uddin 1985). Bivalve mollusk larvae were not digested after 24 hr in the gut of larval turbot [*Scophthalmus maximus* (Liewes 1984)]. The observation of intact-looking zooplankton coming out of the rectum of larval silversides (Bengtson *et al.* 1993), cod ([*Gadus morhua* (Tilseth and Ellertsen 1984)], and other altricial larvae (Pederson and Hjelmeland 1988) (Figs. 7.23 and 7.24) and the assimilation data discussed above (Rust 1995) support that suggestion. It is likely that the utilization of intact structural components is much lower than the utilization of free components. It may be that the energetic cost of completely hydrolyzing the nutrients contained in zooplankton does not justify the incremental gains to first-feeding fish larvae. This may be especially true if the nutrient composition of the zooplankton is not completely nutritionally balanced. In addition, the chitinous exoskeleton of zooplankton may be difficult to digest. First-feeding larvae may be obtaining a significant portion of their amino acids and other nutrients by simply killing the zooplankter in the gut and waiting for the nutrients to leak out.

7.11.5. Feeding and Diet Acceptability

During intensive culture with prepared feeds, altricial larvae often will not eat enough to support growth and development. A second key problem

with artificial diets may relate to the larval fish's inability to identify the diet as food and ingest it (Rust and Barrows 1998; Barrows and Rust 2000; Guthrie *et al.* 2000). Physical properties of the diet or rearing environment, such as color, intensity and wavelength of reflected light, degree of polarization and contrast, and/or chemical properties affecting gustation and olfaction, may be key attributes that stimulate the larvae to approach and ingest the diet in quantities sufficient to sustain growth (Barrows and Rust 2000).

Most fish larvae are visual feeders, although taste buds and olfaction are often also functional at this time (Noakes and Godin 1988; Poling and Fuiman 1997; Utne and Stenevik 1997). This makes vision and chemoreception the two most important sensory systems used by first-feeding larvae to locate and ingest food (Blaxter 1988; Noakes and Godin 1988; Higgs and Fuiman 1998).

At first feeding, most developing larvae lack a duplex retina (Noakes and Godin 1988; Fuiman and Delbos 1998; Hagedorn *et al.* 1998). Most species have cones but may or may not have developed rods in the retina [although the opposite is true in some species (Margulies 1997)]. Three or four types of cones may be operational in fish larvae, each with a different light wavelength of maximum sensitivity. As the larvae develop, visual acuity increases (Blaxter 1988; Noakes and Godin 1988; Utne and Stenevik 1997). More specialized retinal structures such as a tapetum lucidum and macroreceptors (which are adaptations to low light environments) do not develop until much later (Vandenbyllaardy *et al.* 1991; Margulies 1997).

A heart rate conditioning technique used by Hawrysyn and co-workers (1989) has led to the identification of up to four cone types in young rainbow trout that have different regions of maximal photosensitivity. In addition to the adult blue (400- to 450-nm)-, green (500- to 550-nm)-, and red (600- to 650-nm)-sensitive cones, a transient UV-sensitive (340- to 370-nm) cone was also identified. The UV cone was found only in very young trout and was completely absent in adults. This cone has now been identified in a number of other larval fish (McFarland and Loew 1994; Losey *et al.* 1999) using a microspectrophotometric technique. The functional significance of this cone is unknown for most species, but it has been shown to be involved in prey identification and feeding behavior in a number of species feeding on zooplankton (Loew 1993, 1996; Browman *et al.* 1994; Losey *et al.* 1999).

A good example of where an understanding of eye development can positively impact feeding and culture success with larval fish is lingcod (*Ophiodon elongatus*). Lingcod larvae reared outdoors under full-strength sunlight feed and behave much differently than larvae reared indoors under incandescent light. Incandescent light is lacking in the UV and near-UV ranges and high in the visible yellow bands. Fish reared indoors under these lights feed poorly and displayed "nosing" behavior typical of stressed larvae. All larvae reared

indoors died within 3 weeks of first feeding, a period of time only slightly longer than starvation. Larvae reared outdoors feed normally and survived to the juvenile stage, where they were weaned onto conventional salmon pellets (K. Massee, personal communication, 2000).

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8

Nutritional Pathology

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8.1 Introduction

In the wild, nutritional diseases are uncommon. Drought or pollution may limit food availability, and particular phenomena such as the failure of oceanic currents may, on occasion, result in a collapse of planktonic growth and consequent starvation of their predators. Generally, however, wild fish have full opportunity, in their natural environment, to acquire reasonable levels of all their nutritional requirements for survival. This does not mean that, in the normal life cycle of the animal, particularly in temperate zones, seasonal temperature effects, or cyclical changes associated with reproduction, will not produce secondary clinical and histopathological signs of inanition or starvation. The inanition of the Atlantic salmon kelt in winter, after spawning—a fish bearing little resemblance to the magnificent fresh-run salmon—is an obvious example of the effects of hormonally induced starvation.

Dietary provision for farmed fishes may take the form of a complete diet, incorporating the entire nutrient requirement, at appropriate levels for each component. Alternatively, in the case of pond fish, particularly in developing countries in the tropics, provision of a supplementary diet serves to enhance the growth rate of fish, which are expected to obtain at least a proportion of their nutritional complement from the natural productivity of their ponds. These ponds will normally have been fertilized to increase their primary production.

It is usually extremely difficult, under farm conditions, to define nutritional diseases in absolute terms, because it is rare for a single deficiency of one essential nutrient to exist. Nutritionally compromised diets will often render fish more susceptible to infectious conditions. These are more clinically obvious and may make the underlying nutritional basis for the condition extremely difficult to define. Of course deficiency of a necessary component is only one aspect of nutritional disease, which has been defined as the deficiency, excess, or improper balance of the components present in a fish diet (Snieszko 1972). Properly the definition should also include the presence of noxious or toxic components within the food, or endogenous antinutrient factors (DeSilva and Anderson 1995; Jauncey 1998), a problem which has become increasingly significant as intensive aquaculture has developed.

Although most deficiency diseases are associated with a complexity of marginal or absolute deficiencies, it is only by detailed experimental studies

of single-deficiency conditions for each species that a true understanding of nutritional pathologies of fishes can be obtained. In experimental single-deficiency conditions, described by Halver (1972) and co-workers, a wide range of specific and often pathognomonic signs has been demonstrated. In the clinical situation, however, a more general syndrome, often with inappetence, darkening skin color, and poor growth as the only obvious clinical features, is more common (Hardy 2000).

Artificial diets intended as complete sources of nutrients, when produced by a major manufacturer, are usually of high quality. It is only rarely that a particular batch will be responsible for a problem. However, when home mixing of wet diets or trash fish feeding is practiced, the possibility of a deficiency or imbalance syndrome is much higher. Even the high-quality commercially compounded diet, however, is susceptible to degeneration during storage. This is particularly likely if storage is under conditions of high temperature or humidity.

8.2 Principles of Nutritional Pathology

Starvation may be due to complete deprivation of food, to inadequate feeding levels of a diet which in itself is completely satisfactory, or to behavioral, physiological, or mechanical prevention of food intake.

Complete deprivation may occur if fish are accidentally left in a facility. Inadequate feeding levels may be associated with bad husbandry, or, in the case of stocked sport fisheries, overstocking may be responsible. Behavioral starvation is the term used to describe the condition often found when attempts are made to rear larval fishes on artificial diets, in which the larvae do not recognize or accept the diet because of inadequate presentation, texture, or taste. Nonfeeding larvae or fry may, by catabolizing structural nutrients, survive for a considerable time, although eventually they will appear dark and the head will usually be disproportionately large (Fig. 8.1).

When larvae, or other fast-growing fish such as salmon smolts, are starved, they eventually reach a stage at which the degree of body damage induced by the catabolic breakdown is such that, even when normal nutrition is resumed, they cannot recover. This stage, beyond which they cannot be restored, is known as the point of no return (PNR). Larval fish which pass this stage can, however, survive for a considerable time before they inevitably succumb (Ehrlich 1974).

The process of physiological starvation is characterized, as might be expected, by loss of weight and change in morphology. Thus affected fish become thinner and the head becomes relatively larger in proportion. The skin color becomes very dark and eventually the fish lose appetite. At necropsy,

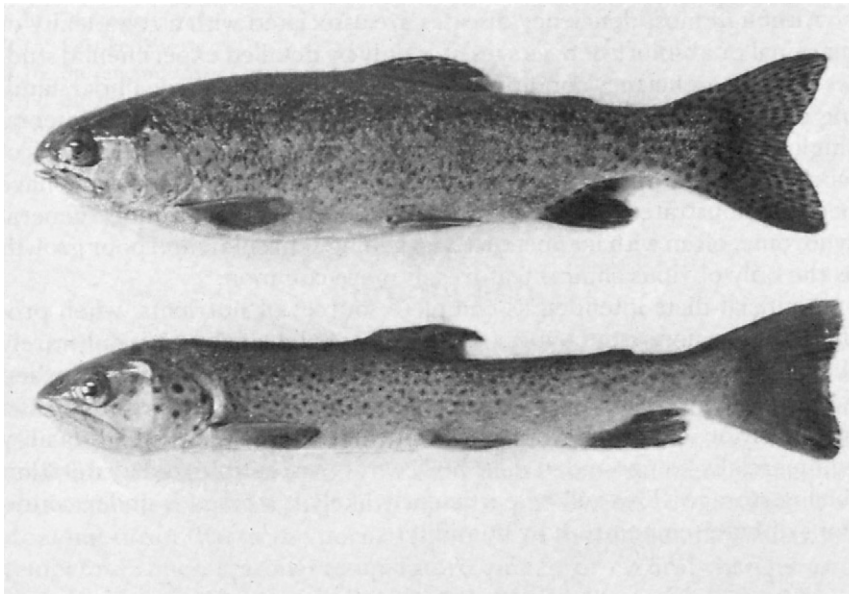

FIG. 8.1

Effects of starvation in (*top*) a normal rainbow trout and (*bottom*) a starved brown trout. From Halver (1972).

there is usually pallor, associated with anemia, and no abdominal lipid is present. The liver is normally shrunken with sharp edges, and where pyloric or intestinal ceca are present, they are upstanding and, because they have no visceral fat around them, are very obvious (Fig. 8.2).

Histologically the principal feature, apart from lack of lipid tissue, is the distinctive granular degeneration of the skeletal muscle fibers, with central migration of sarcolemmal nuclei. They are predominantly pale staining and have their sarcoplasm condensed into strands and granular deposits in a stroma of clear fluid (Roberts 2000). The digestive tract is condensed, with an apparent increased proportion of collagenous, fibroblastic, and nuclear elements. The hemopoietic tissue is dark and condensed, but a distinctive feature is the increased size of the melanomacrophage centers of spleen and kidney, with large deposits of lipofuscin within them, as well as an increase in the melanin content (Agius and Roberts, 1981). A particularly significant feature of the melanomacrophage centers of starved fish is the high level of hemosiderin sequestered there, indicating the premium placed on retaining iron complexes within the body even *in extremis* (Agius 1981).

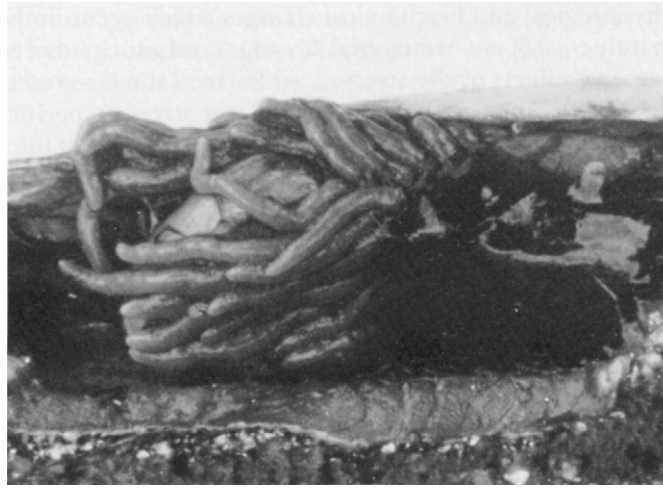

FIG. 8.2

Pyloric ceca of a starved Atlantic salmon showing the complete absence of fat.

Farmed fish, more than any other cultured animals, manifest the classical features of Selye's (1950) stress theory. However, it is Brett's (1958) definition of stress in fish that probably fits the situation observed in most husbandry situations. He describes stress as "a stage produced by an environmental or other factor which extends the adaptive responses of an animal beyond the normal range or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced." In fish husbandry, such factors, associated with density, environmental provision, or other husbandry features, are always present (Pottinger and Pickering 1997).

The primary function of the farmer is to attempt to maintain a system whereby the various components which are capable of acting as stressors are at the lowest possible level compatible with economic success. This has significant implications for growth and feed conversion as well as vulnerability to infection. Stress induces tissue catabolism (Braley and Anderson 1992), and energy required for such degradation and resynthesis is not otherwise available for growth (DeSilva and Anderson 1995). A number of the stressors which can play a role in determining the levels of nonspecific stress in fish are related to food and feeding. As well as frankly inadequate deficient diets, other factors such as irregular feeding, presentation of feed in an unsuitable form, and buildup of waste food detritus can contribute to the sum total of pressures on the adaptive capacity of the animals.

The physiological and biochemical changes which occur in the animal under the influence of environmental stressors, and which serve to moderate the negative effects of the stresses, are termed the general adaptive syndrome (GAS). They are neither species nor stressor specific and are mediated by nervous and hormonal action. The end result, if the adaptive response is stimulated beyond physiological levels and adaptive exhaustion is achieved, irrespective of whether the stimulus has an environmental or husbandry-related origin, is that the fish are made increasingly vulnerable to infection. This may be by opportunist, obligate, or facultative pathogens which are extant in the fish's environment. Thus many nutritional diseases in fish are compounded by bacterial or fungal infection. Indeed it is often the case that inexperienced diagnosticians will assume that the microbial agent is the primary pathogen and fail to recognize the underlying nutritional stressor (Moller 1981, 1984).

Feeding activity is associated with a particular metabolic cost referred to as the cost of specific dynamic action (SDA). This relates particularly to the metabolism associated with digestion, increased circulatory vascular activity in the gut and related organs, and enzymatic and other metabolic acids and the excretion of nitrogenous metabolites. There is considerable uncertainty about precisely which processes contribute to the total SDA effect and this has led Ware (1975) to suggest the broader definition of SDA as an "entropic tax paid during food conversion." Whatever the uncertainties, however, of the exact processes contributing to the metabolic oxygen demand created by SDA, it is well recognized that at high temperatures, when dissolved oxygen levels in water are reduced and even the general resting metabolic oxygen demand by fish is high, feeding to satiation can result in such a high oxygen demand to satisfy SDA that heavy mortality can result.

The clinical features of such losses are typically those of oxygen starvation. Fish die about 2 hr after feeding, with open mouths, and with considerable amounts of undigested food in the gut. The problems of feeding at high temperatures are greatly exacerbated if there is an underlying gill or blood pathology. Often, when hyperplastic or telangiectatic gill damage, associated with pollution, transport, or handling, has resulted in a reduction of gill function, the fish can survive until the dissolved oxygen levels fall. It is frequently a feeding episode which provides the final metabolic oxygen demand which cannot be met by the compromised respiratory system.

Other conditions which can increase the susceptibility to SDA-mediated mortalities include fatty liver syndrome (see Section 8.3.3), which inhibits hemopoiesis, and a number of chronic hemorrhagic conditions such as warfarin poisoning, chronic viral hemorrhagic septicemia (VHS), and infectious salmon anaemia (ISA).

8.3 The Deficiency and Imbalance Diseases

Deficiency diseases are of two types, deficiency or imbalance of the macronutrients in the diet—the protein, carbohydrate, lipid, and fiber—and deficiency of the micronutrients—the vitamins and minerals. In terms of macronutrients it is usually in the lipid component of the macronutrients that the most serious problems arise, in terms of practical diets, whether in relation to deficiency, oxidation, or imbalance. Among the micronutrients, any of a wide range of components can exert an effect, especially in fast-growing, younger fish.

8.3.1. Protein

All fish require relatively high levels of protein as a source of amino acids for protein synthesis and, generally, for gluconeogenesis as well. Thus fish diets must contain high levels of high-quality protein. Since protein is one of the most expensive components of the diet, feed manufacturers have to optimize diet formulae to allow economies while still maintaining an adequate complement of protein. The principal feature determining protein quality for fish nutrition is the level and availability of the essential amino acids (EAA). Deficiency of one or more of these leads to deficiency disease.

Normally essential amino acids within a formulated diet will be conserved, but if amino acid intake is restricted, then they may themselves be metabolized to form nonessentials. Thus to avoid deficiency it is necessary for diets to contain a sufficiency of both essential and nonessential amino acids.

Deficiency diseases can still arise even in the presence of apparent luxus. This is because certain amino acids may be rendered biologically unavailable or inactive, even when still chemically measurable, during the course of processing of the diet. Lysine, for example, may form an addition compound with carbohydrate in the feed, rendering it unavailable to the fish (Hardy 2001).

Several specific amino acid deficiency conditions have been described for fish under experimental conditions. Lysine deficiency has been specifically related to dorsal fin erosion, often with secondary flavobacterial infection (Walton *et al.* 1984). Spinal deformities have been associated with a variety of amino acid deficiencies including tryptophan, leucine, lysine, arginine, and histidine (Ketola 1983; Mazid *et al.* 1978; Walton *et al.* 1982; Halver and Shanks 1980). Lenticular cataract, commonly an early indicator of marginal deficiencies, has been associated with both methionine and tryptophan deficiency.

Under normal farming conditions, however, it is unusual to encounter acute single-amino acid deficiencies and affected fish will usually show a range of clinical features, all associated with poor growth and darkening of the skin. Such deficiency conditions can arise as a result of improper formulation or from using ingredients with intrinsic specific amino acid deficiencies or in imbalanced proportions. They may also result from improper processing of the diet, generally as a result of excessive heat or chemical treatment during preparation.

8.3.2. Carbohydrate

Since fish have a much more limited capacity for carbohydrate metabolism than higher vertebrates, there is little point in increasing carbohydrate levels, and only limited information is available on the effects of high-carbohydrate levels (Jauncey 1982). Many reports have indicated that excessive carbohydrate intake will result in excessive glycogen deposits in the liver, and continued intake results in extensive lipid deposited in the viscera (Halver 1972). Hess (1935) reports that excessive dietary carbohydrate in ornamental cyprinids results in hepatic degenerative changes.

8.3.3. Fats

Dietary disease problems associated with the lipid component of the diets appear to be among the most serious and prevalent of all nutritional problems in fish. Fish tissues contain predominantly fatty acids of the ω-3 series and so fish diets must provide sufficient essential fatty acids of the ω-3 (linolenic) and ω-6 (linolenic) series as well as a general lipid contribution to calorie requirements. Deficiency syndromes result when sufficient levels of ω-3 or ω-6 or longer-chain members of the series are unavailable. Linolenic acid is particularly important for normal growth. The ability to elongate and desaturate 18 : 3 ω-3 (linolenic) seems to vary between species. In freshwater species such as rainbow trout, for example, oleate or linoleate incorporated into the diet can be converted, at least to some degree, to arachidonate (Castell *et al.* 1972). The findings of Cowey *et al.* (1976), however, show that, in turbot at least, little conversion of linoleate to arachidonate is possible when they are fed diets high in such lipid, e.g., corn oil. In such circumstances, major changes also take place in the structure of the fat storage cells of the animal. The fat cells surrounding the lateral lymphatic sinus are particularly affected, with thickening and deformity of the lipid cell walls, increased vascularity, and deposition of a hyaline material between the cells (Fig. 8.3).

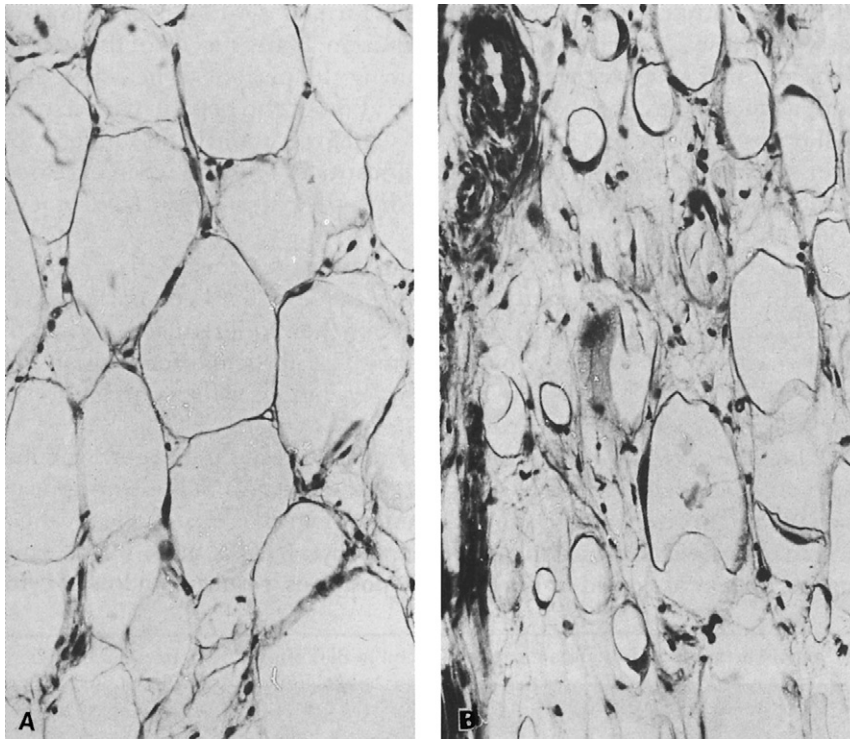

FIG. 8.3

(A) Normal perilymphatic fat cells from a turbot given a diet containing cod liver oil. (B) Perilymphatic fat cells from a fish a given diet containing hydrogenated coconut oil. Extensive thickening of the cell membrane, deposition of hyaline material between cells, and increased vascularity are apparent. H&E, ×500.

The deficiency signs in essential fatty acid deficiency almost always relate to a swollen, pale liver with fatty infiltration, and there is a consistent anemia, putatively associated with the lack of secretion of hemopoietin by the compromised liver. Mortality is also high, particularly in young, fast-growing fish (Castell *et al.* 1972; Takeuchi and Watanabe 1977, 1982; Farkas *et al.* 1977; Takeuchi *et al.* 1983; Bell *et al.* 1985).

The most important problem with the lipid component of fish diets is the propensity which the high concentration of polyunsaturated fatty acids (PUFA), which includes the essential ω-3 and ω-6 fatty acids, has for becoming autoxidized by atmospheric oxygen unless antioxidant protection

is incorporated in the diet. Autoxidation not only reduces the availability of fatty acids to the host, but also is deleterious to the fish in its own right, because rancidin, a product of the oxidation process, induces high levels of free radicals, peroxides, aldehydes, and ketones which are not only toxic to the fish but also react with other dietary components. Control of oxidative rancidity is one of the most serious of all of the problems facing the feed compounder and the pathological effects which develop in fish fed rancid diets can be extremely serious. For this reason, farmed fish diets often include a significant luxus of α-tocopherol as well as other antioxidants.

The most serious and frequently reported clinical sign associated with rancid fat within feeds is the syndrome in farmed rainbow trout known as fatty liver disease, or lipoid liver degeneration. Many species of fish use the liver as a major lipid-storage organ during the periods of heavy feeding, and at such times, extensive lipid infiltration of the hepatic parenchyma is seen. However, in species such as salmonids and mullets, the liver does not play this role to any significant degree, and when excessive unsuitable lipid is incorporated in the diet, the pathological syndrome of lipoid liver degeneration occurs.

Lipoid liver degeneration often occurs in fish fed trash fish or pelleted diets, in which the lipid component is excessive or has been partially oxidized. The rancid lipids exert their effect by their inherent toxicity and also by reacting with the dietary proteins to lower their biological value. They also reduce the activity of vitamins which are not themselves antioxidants. The particular features of each outbreak of lipoid liver disease will vary depending on the contribution of the various products of the oxidation to the pathological effects.

The clinical features of lipoid liver disease include extreme anemia, with a concomitant pallor of the gills of affected fish. The liver appears swollen and bronzed and has rounded edges (Fig. 8.4). Histologically there is extreme lipid infiltration of the hepatocytes. This may, in the later stages, appear as ceroid or lipofuscin depositions, resulting in loss of cytoplasmic staining and distortion of hepatic muralia. In addition, there is inactivity of renal and splenic hemopoietic tissue, and the melanomacrophages are replete with high levels of pale-staining pigment (Fig. 8.5). Depending on the extent to and length of time for which the condition has been extant, the degree of oxidation, and the type of fat in the diet, there may also be varying degrees of infiltration of the liver by macrophages, which also contain ceroid. All salmonids, and possibly other top predators, are susceptible to the condition, although it has been described principally in rainbow trout fed on trash fish. When fish are lightly affected, starvation can lead to complete recovery, but when severe liver or hemopoietic damage is present, fish rarely recover to the level of satisfactory feed conversion. Jackson *et al.* (1984) have

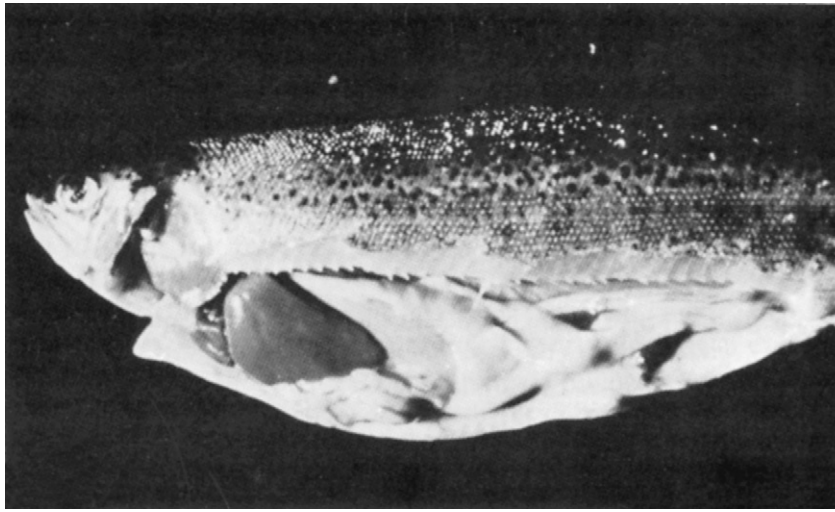

FIG. 8.4

Rainbow trout affected by lipoid liver disease. The liver is swollen, with rounded edges, and there is considerable deposition of abdominal fat.

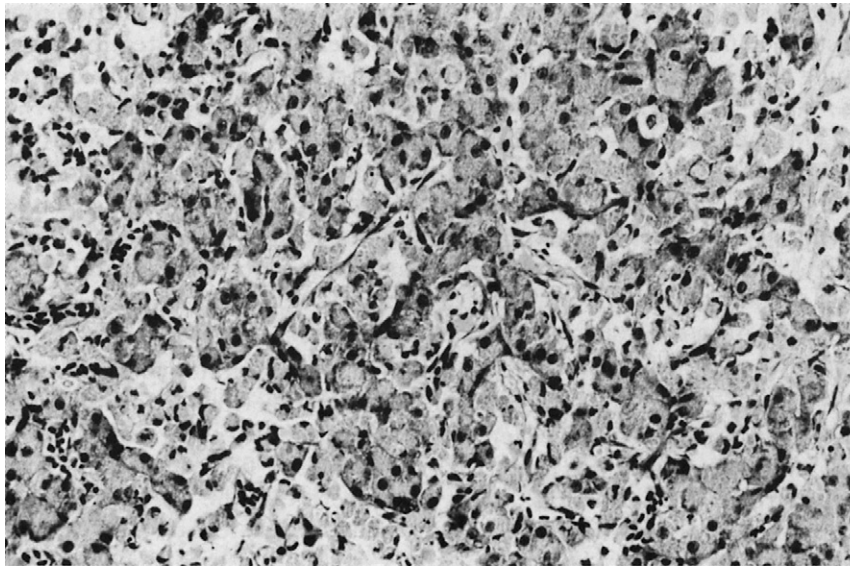

FIG. 8.5

Ceroid infiltration of hepatic cells in lipoid liver degeneration. The hepatic structure is distorted and hepatocytes are loaded with yellow pigment. H&E, ×250.

reported the effect of a range of diets prepared from clupeid silage with and without the antioxidant ethoxyquin. Their findings showed that, at a suboptimal level, those diets with high levels of hydroperoxides and secondary breakdown products (namely, unprotected) consistently induced pathological changes, although these were limited to the morphological appearance and distribution of the eosinophilic granule cells of the intestine.

Other signs associated with the feeding of oxidized lipids include congestion, hemorrhage, splenic hemosiderosis, exophthalmia, steatitis, darkening of the skin, focal hepatic necrosis, and skeletal myopathy (Soliman *et al.* 1983; Murai and Andrews 1974; Park 1978; Moccia *et al.* 1984).

The condition of Atlantic salmon in Norway, known as Hitra disease, is often considered to be associated with oxidized lipid or vitamin E deficiency (Fjolstad and Heyeraas 1985; Poppe *et al.* 1985), although there is now strong evidence that it is associated primarily with pathogenic vibrios (Egidius *et al.* 1981; Hjeltnes and Roberts 1993).

8.3.4. Fiber

The role of dietary fiber in animal nutrition has become a major area of investigation, and Davies (1985) has reviewed the evidence for its significance in fish nutrition. There does not appear to be any pathological effect associated with excessive or low levels of fiber, although it may affect the growth rate. There is, however, concern that the removal of fiber from higher-protein diets, or the semipurified diets used in research, may affect the value of such diets. Some species, namely, catfish, need substantial amounts of fiber in the diet to move nutrients along the absorptive pathways slowly.

8.4 Micronutrients

The micronutrients are, as the name suggests, those components of the diet which, although essential, are required in only relatively small quantities. If these are absent, or insufficient, they give rise to specific deficiency diseases which often have particular clinical and histopathological features. As is the case with most other pathologies in fish, salmonids are the species about which most information is available. In practice, diets are rarely deficient in one specific micronutrient, and usually the clinical signs and histopathological features are not particularly specific. However, a considerable amount of experimental work has been carried out using diets deficient in one specific component, and this has allowed the specific

deficiency pathology to be defined, at least for one or two species. Vitamins, a group of unrelated organic compounds required for the normal metabolism of all vertebrates, have been well studied, particularly for Pacific salmonids (Halver 1957a,b, 1985). They are essential in the sense that they cannot be metabolized at all or else cannot be synthesized fast enough for an animal's needs. They are required only in small amounts, but critically, they may be required at higher levels at times of fast growth or reproduction. They may also be required at significantly increased levels in high-energy diets.

8.4.1. Fat-Soluble Vitamins

The fat-soluble vitamins can be readily stored in the body of the fish and are metabolized relatively slowly. Thus an avitaminosis is usually slow to develop unless the fish is growing very rapidly. Cumulative hypervitaminoses are also possible if relatively massive doses are given. These hypervitaminosis signs are generally manifest by increasing liver necrosis and hepatocyte destruction.

8.4.1.1. Vitamin A (Retinol)

The unsaturated alcohols retinol/retinal and dehydroretinol are not readily deficient in most fish diets. However, in experimental deficiency studies, hypovitaminosis A has been reported to result in reduced growth, exophthalmia, keratomalacia, and thickening, foreshortening, and deformation of the gill arch and operculum (Aoe *et al.* 1969). Hypervitaminosis A has been reported in farmed salmon fed a vitamin A-rich whale liver diet. The effects included choroidal inflammation, corneal edema, and nervous effects. Experimentally it was not possible to repeat these effects, but at very high levels of vitamin A, experimental diets induced extensive necrosis of the tail (Burrows *et al.* 1952). Other effects reported to be due to hypervitaminosis A include splenomegaly, hepatomegaly, epithelial hyperplasia, and osseous dystrophy (Hilton 1983), but unless extreme, the effects are readily reversible.

These reports do not coincide with the findings of Pickering (1978), who carried out a detailed study of the effects of hypo- and hypervitaminosis A on the epidermis of brown trout and failed to show any effects in either case. In practical feeds, hypervitaminosis is unlikely to occur except when unusual ingredients such as whale liver cited above are used or when mistakes are made in feed production. Protected forms of vitamin A (vitamin A acetate, palmitate, and propionate) which are unaffected by oxidizing dietary lipids are normally used in fish diets, and this reduces the risk of deficiency even further.

8.4.1.2. Vitamin D (Cholecalciferol)

Deficiency of this vitamin has been demonstrated experimentally only in salmonids and channel catfish (Brown 1988; Leatherland *et al.* 1980; Lovell and Li 1978). Effects include reduced growth and, no doubt reflecting the metabolic importance of this vitamin for calcium metabolism, reductions in body ash, calcium, and potassium levels. Fish can sequester calcium from the water supply and effects of hypovitaminosis D occur only in very low calcium waters.

Hypervitaminosis has been demonstrated experimentally in brook trout, where it induced hypercalcemia and hemoconcentration (Poston 1969).

8.4.1.3. Vitamin E (α-Tocopherol)

This most biologically active of the tocopherols, α-tocopherol, is normally the form incorporated in practical fish diets and generally a protected form, α-tocopherol acetate, which will not oxidize during feed storage, is used. Tocopherol metabolism is closely linked to that of selenium and generally both are present in excess in practical diets. Vitamin E is particularly important, within the fish, as a component of the protective mechanism against the actions of free radicals, converting them to harmless compounds by donating protons. When fish receive oxidizing lipid in their diet, tocopherol exhaustion can be rapid and it becomes unavailable for its other functions related to the maintenance of stability of the structure of biological membranes.

There is some evidence that vitamin E requirements of Atlantic salmon are greater in seawater than during the freshwater stages. In healthy salmon, liver and plasma vitamin E concentrations increase dramatically soon after transmoltification and they increase at a slower rate thereafter. Temperature also affects the stability of biological membranes. The effects of vitamin E deficiency are exaggerated at lower temperatures and the requirement is also exercise related (Hardy 2000).

A wide range of clinical conditions has been associated with vitamin E deficiency. They involve principally muscle and fat tissue and include muscular dystrophy (Fig. 8.6) (Pearse *et al.* 1974; King 1975), steatitis (Fig. 8.7) (Roberts *et al.* 1979), and cardiac and digestive system pathology (Roy 1990). Erythrocyte membrane stability is particularly affected and the resultant anemia is readily assessed clinically as a measure of the tocopherol status of affected fish (Draper and Csallany 1969).

Probably the most significant group of conditions, economically, involving vitamin E deficiency is the range of polymyopathies in Atlantic salmon, which are all associated with low tissue vitamin E levels, despite the presence

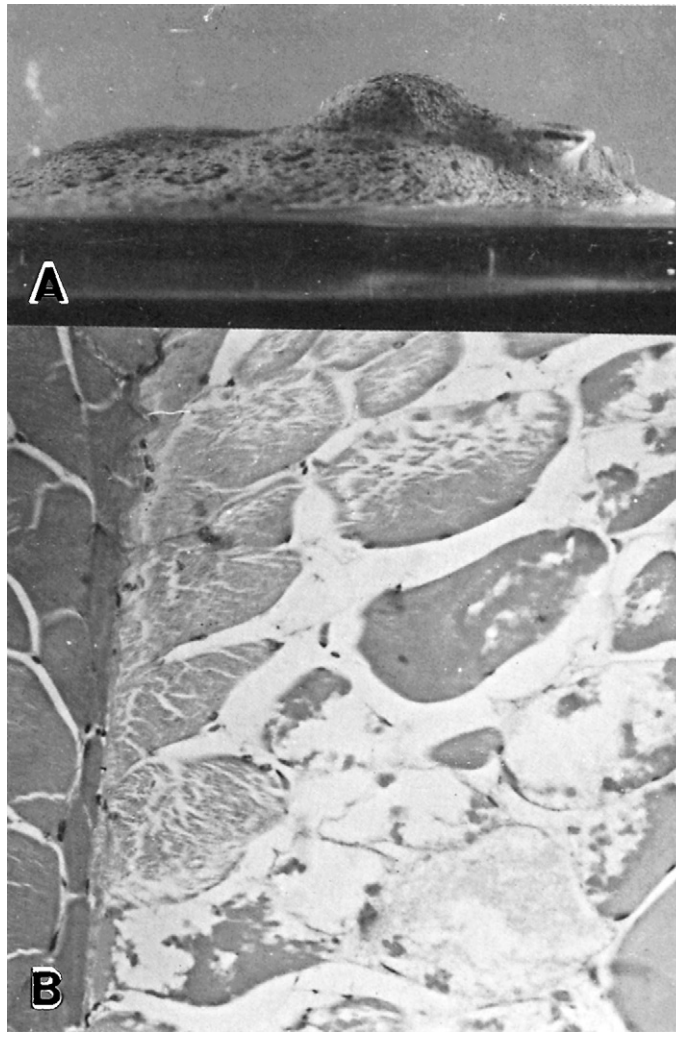

FIG. 8.6

(A) Turbot showing a skeletal deformity associated with vitamin E deficiency. (B) Section of skeletal muscle of the same fish showing bland sarcoplasmic degeneration at the periphery of a myotome. H&E, ×750.

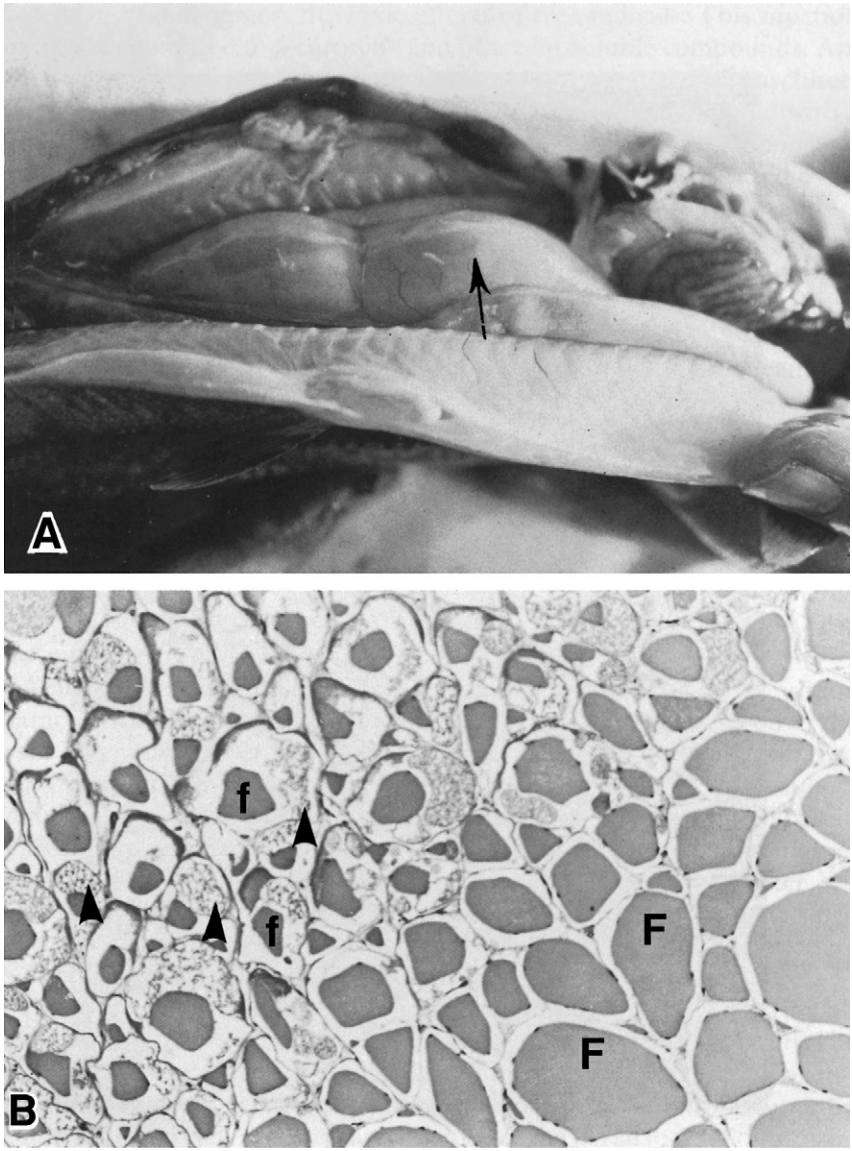

Fig. 8.7

(A) Grossly enlarged and thickened swimbladder of a rainbow trout with pansteatitis. (B) Degenerative white muscle in the same fish, showing shrunken sarcoplasm (f) with granular deposits (arrowheads) within the perimysium and normal fibers (F). H&E, ×70.

of high dietary vitamin E. Pancreas disease (PD), caused by a toga virus, is characterized by a severe round cell infiltration, necrosis and fibrosis of the pancreas, and a variable polymyopathy involving the esophagus, skeletal muscle, and heart (McLoughlin *et al.* 1996; McLoughlin 1996; Ferguson *et al.* 1986) (Fig. 8.8). Fish are also anemic and often unable to swallow. Mortalities are variable but entire populations may be affected and have low blood and liver tocopherol levels, although the selenium levels appear normal. They also have high levels of creatine kinase (Roy 1989).

Cardiomyopathy syndrome (CMS) is a very similar condition of farmed salmon in the sea, except that there are no pancreatic lesions (Ferguson *et al.* 1989). In fish with CMS there is often a very severe cardiac myopathy with acute pericarditis and atrial myonecrosis so severe that atrial rupture may supervene at the least exertion. There are frequently nervous signs including circling, loss of balance, and hyperexcitability associated with focal encephalitis.

In both conditions, the muscle lesions resemble those of the "white muscle" vitamin E/selenium-related myopathies found in poultry and pigs, despite adequate levels of vitamin E in the feed. The metabolic or functional deficiency of tissue vitamin E is therefore presumed to be associated with virus activity, which is certainly the case in PD and suspected in the less common CMS.

Where experimental studies have attempted to replicate the features observed in putative clinical vitamin E deficiency conditions, this has rarely been achieved. Wilson *et al.* (1984), using catfish, and Cowey *et al.* (1981) and Watanabe *et al.* (1981), using rainbow trout, have failed to show more than minimal changes in deficient fish. Moccia *et al.* (1984) demonstrated hematological changes and alteration in splenic hemosiderin levels and hepatic morphology but did not mention muscle changes.

Poston *et al.* (1976), however, feeding very young Atlantic salmon, showed anemia, muscular dystrophy, and biliary retention as well as exudative diathesis and loss of pigmentation. Cowey *et al.* (1984), studying the effects of reduced vitamin E levels combined with the presence of the moderately oxidized oil, showed that while the presence of the moderately oxidized oil did not affect the results, vitamin E deficiency in either group resulted in severe pathological changes. These included myofibrillar hyaline degeneration, cellular infiltration of subepericardial areas, and fibroblast proliferation. The contrast of these findings with those of Cowey *et al.* (1981), Hung *et al.* (1981), and Watanabe *et al.* (1981) was explained by the fact that all three groups had carried out their experiments at 15°C or above, whereas Cowey *et al.* (1984) had carried out their studies at 12–6°C. Over this range of ambient water temperatures considerable variations take place in the hardness of different unsaturated lipids so a cellular membrane structural effect

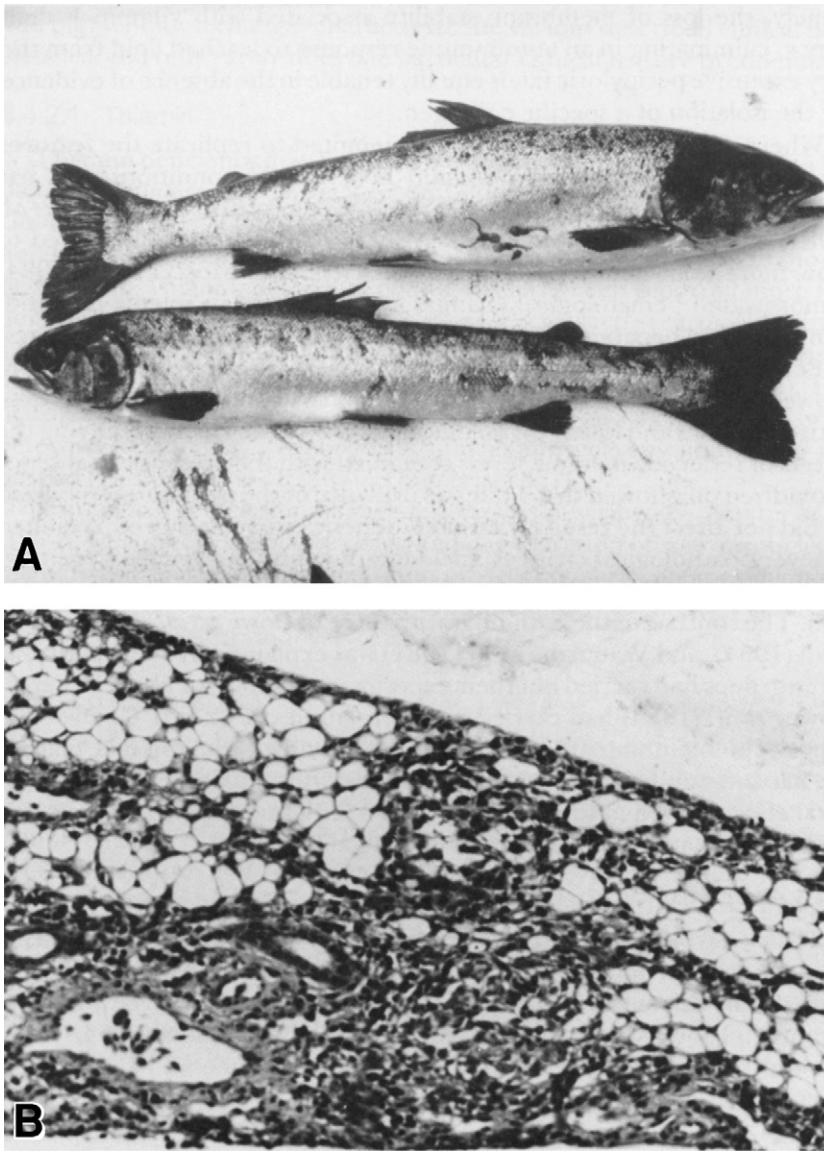

FIG. 8.8

(A) Atlantic salmon with pancreas disease (PD) showing loss of condition and wasting. (B) Degeneration of lipid tissue of the pancreas and replacement of pancreatic acini. H&E, ×150.

related to the polyunsaturated fatty acids in the membrane is both feasible and requires further investigation.

8.4.1.4. Vitamin K

Deficiency of the two forms of vitamin K, phylloquinone and farnoquinone, appear to have significance only for the blood-clotting mechanism. In the channel catfish there is some doubt about its actual requirement (Duprée 1966; Murai and Andrews 1977) but Poston (1964, 1976) showed that its deficiency increased the clotting time and reduced the microhematocrit of trout.

8.4.2. Water-Soluble Vitamins

Deficiencies of an individual water-soluble vitamin (B or C-group) are somewhat uncommon in practical diets. A wide range of specific clinical features has, however, been described in experimental studies.

For some water-soluble vitamins such as vitamin C distinctive clinical signs have been defined. For others, poor growth and darkening of color may be the only features. Since there are great differences in the tissue turnover times of the different water-soluble vitamins, the time required to induce a specific deficiency is also variable. Some develop rapidly, whenever a deficient diet is fed. Others may take months to develop. Another important feature of water-soluble vitamin deficiencies is that they take two or three times longer to develop in older fish compared to fry, fingerlings, or, in the case of salmonids, smolts. This is because these stages have much lower tissue reserves and higher growth rates, especially at high water temperatures on high-energy diets (Hardy 2001).

8.4.2.1. Thiamin

Thiamin deficiency may be actual or induced by the presence within the diet of thiaminase, an enzyme capable of destroying thiamin (Seacock and Goodland 1944). Thiaminases are common in the tissues of certain fish, notably the cyprinids and cluepeids, and in certain legumes. These should, therefore, not be included in diets in uncooked form. Thiamin deficiency conditions have been reported more frequently in salmonids and eels than in species such as carps which have a higher proportion of carbohydrate in their diet, despite the importance of thiamin in carbohydrate metabolism (Murai and Andrews 1978a). The features of thiamin deficiency in these animals are also more marked, with petechial hemorrhage, fin necrosis, ataxia, and nervous signs, particularly hyperexitability, due to more rapid depletion of thiamin from the brain than from other tissues, being reported (Hashimoto *et al.* 1970; Duprée 1966).

Histological correlation of such lesions was described by Blaxter *et al.* (1974), who reported hemorrhage and degeneration of specific nuclei of the periventricular areas of the brain of Atlantic herring experimentally fed a diet deficient in thiamin, a condition readily prevented by enhancement of dietary thiamin levels (Fig. 8.9).

8.4.2.2. Riboflavin (B_2)

Since riboflavin is particularly active as a coenzyme for oxidase systems, it is important in the respiration of poorly vascularized tissues such as the cornea and lens of the eye. In fish, it has also been associated with petechial hemorrhages in carp (Ogino 1967) and with congenital dwarfism in channel catfish (Murai and Andrews 1978b). In the eye, the corneal lesion is similar to that of higher animals, namely, cloudiness, with growth into the substance of the cornea by limbal capillaries and hemorrhage. The cataract is usually bilateral and results in blindness. Barash *et al.* (1982) have shown that the electrophorectic properties of the proteins of the lens of riboflavin-deficient fish are distinct from those of normal fish and, indeed, from those of fish with cataracts of different origins.

8.4.2.3. Pyridoxine

Pyridoxine is widely distributed in the feed components which are normally utilized in fish feed compounding. Thus it is rare for deficiencies to occur in fish fed normally formulated diets. However, Herman (1985) described several histopathological features, including renal, ovarian, and hepatic degenerative changes, thyroid colloidal deficiency, and hemopoietic hyperplasia, in farmed Atlantic salmon fingerlings, following accidental exposure to a pyridoxine-deficient diet. The clinical signs of hyperexcitability and poor growth were similar to those described in the experimentally induced condition in salmonids (Halver 1957a), eels (Arai *et al.* 1972), turbot (*Scophthalmus maximus*) (Adron *et al.* 1978), and gilthead bream (*Sparus aurata*) (Kissil *et al.* 1981). Blood changes associated with pyridoxine deficiency and probably correlated with the hemopoietic changes have been described by Smith *et al.* (1974) in rainbow trout. Since the active form of pyridoxine is rapidly depleted, deficiency signs will develop in a period as short as 3–4 weeks.

8.4.2.4. Pantothenic Acid

Pantothenic acid deficiency in young fish is one of the most frequently described vitamin B-group deficiencies in farmed fish. In almost all species it is associated with a lamellar epithelial hyperplasia, progressing proximally from the periphery of the affected gill as the condition develops (Fig. 8.10). It precedes or coincides with the onset of anorexia (Karges

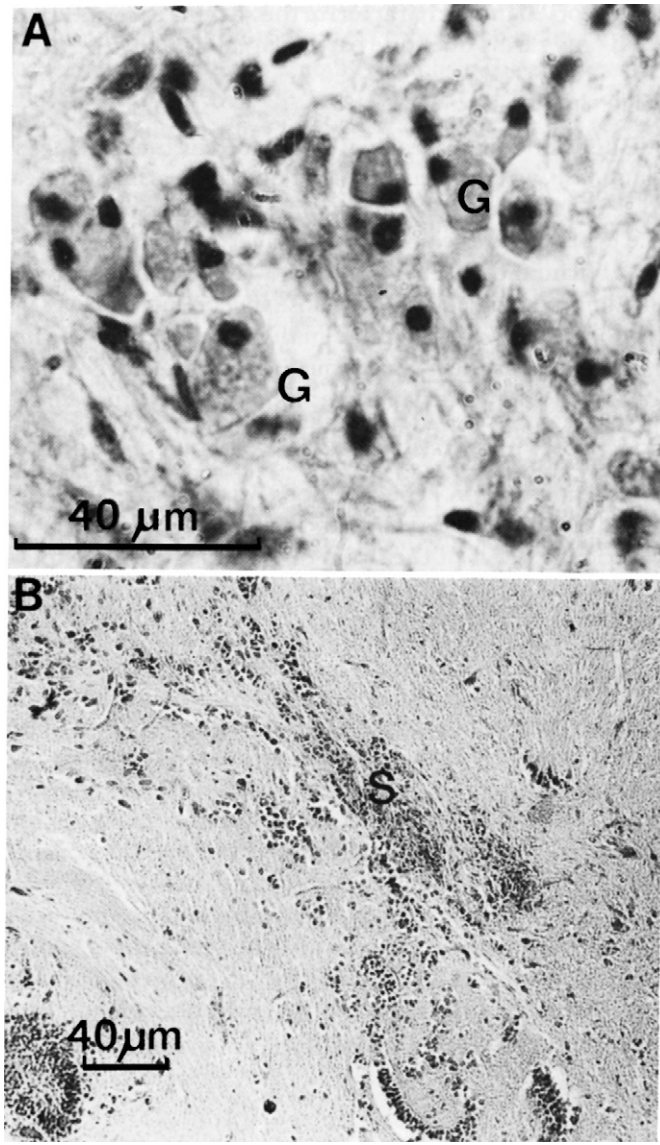

FIG. 8.9

Cranial lesions associated with thiamin deficiency in an Atlantic herring. (A) Gemastocytes (G) around an area of neuronal necrosis. They are large, clear vacuolated cells with a foaming cytoplasm and a large eccentric nucleus. (B) A glial scar (S) in the thalamus of a recovered, clinically normal herring. H&E, ×100.

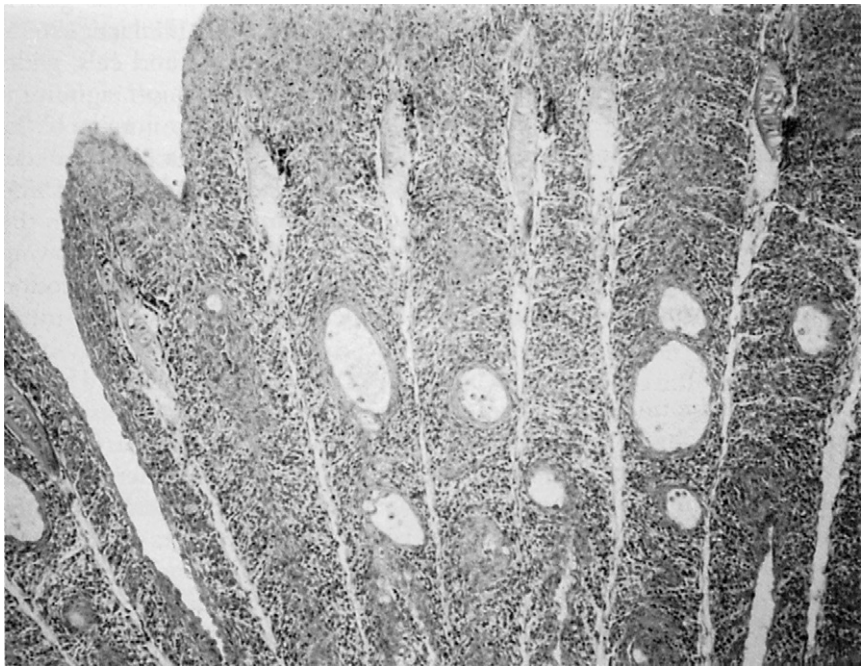

Fig. 8.10

Gill from a pantothenic acid-deficient rainbow trout. Severe primary lamellar hyperplasia has resulted in fusion of respiratory surfaces. H&E, ×20.

and Woodward 1984) and may be overlaid by bacterial infection, although it should not be confused with so-called bacterial gill disease, a proliferative response to flavobacterial infection which can occur without any evidence of pantothenic acid deficiency. Other clinical features associated with pantothenic acid deficiency include erosion of fins and barbels and superficial skin ulceration. Anemia has also been reported by some workers, but only toward the end of experimental studies, where it is probably a result of the concomitant anorexia.

8.4.2.5. Inositol

No specific syndromes have been reported in association with deficiency of this B-group vitamin. Aoe and Masuda (1967) and Coates and Halver (1958) have, however, shown that growth is impaired in its absence and there have been reports of mild ulcerative lesions of the integument which respond to its supplementation (McLaren *et al.* 1947).

8.4.2.6. Niacin

Niacin deficiency in fish has been associated principally with dermatoses, although in salmonids, muscle spasms and edema of the gastric submucosa have been described in experimental studies (Halver 1957b; Poston and Di Lorenzo 1973). In cyprinids, catfishes, and eels, widespread hemorrhages are found experimentally, but the most significant feature of niacin deficiency is the predilection it creates for erosion of the dorsal epidermis following exposure to ultraviolet light, a condition described as "back peel" by De Long *et al.* (1958). Poston and Wolfe (1985) have demonstrated that at least 18 ppm niacinamide is necessary in the diet of the rainbow trout to avoid the induction of skin damage following exposure to UV light. At lower levels, or in its absence, extensive erosion of the caudal and pectoral fins and of the head and snout occurs.

8.4.2.7. Biotin

Although biotin has important roles as a coenzyme for many aspects of lipid and carbohydrate metabolism, it is usually readily available from the normal ingredients of fish diets (Castledine *et al.* 1978). When it is deficient in experimental salmonid diets, the usual range of clinical deficiency effects, such as darkening, anorexia, a reduction in growth, poorer feed conversion, and increased mortality, occurs (Poston and McCartney 1974; Poston and Page 1982). A feature reported in a number of experimentally deprived fish studies, which was variable between species, was the modification of epithelial mucous cells or mucin production. This was recorded by Ogino *et al.* (1970) in the common carp (*Cyprinus carpio*), where there was a marked increase in the numbers of mucous cells in the epidermis. It was also recorded in the rainbow trout by Castledine *et al.* (1978), who showed marked changes in the structure of the epithelium of the primary lamellae, with hypertrophy and hyperplasia of mucous cells and a heavy tenacious mucous overlay. In addition, they demonstrated distinctive glycogen accumulation within hepatic parenchymal cells. These did not, however, completely correlate with the clinical observations of Phillips and Brockway (1957), who ascribed an epidermal and cuticular condition described in rainbow trout to biotin deficiency.

8.4.2.8. Choline

The choline requirement of fish is high and its absence results in poor growth and conversion (Halver 1957a; Coates and Halver 1958; Ketola 1976). There are, however, some variations in the limited pathological features recorded. Renal hemorrhages consistently observed in experimental choline deficiency in chinook salmon could not be replicated in silver

salmon. Sturgeon fed a choline-deficient diet developed a pronounced thinning of the intestinal muscularis and focal pancreatic degeneration (Hung 1989).

8.4.2.9. Cyanocobalamin

Halver (1953, 1957) demonstrated that in coho salmon, the absence of cyanocobalamin results in anemia and poor growth accompanied by darkening and variable erythrocytic depression. John and Mahajan (1979) showed, in the Indian carp (*Labeo rohita*), that cyanocobalamin deficiency led to a significant leukocytopoiesis, related mainly to thrombocyte and, to a lesser degree, eosinophil numbers, with neutrophil and large lymphocyte numbers significantly depressed.

8.4.2.10. Folic Acid

Folic acid deficiency in fish is related principally to an abnormal blood picture. Smith (1968) and Smith and Halver (1968) reported a megaloblastic, normochromic, macrocytic anemia in coho salmon, a finding confirmed by John and Mahajan (1979) in *Labeo rohita*. This differed from the latter authors' findings in the air-breathing fish *Channa punctata*, which had a hypochromic anemia under similar conditions (Mahajan and John 1976). Those authors also showed that a combined deficiency of folic acid and cyanocobalamin produced a more severe anemia than each did separately.

8.4.2.11. Vitamin C

Ascorbic acid deficiency has been studied extensively in fish. It is a cofactor for the hydroxylation of proline to hydroxyproline, an essential component of collagen, and thus it is vital to the connective tissues, to bone matrix, and to scar tissue in wound healing (National Research Council 1993). In all fish species studied, two related syndromes are associated with vitamin C deficiency: a marked reduction in wound-healing capacity and a skeletal malformation syndrome including spinal lordosis and scoliosis, spinal fracture, deformity of the mandible and its articulation, and foreshortening of the operculum (Figs. 8.11 and 8.12). Additionally there is a tendency to hemorrhage and to secondary infection. All these features appear to be related to failure of collagen synthesis.

Studies by Kitamura (1965), Poston (1967), and Halver *et al.* (1969), working with rainbow trout, brook trout, coho salmon, and tilapias, respectively, all showed, after a varying period of time on a deficient diet, the development of a deforming diathesis of skeletal cartilage and osteoid replacement of many bony tissues (Fig. 8.13). Halver *et al.* (1969) showed extensive hemorrhage in conjunction with the skeletal distortion in coho salmon, but in

FIG. 8.11

Scoliosis (upper) and lordosis (lower) in coho salmon fingerlings deficient in ascorbic acid for 22 weeks. The middle fish is a normal coho fed a complete test diet. From Halver (1972).

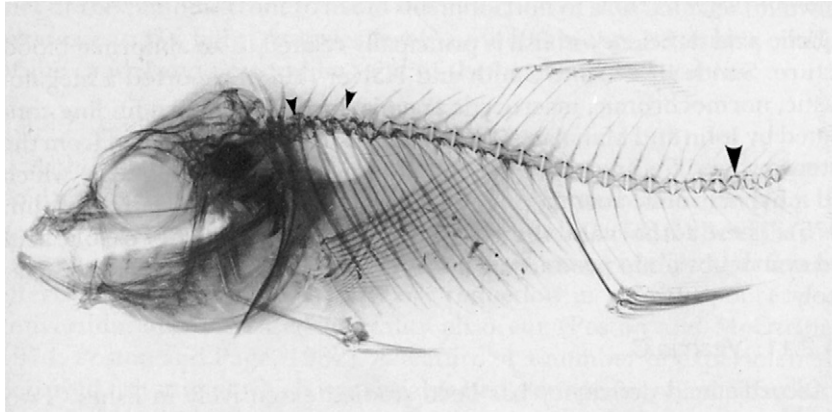

FIG. 8.12

X-Ray of osteoid deformation and fracture (arrowheads) in thoracic vertebrae of a vitamin C-deficient *Oreochromis niloticus* and associated deformation of the body and articulation of posterior vertebrae (arrowhead).

FIG. 8.13

Gill cartilage metaplasia (O) associated with vitamin C deficiency in a cultured tilapia. H&E, ×70.

tilapias at tropical temperatures, where the onset of the clinical signs was earlier, the hemorrhage was not a necessary concomitant (Soliman *et al.* 1983). Radiographically, scorbutic fish show deficient mineralization, occasionally with a focal increase in density. The area of the spine just posterior to the costal articulations was the most frequently damaged and deformed.

Studies on wound healing in fish in relation to ascorbic acid deficiency were first developed by Halver *et al.* (1979), who induced lesions 1 cm long in the dorsal or abdominal musculature and closed them with gut sutures. Controls healed well within 3 weeks, whereas deficient fish failed to heal, and where abdominal lesions penetrated the abdominal wall, there was an infusion of water into the abdominal cavity. Soliman *et al.* (1985), using somewhat smaller lesions in tilapias, showed a similar response, characterized by failure of the dermal collagen of the stratum compactum to proliferate in the normal healing response. In their study, however, it was obvious that the epidermal closure by rapid epithelial cell migration from the periphery of the lesion was unaffected by the deficiency, with securing of waterproofing being achieved as a priority, irrespective of the vitamin C levels (Fig. 8.14).

Clinically, vitamin C deficiency is a particularly important condition in commercial catfish culture. Meyer (1975) described a widely occurring

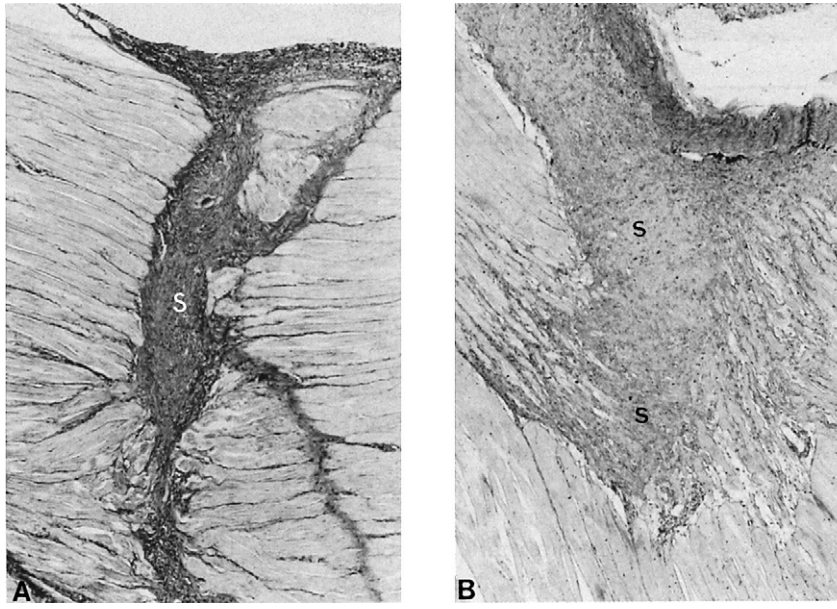

FIG. 8.14

(A) Contracted collagenous scar(s) in the dermis and muscle of a tilapia fed a normal diet 13 days after induction of a lesion. The epidermis is normal and the scar of collagenous tissue has contracted to align the adjacent myofibrils. (B) A similar lesion in a vitamin C-deficient fish 16 days after induction. The lesion is still cellular, and there is no dense, contracted collagen within the fibrosis scar (S). H&E, ×60.

condition known as broken back syndrome in channel catfish (*Ictalurus punctatus*). The anomaly is characterized by damage to the spinal column, depigmentation, a reduced growth rate, and mortalities. Often the condition was induced by sublethal toxaphene in the water or diet, thus increasing the dietary ascorbate requirement. Lim and Lovell (1978) have replicated this syndrome using artificially prepared diets in farmed channel catfish. They found that gill malformation was the earliest and most sensitive response to deficiency. In the Asian catfish (*Clarias lazera*) in culture, there is a very marked and serious vitamin C deficiency syndrome, known as "crackhead" (Kamonporn *et al.* 1981), characterized by spinal malformation and, particularly, infection of the posterior cranial symphysis—the "crackhead." The bones of the head are lighter than those in normal fish and a hollow sound is produced when it is tapped against a hard surface.

In rapidly growing Atlantic and Pacific salmon on high-energy diets at high temperatures, especially where these coincide with smoltification, a

FIG. 8.15

Atlantic salmon with screamer disease. There is a deformed jaw due to fusion of the articulation and a permanently open mouth. The disease is associated with vitamin C and phosphate marginal deficiency in fast-growing fish. Courtesy of Dr. Patricia Salas.

combined vitamin C/phosphate deficiency can arise. Fish have a distinctive appearance and are known as "screamers" because of the supposed resemblance to the painting "The Scream" by Munch (Fig. 8.15). Lesions are particularly associated with the jaw and operculum, though spinal lesions also occur. Histologically there is evidence of gill, jaw, and opercular cartilage metaplasia and deformity associated with osseous dystrophy (Roberts *et al.* 2001).

8.5 Mineral Deficiencies and Imbalances

Although almost all necessary minerals are available in most practical fish diets and fish can also absorb minerals from the surrounding water, mineral deficiencies do, on occasion, arise in farmed fish. Almost invariably they are associated with a reduced bioavailability of the mineral rather

than a frank deficiency. The reduced bioavailability may be associated with a dietary imbalance or with presentation of the element in an unsuitable form. It may also be associated with interaction with other dietary ingredients, such as vitamins and fibers (Tacon and De Silva 1983). The absence of an acid-secreting stomach in cyprinid fish prevents the absorption of organically bound minerals, especially phosphorus, and within plant proteins the presence of phytic acid may chelate trace elements such as iron, copper, and zinc (Satoh *et al.* 1989).

Phytate is the storage form in which phosphorus is retained in seeds. Grains and storage products used in fish diets are therefore often rich in phytate-phosphorus, but this is unavailable to fish.

Fish meals used in practical diets contain high levels of calcium, which, particularly in the case of whitefish meals, greatly reduces the bioavailability of trace elements, and clinical outbreaks of mineral deficiencies can occur. For this reason experimental studies using whitefish meals with relatively high inherent trace element levels, but without trace element supplementation, have been shown to induce typical deficiency signs of reduced growth, loss of appetite, and cataract in salmonids and common carp (Watanabe *et al.* 1983; Yamamoto *et al.* 1983).

8.5.1. Iodine

Fish meals contain iodine so modern diets incorporating fish meals do not normally exhibit iodine deficiencies. However, when salmonid diets were mainly of mammalian origin, goiters (thyroid hyperplasia), readily controlled by iodine therapy, were common. Originally such lesions were described as thyroid adenocarcinomata (Gaylord *et al.* 1914) but their goitrous nature was defined by Marine and Lenhart (1910). The confusion between goiter and neoplasia is readily understandable because, as Davis (1953) has pointed out, the salmonid thyroid, unlike that of the human, is diffusely scattered around the ventral aorta and so, when goitrous, strongly resembles an invasive adenocarcinoma (Fig. 8.16).

8.5.2. Iron

Iron is more often a problem in relation to its toxicity at high levels in certain water systems rather than as a deficiency, and the melanomacrophage system of the hemopoietic tissues plays a particularly avid role in the retention of iron in the body following hemorrhage or infection (Roberts 2000). Iron deficiency anemia has been reported in brook trout (Kawatsu 1972), yellowtail (Ikeda *et al.* 1973), red sea bream (Sakamoto and Yone 1978), carp and eel (Nose and Arai 1979), and catfish (Gatlin and Wilson 1986).

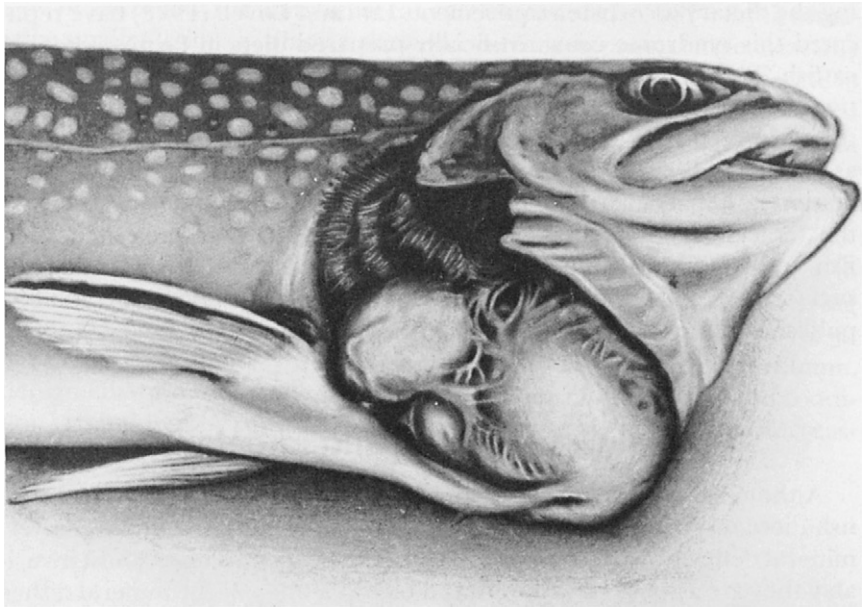

FIG. 8.16

Advanced thyroid "tumors" (actually iodine deficient) in a rainbow trout (*Salmo gairdneri* Richardson). From Halver (1972).

8.5.3. Copper

Probably because of the ease with which fish can absorb copper from water, copper deficiency *per se* has not been described, although Murai *et al.* (1981) attempted to induce it in channel catfish. They did, however, succeed in showing a slight anemia when fish were fed high supplementary levels. The toxicity of high levels of environmental copper is well recognized and induces gill pathology similar to that of the other environmental heavy metal ions. Satoh *et al.* (1983) reported that carp fed high-ash diets without copper supplementation developed characteristic cataracts. One particularly important feature of high copper levels, whether of dietary or environmental origin, is the facilitative effect it has been shown to have on the pathogenesis of *Vibrio anguillarum* infection in salmonids (Hilton and Hodson 1983).

8.5.4. Manganese

Manganese is one of the essential elements in whose absence the usual features of poor growth and dwarfism are complemented by the occurrence

of cataracts. Satoh *et al.* (1983) and Yamamoto *et al.* (1983), working with rainbow trout and carp, showed that manganese supplementation in an available form was essential where fish meals were a major component of the diet. Ishac and Dollar (1968) showed that in *Oreochromis mossambicus,* the absence of manganese resulted in poor growth, reduced food consumption, and loss of equilibrium, but they did not report cataract development. Skeletal abnormalities associated with manganese deficiency have been reported for rainbow trout (Ishac and Dollar 1968), carp (Ogino and Yang 1980), and tilapias (Yamamoto 1983).

8.5.5. Zinc

Zinc deficiency is normally related to the presence in the diet of zinc-binding agents such as ash, phytic acids, and calcium (Ketola 1979; Hardy and Shearer 1985). General features of zinc deficiency have been described in most farmed species, but the usual deficiency signs of poor growth and skin and fin lesions are complemented by the important feature of bilateral lenticular cataract development (Fig. 8.17). Other clinical signs of zinc deficiency in salmonids include erosion of fins, short-body dwarfism, and poor egg hatchability (Hardy 2000).

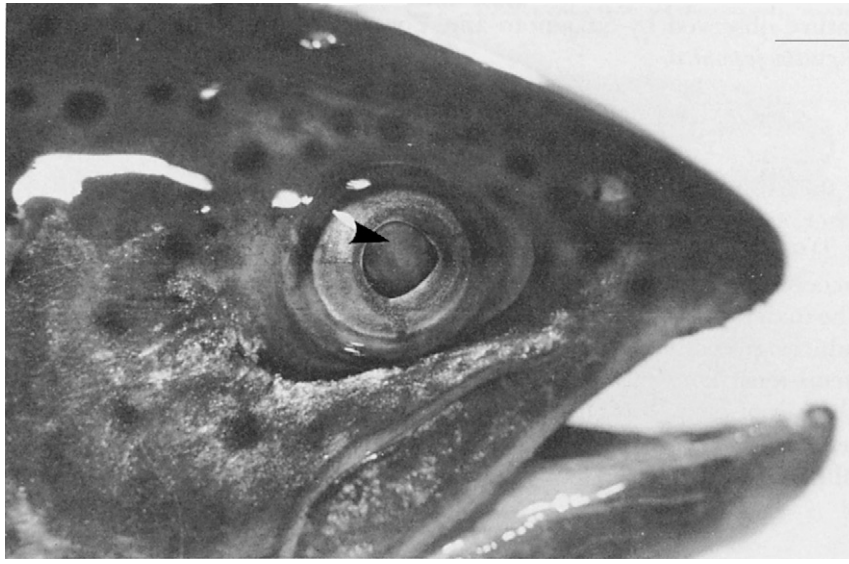

FIG. 8.17

Zinc deficiency cataract in a salmon showing the delicate gray translucent lens (arrowhead).

Cataract development is an important feature of many marginal deficiency conditions in fish. In salmonids, in particular, there is an extensive range of cataracts described. These are associated not only with zinc deficiency but also with riboflavin, (Phillips and Brockway 1957), methionine (Poston *et al.* 1977), and a particularly significant but as yet undefined deficiency associated with withdrawal of components of animal origin, such as blood meal, from high-energy salmon diets (Wall 1998).

The pathology of each type of deficiency cataract is distinctive and Barash *et al.* (1982) have shown that each type also has its own characteristic protein banding in electrophoresis.

8.5.6. Phosphorus

Phosphorus is one of the few minerals which fish cannot supply from their aquatic medium. Nevertheless, phosphorus deficiency is rare because most fish diets have high levels of fish meal, which is rich in this element. As fish meal levels are being progressively reduced, however, and replaced by plant proteins in high-energy feeds, both for economic reasons and to reduce the phosphorus excretion levels from fish farms, it is becoming more frequent. Aside from the normal deficiency features of anorexia and dark coloration, phosphorus-deficient fish also show signs directly related to the role of phosphorus in bone mineralization, principally softening and deformities of the head, vertebrae, and ribs (Ogino and Takeda 1976; Shearer and Hardy 1987; Skonberg 1997; Baeverfjord *et al.* 1998).

8.6 Dietary Mineral Toxicity

Heavy metal contamination of feeds resulting from storage vessel leaching or from use of unusual feed sources can cause significant growth inhibition. Tacon (1985) has documented copper contamination of distillery and brewery wastes, zinc accumulation in shellfish and feathermeal, and selenium in serpentine soils. Generally the features are those characteristic of a nutritional disease, namely, reduced growth and poor food conversion, but cadmium excess is associated with hyperactivity, hypocalcemia, and osteoporosis (Roch and Maly 1979). Lead toxicity in the diet has been associated with development of black tails and spinal curvature (Sippel *et al.* 1983).

The influence of dietary calcium levels on the toxicity of metals, as well as the deficiency diseases, is documented in relation to both lead and zinc

(Takeda and Shimma 1977; Varanasi and Gmur 1978). Calcium greatly reduced the toxicity of both metals in salmonids, and in the case of lead it was suggested that the calcium status of the diet is the key factor in determining the biological fate and hence the toxic potential for ingested lead.

8.6.1. Selenium

The interrelationship between selenium and tocopherol (vitamin E) makes for great confusion in the dietary role of this metal in animals. In fish, or at least the salmonids, its deficiency in the diet is linked with that of vitamin E in the prevention of bland muscular dystrophy (Poston *et al.* 1976) and its supplementation with vitamin E greatly enhances tocopherol activity in liver but not in muscle. At higher levels of dietary carbohydrate, it has also been associated experimentally with the occurrence of nephrocalcinosis, although it is unlikely that it is ever the cause of nephrocalcinosis in intensively produced fish in normal circumstances (Hicks *et al.* 1984). Evidence also exists for significant selenium–copper interaction, which may affect secondary infection susceptibility (Hilton and Hodson 1983). In pancreas disease (PD) (*vide supra*), selenium levels are not reduced even though vitamin E levels may be minimal (Roy 1990).

8.6.2. Calcium

The role of calcium in mineralization of bones and scales, as well as in ionic control of blood electrolyte levels, indicates a high requirement, but fish readily absorb the ion through the gill. Therefore the main pathologies associated with calcium metabolism relate to its metal-chelating capacity within the diet, leading to deficiencies, the effects of metastatic calcification, and osseous decalcification in association with other dietary conditions. Decalcification, osteoporosis, and osteoid dystrophy have already been described in association with vitamin C deficiency.

Metastic calcification can occur in association with a number of pathological processes; indeed in fish as in other animals it frequently forms the final stage in the successful resolution of chronic inflammatory conditions such as tuberculosis and metazoan parasite encystment. The two most common problems occur in the salmonids, where two specific conditions, visceral granuloma and nephrocalcinosis, can cause serious mortalities.

Visceral granuloma is a specific condition confined to the digestive tract of salmonids where focal granulomata develop in the stomach wall and become mineralized, often to result in large metastatic calcified nodules

which can be palpated through the body wall. The initial lesion is a typical foreign body-type granuloma with epithelioid and giant cell formation, but although a dietary component, possibly a mycotoxin, is suspected of initiating the phenomenon, its etiology is unknown. Dietary calcium or phosphorus levels do not appear to be related to it in any way (Fig. 8.18). Nephrocalcinosis is the precipitation within the renal tubules of calcium complexes. The phenomenon can arise in a number of circumstances and the chemical nature of the urolith material—much softer and rarely particulate like the renal calculi of higher animals—varies depending on the origin. The most common form of urolithiasis is associated with excessive levels of dissolved carbon dioxide in the water of rainbow trout (Harrison and Richards 1979; Smart *et al.* 1979). It has, however, also been associated with magnesium deficiency (Knox *et al.* 1981), selenium toxicity (Hilton and Hodson 1983), and nephrotoxic heavy metal complexes in binders used in wet feeding (Fig. 8.19).

Histologically, urolithiasis generally begins with the accumulation of calcified inclusions within vacuolated proximal tubular epithelium and sloughing of tubule cells into the lumen, where a dense, dark-staining precipitate

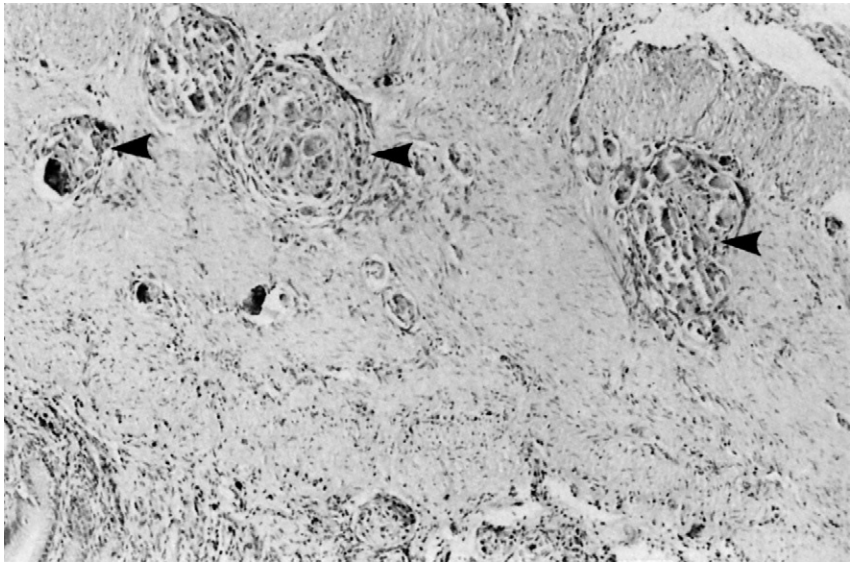

FIG. 8.18

Visceral granulomata (arrowed) in the wall of the stomach of a brook trout. H&E, ×100.

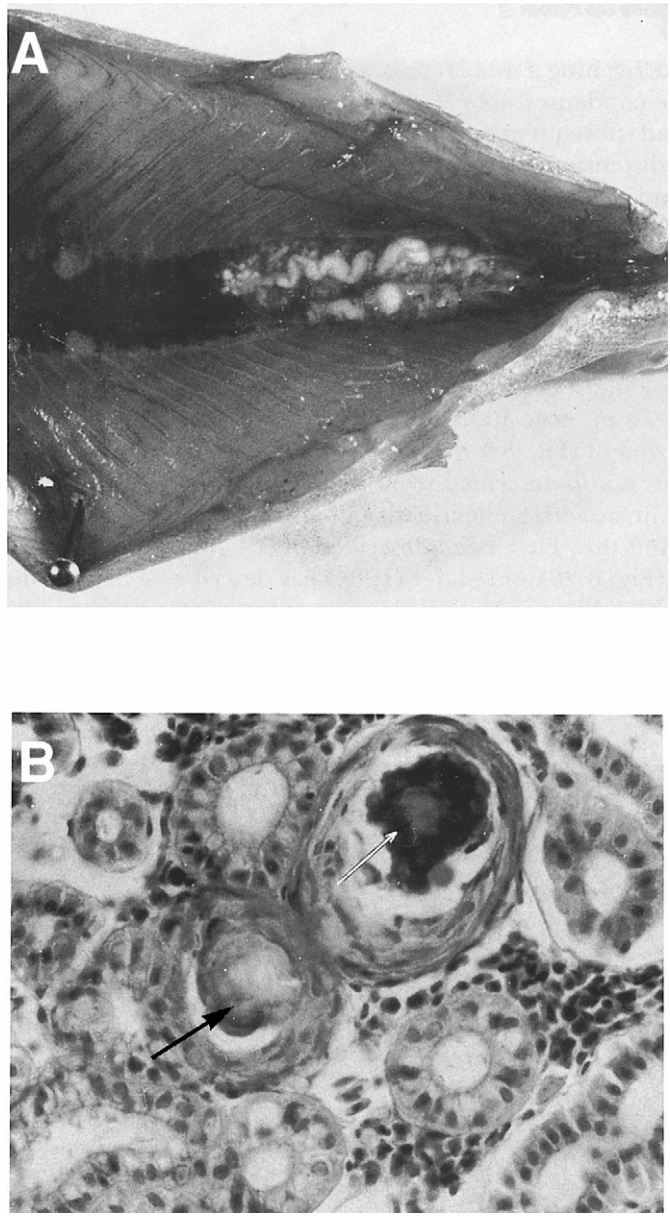

FIG. 8.19

Nephrocalcinosis. (A) Gross lesion showing white deposits in ureters and in the kidney substance of a rainbow trout. (B) Histological section of chronic nephrocalcinosis showing tubules and ducts (arrows) in various stages of calcification.

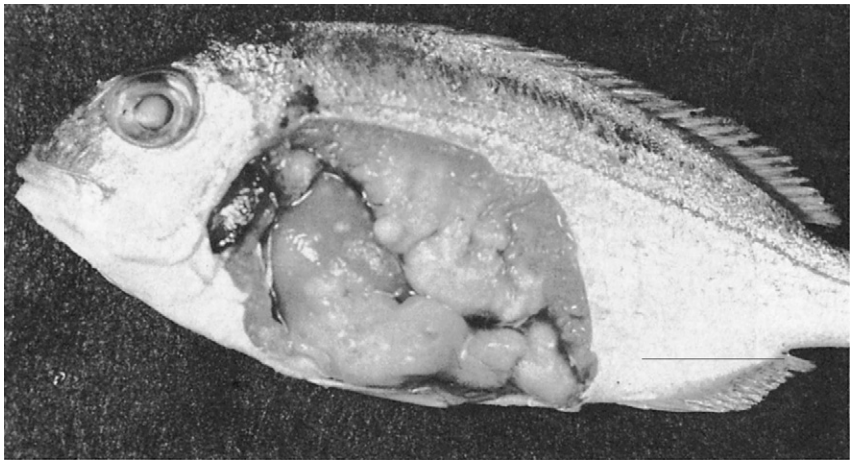

FIG. 8.20

Ectopic calcified granulomata in the viscera and eye of a gilthead bream. Courtesy of Dr. I. Paperna.

forming a tubular cast is elaborated, around which the degenerating tubule condenses. Obstruction of urine flow leads to retrograde distension and resultant degeneration of tubules and shrinkage of glomeruli with distension of the capsule. Subsequently there is peritubular fibrosis and destruction of hemopoietic tissue, and the kidney becomes swollen and gray, with the ureters clearly demarcated by their white content.

Ectopic calcified granulomata have also been described occasionally in other fish species, a particularly dramatic example being the development of large pisiform calcific nodules throughout the body and even in the brain and eyes of gilthead bream. This condition was first described by Paperna *et al.* (1980), in association with an experimental wet-feed diet of a high mineral content (Fig. 8.20).

Lithiasis has also been described from the bile duct of fishes. Again, as with cholelithiasis in higher animals, the causes are often obscure, but they have been described in Atlantic and Pacific salmon on wet diets (Fig. 8.21), and Maier (1984) has described experimental induction of cholelithiasis in the tilapia. The connection with calcium metabolism, if any, is not known. Histologically the condition results in biliary obstruction, which stimulates biliary fibrosis, hyperplasia, and ultimately cirrhosis, with obstructive jaundice often being demonstrable, accompanied by high blood bilirubin levels.

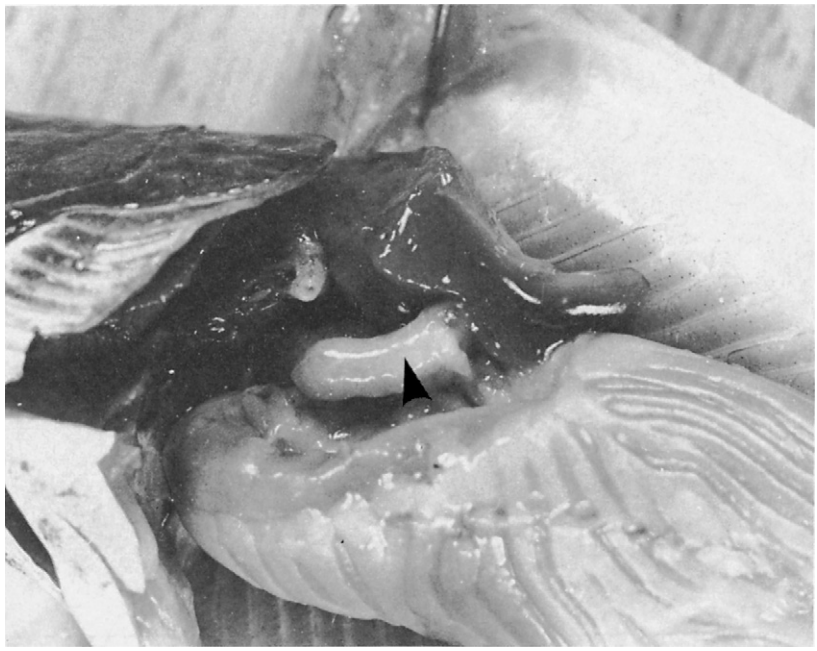

FIG. 8.21

Cholelithiasis in an Atlantic salmon fed a wet diet.

8.7 Mycotoxins

Probably the most important of the natural contaminants are the mycotoxins, metabolic products of fungal contaminants of feed components. The most significant of these toxic metabolites, in terms of fish diets, are the aflatoxins, substances produced by toxic mutants of the blue–green mold *Aspergillus flavus,* which is a common contaminant of oil seeds such as cotton and peanut. The toxicity of the aflatoxins was first described by Ashley *et al.* (1964) and Halver (1965). They showed that the aflatoxins were powerful carcinogens responsible for the widespread occurrence of hepatocarcinoma in cultured trout (Fig. 8.22). The hepatocarcinomata usually reach a clinical level after 4–6 months of feeding of the contaminated meal. The amount of contamination can be very small, as little as 0.01 ppb in the total diet. When fed experimentally at high levels, 80 ppb or more, the toxin produces an acute toxic syndrome, severe or even massive focal hepatic necrosis, and branchial edema, as well as generalized punctate hemorrhage (Ashley 1970).

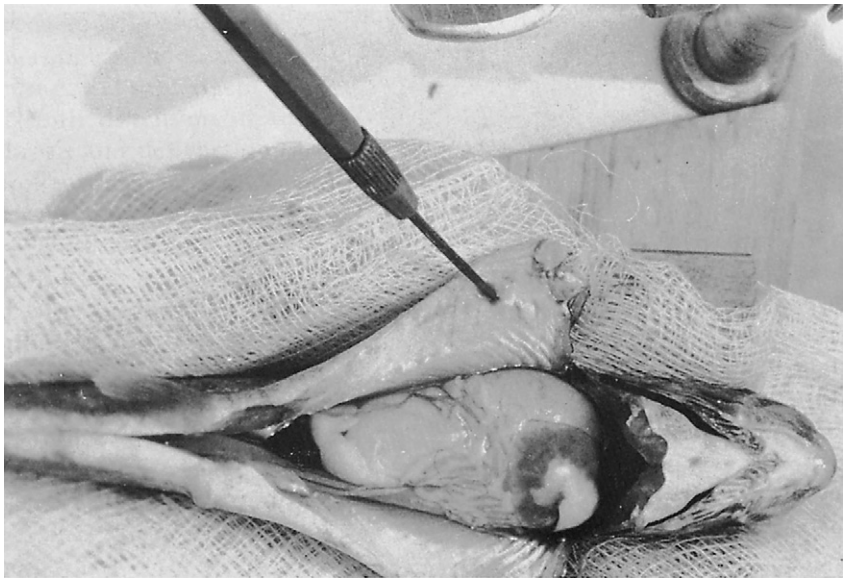

FIG. 8.22

Acute aflatoxicosis in a rainbow trout liver showing a necrotic area (white), other pallid areas near the margin, and several dark hemorrhagic areas. From Halver (1972).

Histologically, the naturally occurring lesions are invasive malignant trabecular hepatocarcinomata, very obvious in sections even in early stages because of the focal, darker-staining zones of malignancy (Fig. 8.23), which can extend to the capsule and involve the biliary and vascular tissue. In later stages the rapidly growing friable and highly vascular tumor tissue may develop central infarctive necrosis and, in the event of handling, sudden massive hemorrhage, which may result in chronic anemia or be immediately fatal.

Aflatoxicosis was a problem principally of rainbow trout, which appear to be the most sensitive to the toxin, and was a significant economic problem in culture during the 1960s. Following the work of Ashley *et al.* (1964), Halver (1965), Halver *et al.* (1969), and Wales and Sinnhuber (1966), it is now rare in rainbow trout culture in the developed world because of the considerable care that is now given to screening the oil seeds. In developing countries, however, it is still a major problem because of the difficulties with storage of oil seeds in the humid tropics. In tilapia culture a wide range of neoplasms has been associated with high aflatoxin levels (Haller and Roberts 1980), including renal tubular carcinoma (Fig. 8.24) and lymphoma, but the more important role of aspergillomycosis probably masks the potential for a higher level of aflatoxicosis.

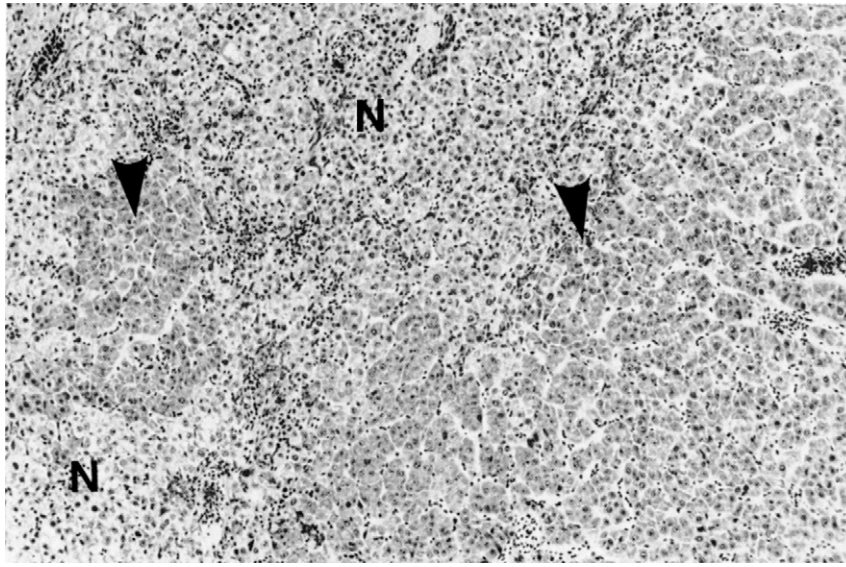

FIG. 8.23

Focal neoplastic tissue (arrowheads) within the liver of a trout with aflatoxicosis. The normal tissue (N) is paler-staining and has a marked inflammatory infiltrate. H&E, ×100.

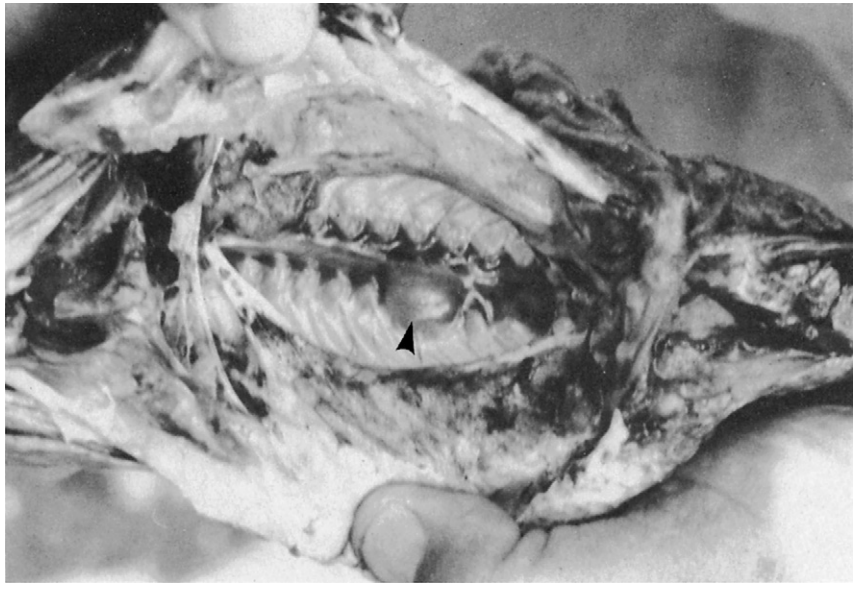

FIG. 8.24

Renal tubular carcinoma in a tilapia fed a moldy diet.

8.8 Toxic Algae

Algal blooms in the water kill fish by removing oxygen from the water. However, many of the freshwater algae have been shown to be toxic to humans or animals, so Phillips *et al.* (1985) have investigated the comparative toxicity of exposure of rainbow trout and tilapias to known toxic strains of *Microcystis aeruginosa* and nontoxic *Anabaena*. The fish consumed both the toxic and the nontoxic extracts without ill effects, but intraperitoneal injection of toxic extracts induced rapid mortality. This suggests that the toxin is not absorbed by the digestive tract. Toxic marine algae, the classic "red-tide" organisms, are associated generally with acute necrotizing branchial lesions, but Roberts *et al.* (1983) have shown clinically and experimentally that, at least in the case of *Gyrodinium aureolum,* one of the most common, they are also ingested and cause severe enteric and hepatic necrosis.

8.9 Cottonseeds

Apart from being one of the most common substrates for aflatoxicosis, cottonseed also contains two toxic components. Gossypol, a yellow product of the pigment gland, causes sudden anorexia and deposition of large sudanophilic globules within the liver and kidney, around which a chronic granulomatous response is laid down (Herman 1970). In the kidney it also produces glomerular nephritis and tubular necrosis.

A glandless unpigmented cottonseed kernel derived from specially selected strains of cotton is also available, but there is a second toxic component in the form of cyclopropenoid fatty acids within the kernel (Fig. 8.25). In rainbow trout these fatty acids are powerful synergists of aflatoxin B_1 and its metabolites. They may also be carcinogenic in their own right, although at a lower level (Hendricks *et al.* 1980; Hendricks and Bailey 1987).

8.10 *Senecio* Alkaloids

These components of the common field plant *Senecio jacobea,* or ragwort, could conceivably be incorporated in fish diets, but they have been investigated only in terms of experimentation. Hendricks *et al.* (1981) fed a complex of pyrrolizidine (*Senecio*) alkaloids as a component of the diet of rainbow trout and induced severe megalocytosis of hepatocytes, with a bizarre nuclear morphology and individual cell necrosis and nodular regeneration.

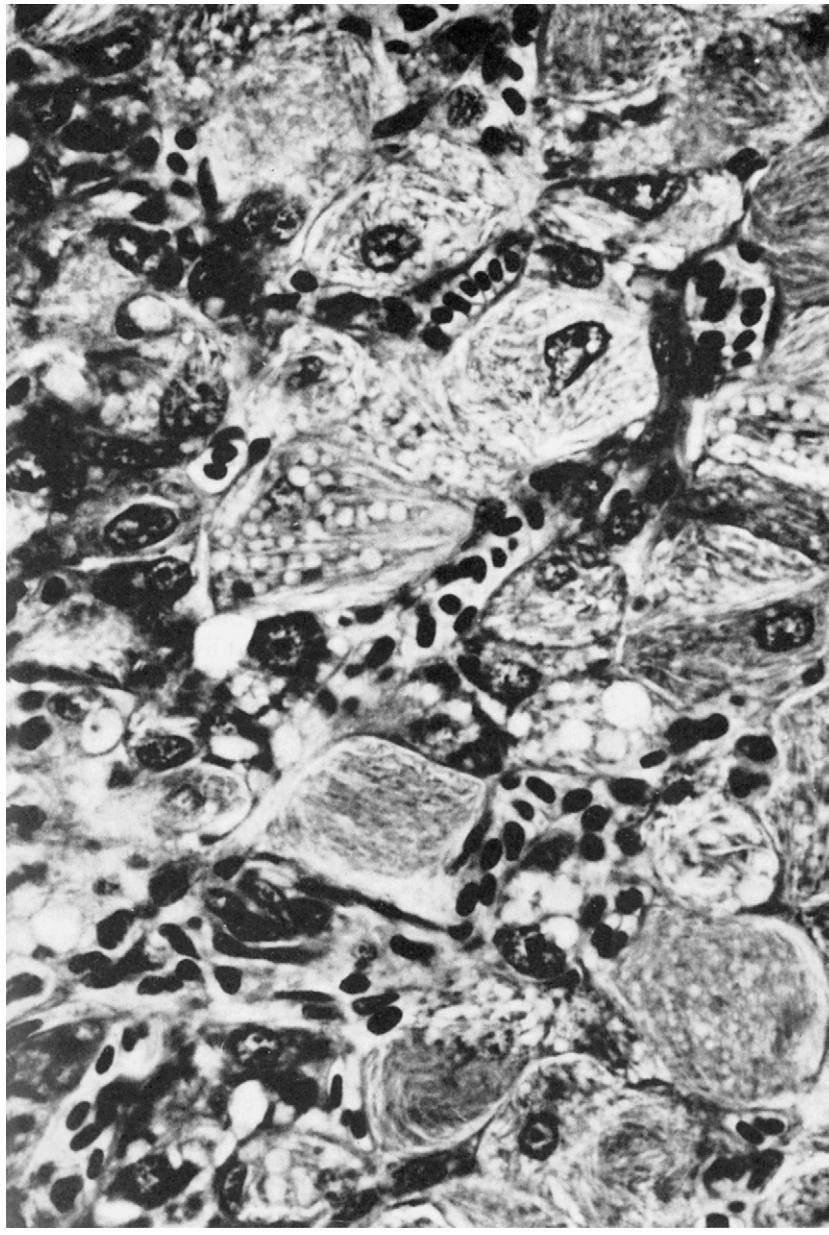

FIG. 8.25

Rainbow trout liver showing the bizarre fibrillar cytoplasm of hepatocytes following feeding of cyclopropenoid fatty acids from cottonseed meal. H&E, ×400. From Halver (1972).

Later there were bile duct hyperplasia and biliary and trabecular cirrhosis. Affected fish had hard, shrunken, mottled, livers. There was a distinctive venous occlusive obstruction of the centrolubular and portal vascular tree, with destruction of the endothelium and intima, and the renal glomerular basement membrane was also thickened.

8.11 *Leucaena* Toxins

The seed pods of *Leucaena,* or ipil-ipil, a leguminous shrub, are particularly valuable as a protein source for fish in developing countries in Africa and Southeast Asia. However, they generally contain high levels of the toxic amino acid mimosine. Studies on the dietary incorporation of *Leucaena* containing mimosine have shown that it causes poor growth, poor appetite, and a general cachexic condition (Jackson *et al.* 1982).

8.12 Anthropogenic Chemicals

The widespread use of pesticides on agricultural crops has meant that residues of substances such as the organochlorines and the organophosphates have become available in feedstuffs or, in the case of wild fish, within the food web. Normally the levels are unlikely to cause pathology, in properly controlled systems, but occasionally accidental overdosing or dietary contamination can result in acute toxic episodes. In such circumstances the deaths are usually rapid and associated with massive liver and renal tubular necrosis, often with hemorrhage and behavioral aberrations. Unlike the situation with heavy metal toxicity, in which the other components of the diet moderate the actual degree of toxicity, in the case of most pesticide compounds diet has little influence on their acute pathogenicity (Marking *et al.* 1984). In chronic toxicity studies, however, there is evidence, at least in the case of the organochloride benzene hexachloride, that the type of diet within which it is incorporated can drastically affect its long-term effect (Phillips and Buhler 1979).

8.13 Binders

Many experimental or production diets for marine fish depend on the use of chemically substituted polycellulose binders to hold the components

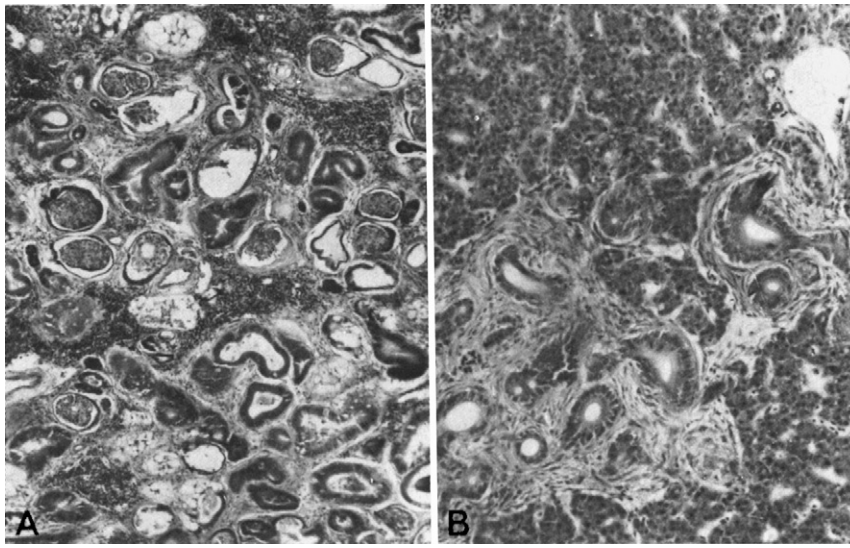

FIG. 8.26

Hepatorenal syndrome in a turbot. (A) Kidney in latter stages showing condensation of the tubular epithelium and peritubular fibrosis. (B) Liver from the same fish showing severe peribiliary cirrhosis. H&E, ×150.

together in water. High levels of such binders in the diet have been associated with a chronic degenerative condition known as hepatorenal syndrome. The condition is characterized principally by vacuolation, necrosis, and ablation of the renal tubules. There is also fibrosis of hemopoietic tissue, cast formation, and a parallel proliferative biliary cirrhosis (Fig. 8.26). Affected fish grow slowly, show poor appetite, become darkened, and eventually succumb (Anderson *et al.* 1976). The exact component of the binders responsible for the condition is not known but metallothiol complexes are suspected.

8.14 Photosensitizers

A number of chemical compounds which may be incorporated into, or contaminate, fish diets have the property, in the presence of ultraviolet irradiation, of releasing electrons and inducing a localized tissue necrosis. Thus, when passing through blood vessels near the skin surface, they can,

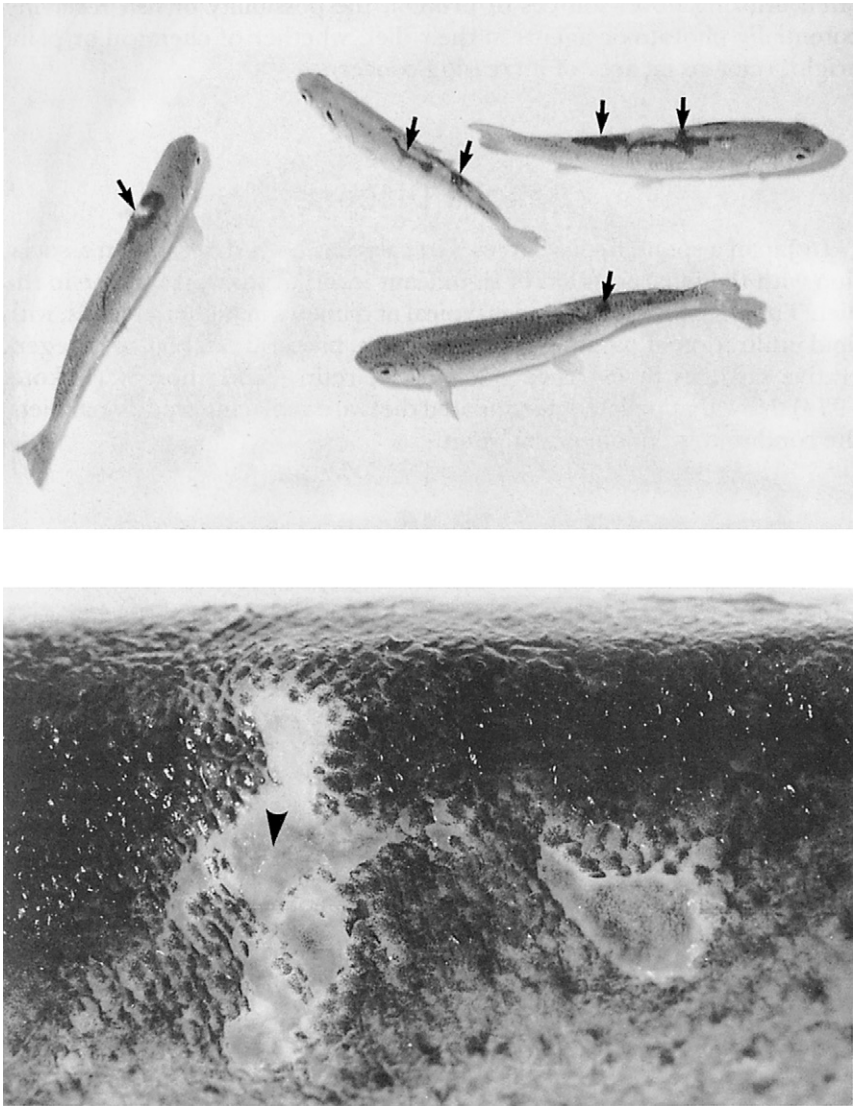

FIG. 8.27

(*Top*) Photosensitization lesions (arrows) in rainbow trout. This can be seen as dark areas on the dorsum of each fish. (*Bottom*) Close-up of an ulcerated lesion showing the exposed underlying dermis and musculature.

when energized by UV, cause severe tissue necrosis and ulceration. The most commonly implicated compound associated with this condition in fish culture is phenothiazine, a drug used for the control of the intestinal parasite *Octomitus*. This was first reported by Rucker (1957), when he referred to the phenomenon of "sunburn" or "backpeel" affecting fish treated with the compound. More recently Bullock and Roberts (1979) have demonstrated its phototoxic capability in Atlantic salmon, whereby fish fed with phenothiazine-coated pellets readily succumbed to sloughing and necrosis of the dorsal skin following irradiation with the erythematous wavelengths of the ultraviolet spectrum, termed ultraviolet-B (Fig. 8.27).

More intriguing, however, was the finding that the ingestion of phenothiazine in association with exposure to ultraviolet-A, the tanning wavelengths, produced a highly specific skin lesion characteristic, in section, of that observed in the salmon disease known as ulcerative dermal necrosis (UDN) (Roberts 1993). This condition has been extant in natural populations of salmon since the late nineteenth century, and despite extensive research no infectious etiological agent has been isolated. It now seems possible that its etiology could well have a phototoxic component. Potential photosensitizers are not uncommon in the sea. Mangum and Dales (1965) reported that many tube-dwelling and burrowing annelids normally accumulate an excess of porphyrins, substances well recognized as capable of inducing photosensitization, in various tissues and blood.

Although reports of photosensitization are relatively sparse in fish culture, it is important to bear in mind that in higher animals the condition is by no means uncommon and is associated with an extensive variety of natural and anthropogenic compounds. With the formulation of increasingly sophisticated diets, often utilizing novel sources of protein, the possibility of fish receiving potentially phototoxic agents in their diet, whether of chemical or plant origin, remains an area of increasing concern.

8.15 Sekoke Disease

In Japan a spontaneous diabetes in carp has been described in association with the incorporation of significant levels of silkworm pupae in the diet. The pathological picture is typical of diabetes in higher animals, with lipid infiltration of parenchymatous organs, bilateral cataract, and degenerative changes in extrinsic eye muscle, retina, and choroid (Yokote 1974). Now that pelleted, formulated diets are replacing traditional diets, the condition is no longer common.

8.16
Spleen- and Liver-Induced Cataracts

Feeding of minced animal viscera as a major component of the diet was a regular feature of earlier salmonid culture practices, and particular

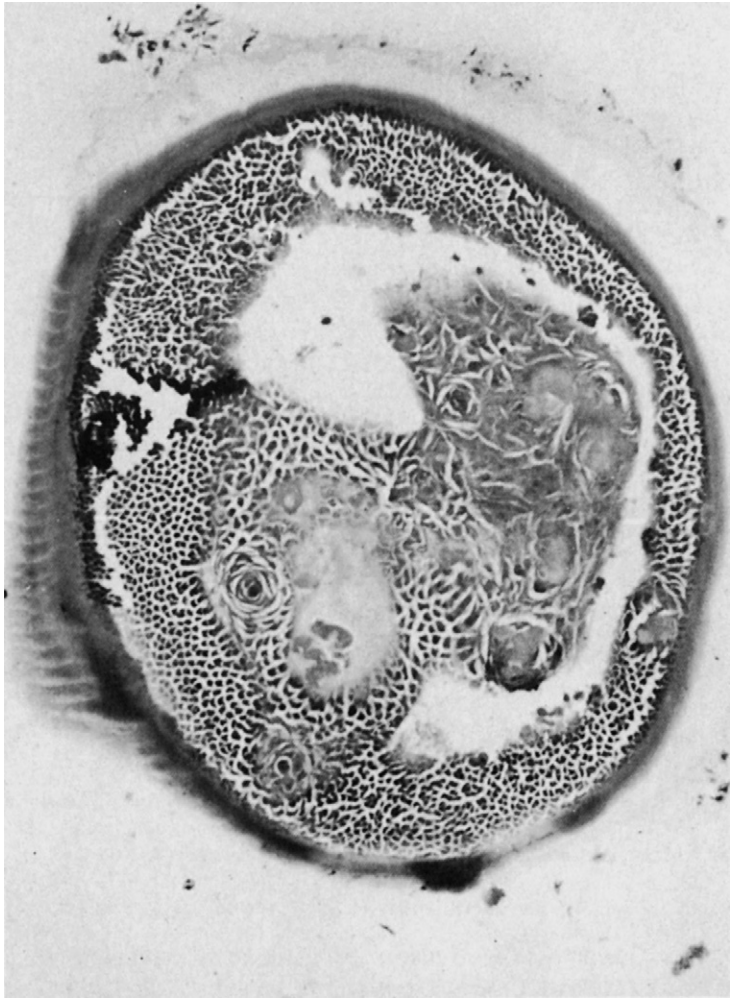

FIG. 8.28

Cataract in a brook trout fed horse meat showing proliferation of the lens cortex. H&E, ×90. From Halver (1972).

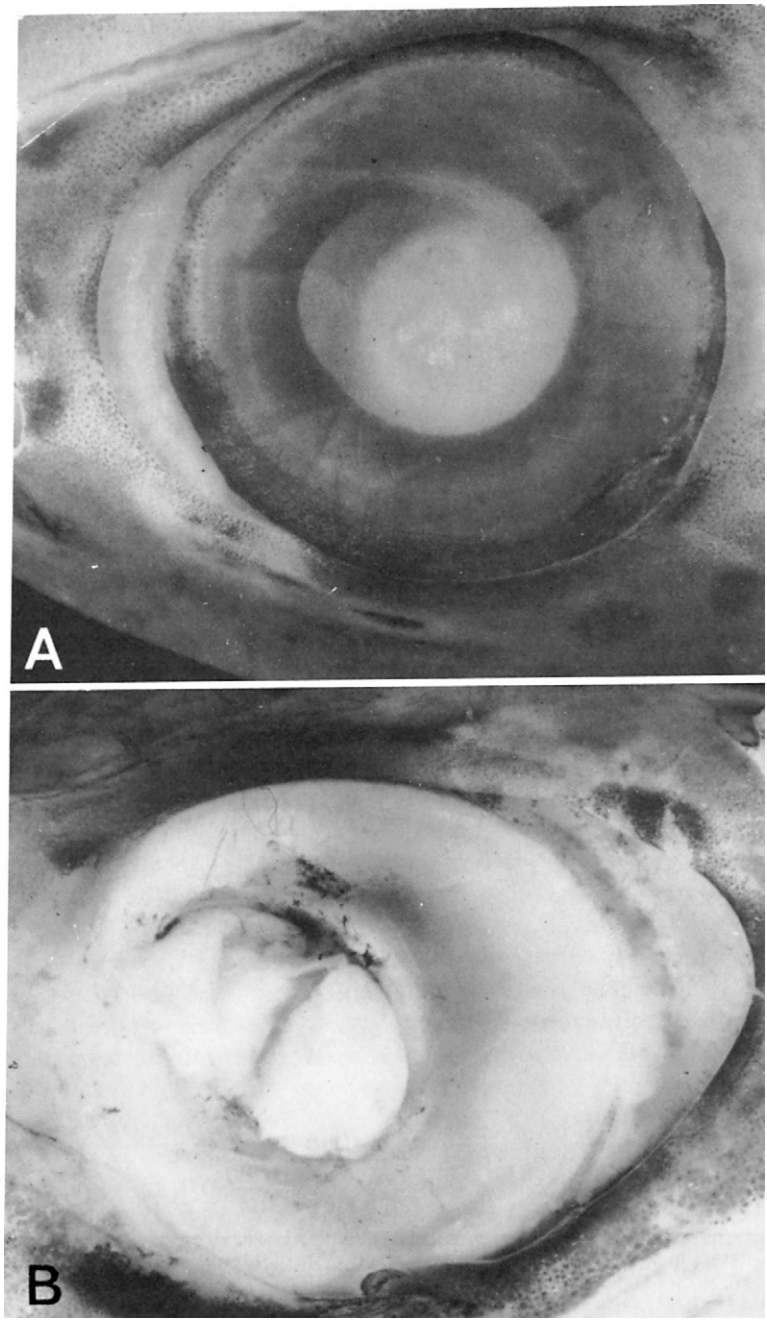

FIG. 8.29

Early (A) and late (B) stages of a nutritional cataract in a rainbow trout fed exclusively on offals.

problems, whether associated with a specific toxicity or, more likely, with a true or conditioned deficiency, were regularly associated with feeding of pig spleen or horse liver. The cataract was generally highly cellular and proliferative, unlike zinc or magnesium cataracts (Fig. 8.28) (Allison 1950).

A similar, but more severe condition associated with the feeding of wild animal viscera was reported by Lee *et al.* (1976), who demonstrated a severe bilateral cataract, with ultimate evulsion of the lens through the ulcerated cornea, in rainbow trout in Central Africa (Fig. 8.29).

8.17 Single-Cell Protein Lesions

Single-cell protein (SCP), derived from unicellular organisms grown on methanol or other hydrocarbon feedstocks, has been incorporated in fish diets to varying degrees for a decade or more. Hoffman and Gropp (1985) have, however, shown, at least for two particular types of bacterial-derived SCP, that feeding at high levels induced varying levels of a distinctive neoplastic and ultimately highly invasive biliary carcinoma.

8.18 Antibiotic and Chemotherapeutic Toxicity

Antibiotics and chemotherapeutics are frequently incorporated in fish diets and normally serious pathology is induced only if excessive dosage or prolonged incorporation occurs. Erythromycin and sulfonamides are most commonly associated with pathological effects. Hicks and Geraci (1984) carried out a histological assessment of such damage in rainbow trout fed a therapeutic dose of erythromycin over various periods. They showed specific toxic vacuolar degeneration of the proximal renal tubular epithelium in the absence of lesions in any other organ.

Prolonged sulfonamide therapy in salmonids has been reported to induce retardation of growth (Gutsell and Sniezko 1949), and renal tubular casts, focal hepatic necrosis, and visceral arterial sclerosis were recorded by Wood *et al.* (1957).

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9

Diet Formulation and Manufacture

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9.1 Introduction

Diet formulation and manufacture are exercises in compromise between the ideal and the practical. The perfect feed formulation that meets the nutritional needs of an animal or fish must always be modified to be less than ideal so that it can be manufactured. Similarly, the perfect feed mixture for producing pellets or some other type of feed particle must always be modified to account for the nutritional needs of the animal. Thus, feed formulation and manufacture, intellectual and physical activities, must be combined to produce animal diets. Together, formulation and manufacture involve the selection and combination of feed ingredient to form a mixture that can be manufactured into a product that delivers the nutrients needed to meet production goals in animal and fish husbandry. Production goals differ depending on the situation, and, in addition, production goals may be, to some extent, mutually exclusive. For example, production goals may be rapid, efficient weight gain and successful maturation and reproduction. In fish farming, these production goals might have to be attained without adding enriching nutrients to the aquatic environment, necessitating the use of certain ingredients that increase the cost of feed or lower its efficiency. This illustrates how feed formulation is an exercise in compromise. Feeds used in research are designed with specific goals, such as the induction of a vitamin deficiency or establishment of a minimum dietary requirement. Such feeds are often comprised of highly refined ingredients containing very low levels of the nutrient(s) being studied; regular feed ingredients contain an array of essential nutrients. Naturally, such research feeds are expensive and not used in production settings.

Diet formulation and manufacture are not independent activities, as mentioned above. When feeds are formulated, the pelleting characteristics of mixtures are just as important as the nutritional content of the mixture. Production diets must be economical to manufacture, ship, store, and deliver to the fish. Pellets must remain intact in water until fish consume them, not only to ensure adequate intake of nutrients that might otherwise leach out of the pellets, but also to minimize water pollution caused by disintegrating pellets. Thus, the way in which feed ingredients are chosen, prepared,

combined, and processed is influenced by many factors, and compromise between the ideal and the practical is a necessity requiring a solid foundation of knowledge and experience.

During the last several decades, enormous changes have occurred in the formulation and preparation of efficient feeds for farmed fish, and these changes have been driven primarily by the demands of the farming industry. New methods of feed and ingredient production, changes in the availability and quality of ingredients used in feeds, the development of new feed ingredients, the cultivation of new fish species, and advances in our knowledge of fish nutritional requirements have all influenced fish feed formulation and manufacture. Rising concerns about the effects of fish farming on the aquatic environment, coupled with concerns about the dependence of fish feeds on fish meal produced from fully utilized or overutilized wild fish stocks, have also influenced fish feed formulation and manufacture. Further expansion of aquaculture production can occur only if efforts to reduce pollution and to utilize sustainable feed ingredients are successful. Both of these issues greatly affect feed formulation and manufacture. Industry-driven advances in fish feeds over the past decade have significantly improved the efficiency of aquaculture, especially salmon farming. Over the next decade, further improvements are necessary so that society accepts aquaculture as a socially beneficial enterprise.

9.1.1. History of Diet Formulation and Manufacture

Fish diet formulation and manufacture has developed greatly since it began several hundred years ago. Prior to the beginning of the 20th century, fish production was mainly extensive, depending on natural food production, often stimulated by pond fertilization, and on supplemental feeding. Early studies on trout feeding showed that feeds based on combinations of animal, fish, shrimp, and vegetable meals reduced growth and impaired health (Embody 1918). However, when 15% of the feed consisted of fresh liver or kidney, these problems were eliminated. Vitamins had not yet been discovered and were not supplemented to early complete diets; fresh liver and kidney supplied the missing vitamins to early feeds.

In the early stages of diet preparation, biologists investigated the natural diet of trout (which varied somewhat with location and season), enumerating the species of aquatic and terrestrial creatures they consumed, along with the relative proportions of natural prey items in the total diet of the fish. They used this knowledge as a guide to arrive at the proper nutritional profile of artificial diets. These efforts were summarized by Embody and Gordon (1924), who also calculated the proximate composition of the natural diet of wild trout. The proximate composition was remarkably similar to that of diets fed to young salmon and trout today; i.e., 49% crude

protein, 15–16% fat, 8% crude fiber, and 10% ash, expressed on a dry weight basis.

9.1.1.1. Wet Feed

During the 1920s and 1930s, salmon and trout were fed a variety of diet formulations based on the availability of feed ingredients in the vicinity of hatcheries and farms. Hatcheries and farms generally produced their own feed on-site each day. The ingredients included salmon eggs; fresh, canned, or frozen fish; oilseed meals; and brewer's yeast; combined with beef liver, spleen, horse meat, chicken eggs, and cottage cheese. Mixtures such as one-third beef liver, one-third hog liver, and one-third salmon viscera were chopped and mixed at the hatchery, 2% salt was added to congeal the mixture, and it was delivered to the fish by a hatchery worker using a spoon or ladle. This process was labor intensive, and since the mixtures were wet and runny, the water quality was low. Feed for small fish was sometimes dropped through an air-blower fan to reduce the particle size. These feeds contained about 60% moisture and had a texture similar to moist sawdust (Hastings and Dickie 1972). The feed cost per pound of fish ranged from $0.032 to $0.57, and feed conversion ratios ranged from 2.0 to 8.0 (Donaldson and Foster 1937).

9.1.1.2. Wet–Dry Feed Mixtures

In the 1940s, the demand for ingredients used in wet fish feeds increased due to increased hatchery production and to competition from other users. To extend the traditional ingredients, meat–meal mixtures were developed. These diets were blends of slaughterhouse by-products and dry, commercially available feed ingredients. Cortland dry feed mixture No. 6, consisting of 24% each of dry skim milk, cottonseed meal, white fish meal, and wheat middlings, with 4% salt added, was a typical dry feed (Phillips *et al.* 1940). This mixture was blended with an equal weight of beef liver and hog spleen, water was added, and the mixed material was squeezed through a potato ricer to feed small fish in troughs. Versions of the potato ricer were adopted to use compressed air for large-scale applications to feed fish in large production raceways (Pelnar 1947). Cortland dry feed mixture No. 10 was the same as No. 6, except that soybean meal replaced the cottonseed meal. Feeding trials using brook trout at the Cortland hatchery in New York indicated that growth rates equivalent to those obtained using a meat diet were achieved using the meat–dry meal mixtures. The feed cost per unit of production was reduced by about one-half (Tunison *et al.* 1941).

Meat–meal mixtures are still in use in many parts of the world. In northern Europe, fish processing waste and fish silage combined with dry meal mixes at 50:50 to 90:10 proportions constitute wet feeds for salmonids. The dry

mixes contain fish meal, cooked starch, vitamin and mineral premixes, fish oil, and an alginate binder. These feeds are made at the fish-farming site and fed within several days. In Japan, wet diets containing ground fish combined with binders (90:10 ratio) are prepared for yellowtail and salmon. In Asia, combinations of ground fresh fish, fish meal, and a dry meal containing vitamins and binders are produced by small-scale farmers as an alternative to purchasing ready-made feed. Generally, these types of wet diets support excellent growth of fish. They are practical when the availability and cost of fresh fish or fish processing waste and the cost of feed preparation warrant their use.

9.1.1.3. Semimoist Pelleted Feeds

During the late 1940s and 1950s, studies were conducted by the Oregon Fish Commission and the Seafood Laboratory at Oregon State University to develop a wet mixture–dry mixture combination salmon feed that would not transmit disease to young salmon. At that time, fish tuberculosis, a disease caused by *Microbacterium pisum,* was a major problem in Pacific salmon hatcheries. Although this was not a fatal disease of salmon fingerlings, on their return to hatcheries as adults, infected fish could not be used as spawners because their gonads were not developed. The practice of feeding meat–meal diets containing chopped salmon carcasses or salmon viscera was found to be the primary way in which young salmon fingerlings were infected with fish tuberculosis (Wood 1979). In other words, the disease was transmitted from adult to offspring (vertical transmission) via the wet fish portion of the diet. Disease transmission via the diet was eliminated by pasteurizing the wet fish before it was used in the diet.

The next stage of development of semimoist feed, now known as the Oregon moist pellet (OMP), involved testing various ingredient combinations to produce a mixture that could be easily formed into feed particles. By reducing the proportions of pasteurized fish waste material and increasing the dry meal, and by using ingredients in the dry mix that held the finished product together, a semimoist (~32% moisture) mixture of improved quality was developed.

The final refinement in semimoist feeds was the introduction of a practical method of forming pellets. Various methods to form feed particles were evaluated. Potato ricers gave way to grease guns and, finally, to meat grinders in which 50% of the holes in the die were plugged. The resulting "noodles" were chopped first by hand and then by a slow-turning fan which made them into small particles. The drawbacks were that the particles were not of a uniform size and many fine and undersized particles were created. The final step involved the use of commercial noodle machines with external cutting knives that chopped the noodles into the proper

Table 9.1

Oregon Moist Pellet Specifications

Ingredient	Percentage in diet		
	Oregon Mash (OM-3)	OP-4	OP-2 ($\frac{1}{8}$-inch and larger pellets)
Herring meal (or anchovy or hake meals up to $\frac{1}{2}$ fish meals) except mash	49.9	47.5	28.0
Wheat germ meal	10.0	Remainder	Remainder
Cottonseed meal (48.5%)	—	—	15.0
Dried whey product or dried whey	8.0	4.0	5.0
Corn distillers' dried solids	—	—	4.0
Sodium bentonite	—	3.0	—
Trace mineral premix	0.1	0.1	0.1
Vitamin premix	1.5	1.5	1.5
Choline chloride (70%)	0.5	0.5	0.5
Wet fish	20.0	30.0	30.0
Fish oil	10.0	6.5–7.0	6.0–6.75

length for feeding. With this refinement, salmon diet preparation moved from individual hatcheries to commercial plants where pellets were mass-produced, quick-frozen, sacked, and shipped frozen. As the vitamin requirements of salmon were determined, vitamin premixes were developed which ultimately resulted in the elimination of beef or fish liver from the diet of young Pacific salmon.

The OMP formula continued to be modified and refined by the Oregon Department of Fish and Wildlife, and eventually there were three OMP formulations, a mash for fry, and two production formulations (Table 9.1). In 1985, the State of Oregon closed its fish feed development program, and further refinement of the open-formula OMP ceased. Several commercial feed producers assumed responsibility for further evolution of the semimoist feed, and today, semimoist feed is available from a single company in North America. This feed is a closed-formula feed, meaning that its formulation is the property of the feed company, but its basic formulation and manufacture is similar to the OMP. Manufacturing processes for both moist and dry pellets are described later in this chapter.

9.1.1.4. Dry Pelleted Feed

During the period that semimoist feeds were being developed for salmon, progress was being made in the development of dry compressed pellets for

trout. The first report on the successful use of dry pellets for trout rearing appeared in 1956, building on decades of work at the Cortland laboratory. Grassl (1956) found that trout hatchery production could be increased by 60% while feed costs were reduced by 40%. His formulations did not include a supplemental vitamin premix, so he fed beef liver to the trout once every 3 weeks. The 10-cm fish reached a stocking size of 18 cm after 7 months of feeding, less than half the growth rate expected at trout farms today.

The addition of a vitamin mixture to the dry formulation permitted the successful rearing of trout to spawning and subsequent rearing of fry entirely on manufactured pellets (Phillips *et al.* 1964). The formulations of Phillips *et al.* (1964) for trout and Fowler and Burrows (1971) for fingerling Pacific salmon provided the basis for the development of dry pellet formulations produced throughout the world.

Open-formula diets for salmon and trout are diets for which the formulations are available to anyone, in contrast to closed-formula diets, which are the proprietary property of feed companies. Open-formula diets are periodically updated and tested by the various governmental agencies that develop them. Current specifications for severalof these diets are listed in Tables 9.2

Table 9.2

Open-Formula Diet Specifications for Abernathy Salmon Diets[a]

Ingredient	Percentage in diet		
	Starter S9 (92)	Crumbles A2-2 (92)	Pellets A3-2 (92)
Herring meal	58.0	50.0	46.5
Dried whey	5.0	5.0	5.0
Blood flour (or meal)	2.5	2.5	2.5
Wheat germ meal	—	5.0	5.0
Feather meal	7.5	10.0	10.0
Condensed fish solubles	3.0	3.0	3.0
or			
Poultry by-product meal	1.5	1.5	1.5
Wheat middlings mill-run or shorts	Remainder	Remainder	Remainder
Vitamin premix	1.5	1.5	1.5
Choline chloride (60%)	0.58	0.58	0.58
L-Ascorbyl-2–polyphosphate	0.2	0.2	0.2
Trace mineral mixture	0.05	0.1	0.1
Lignon sulfonate pellet binder	2.0	2.0	2.0
Calcium propionate		0.125	0.125
Fish oil or soybean lecithin (max. 2%)	12.0	9.0	9.0

[a] Year 2000 specifications.

Table 9.3

Open-Formula Diet Specifications for Salmonid Diets (MNR-98HS)[a]

Ingredient	Percentage in diet		
	Starter (HND)	Grower (HND)	Broodstock (19-2)
Fish meal (70% CP)	30.0	18.0	25.0
Blood meal, animal (>85% CP)	7.0	—	—
Poultry by-product meal (65% CP)	6.0	13.0	15.0
Whey, dried (12% CP)	9.0	9.0	9.5
Alfalfa meal (15% CP)	—	—	11.0
Soybean meal (49% CP)	—	—	13.0
Brewer's yeast (45% CP)	5.0	—	—
Corn gluten meal (60% CP)	25.0	37.6	17.0
Lysine hydrochloride	0.5	1.4	—
Vitamin premix	1.0	0.5	1.0
Mineral premix	0.5	0.5	0.5
Fish oil	16.0	20.0	8.0

[a] Ontario Ministry of Natural Resources (1998).

and 9.3. Dry, compressed pellets have several advantages over moist pellets. First, dry pellets do not require frozen storage; room-temperature storage is sufficient for at least 90 days after manufacture. Second, dry pellets can be used in inexpensive, platform-type demand feeders (moist pellets do not flow sufficiently to be used in most demand feeders). Third, dry pellets are less expensive than moist pellets, especially when the cost of moist pellets is expressed on a dry weight basis. Dry, compressed pellets also have several disadvantages. First, some species of young salmon start feeding less readily on dry feed than on moist feed, particularly in cold water (<7°C). Second, dry, compressed pellets do not float in water, unlike extruded pellets. Third, there is a limit to the amount of fat that can be included in the feed to be pelleted (<7%). However, by spraying on after pelleting (top-dressing), additional fat can be added to obtain levels of 16–20%.

Cooking extrusion is the most recent development in pelleted fish feed manufacture. These pellets are formed by extrusion of a moist mixture (20–24%) followed by drying to reduce the moisture content to 10% or less. Extruded pellets are used by the catfish, salmon, trout, and shrimp industries and by many other sectors of aquaculture, particularly for fish farmed in sea cages. The extrusion process expands starch in the feed mixture, which lowers the pellet density. Extruded pellets can be made to float, sink slowly, or sink rapidly in water, depending on the conditions of manufacture. Since

Table 9.4

Semipurified Diet Formulation for salmonids (H-440) and Oregon Test Diet (OTD) and Guelph Test Diet for Trout

Ingredient	H-440	OTD	Guelph (100)
Vitamin-free casein	38.0	49.5	40.0
Gelatin	12.0	8.7	4.0
Dextrin	28.0	15.6	9.0
Starch	—	—	11.5
α-Cellulose	—	7.7	3.0
Carboxymethylcellulose	—	1.3	—
DL-Methionine	—	—	0.5
L-Arginine	—	—	1.0
Fish oil (marine origin)	—	10.0	15.0
Vitamin E	—	0.2	In vitamin premix
Choline chloride	—	1.0	In vitamin premix
Mineral mix	4[a]	4.0[d]	8.0
Vitamin premix	9[b]	2.0[e]	3.0
Oil premix	9[c]	—	

[a] Mineral mix contains the following (g/kg premix): calcium biphosphate, 135.7; calcium lactate, 326.9; ferric citrate, 29.7; magnesium sulfate, 132; potassium phosphate (dibasic), 239.8; sodium biphosphate, 87.2; sodium chloride, 43.5; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $CuCl_2$, 0.1; $MnSO_4 \cdot H_2O$, 0.8; $CoCl_2 \cdot H_2O$, 1.0; $ZnSO_4 \cdot H_2O$, 3.0.

[b] Vitamin premix contains the following (g/kg premix): α-Cellulose, 893; choline chloride, 56; inositol, 22; ascorbic acid, 11; niacin, 8.4; calcium-pantothenate, 5.6; riboflavin, 2.2; menadione, 0.45; pyridoxine hydrochloride, 0.56; thiamin hydrochloride, 0.56; folic acid, 0.17; biotin, 0.06; vitamin B_{12}, 10 g.

[c] Oil premix contains the following (g/kg premix): corn oil, 664.7; cod liver oil, 331.9; *dl*-α-tocopheryl acetate, 4.4.

[d] Mineral mix contains the following (g/kg premix): $CaCO_3$, 21; $CaHPO_4 \cdot 2H_2O$, 735; K_2HPO_4, 81; K_2SO_4, 68; NaCl, 30.6; $Na_2HPO_4 \cdot 6H_2O$, 21.4; MnO, 25.0; $FeC_6H_5O_7 \cdot 3H_2O$, 5.58; $MnCO_3$, 4.18; $2CuCO_2 \cdot Cu(OH)_2$, 0.34; $ZnCO_3$, 0.81; KI, 0.01; NaF, 0.02; $CoCl_2$, 0.2; citric acid, 6.88.

[e] Vitamin premix contains the following (g/kg premix): thiamin hydrochloride, 3.2; riboflavin, 7.2; niacinamide, 25.6; biotin, 0.08; calcium-pantothenate, 14.4; pyridoxine hydrochloride, 2.4; folic acid, 0.96; menadione, 0.8; vitamin B_{12}, 2.67; inositol, 125; ascorbic acid, 60.0; *p*-aminobenzoic acid, 20; vitamin D_2, 0.4; BHA, 0.75; Celite, 735.8.

they are dry, extruded pellets can be used in automatic and demand feeders. Extruded pellets are relatively porous and can soak up sprayed oil to reach levels of over 35%, typical of feed for Atlantic salmon. The cost of production is slightly higher for extruded pellets than for compressed pellets, but their advantages outweigh the additional cost in many aquaculture applications.

9.1.1.5. Semipurified Research Feeds

The adaptation of semipurified research diets for animals for use with trout (McLaren *et al.* 1947; Wolf 1951) and subsequent refinement of these diets for Pacific salmon (Halver 1957) and catfish (Dupree 1966) made possible the determination of the essential nutrient requirements of many species of fish. Semipurified diets contain highly refined ingredients, such as casein, gelatin, egg white, and dextrin. These ingredients have very low levels of vitamins and fairly low levels of the essential minerals required by fish, so essential vitamins and minerals must be added to the diet to ensure optimum growth and health (Table 9.4). In typical requirement studies on fish, all nutrients are present in sufficient amounts except for the one being studied. It is added at incremental amounts to test diets, and the response of the fish is measured. In amino acid requirement studies, a mixture of crystalline amino acids replaces a portion of the casein and gelatin, with the amino acid being studied being added at incremental levels. Using this approach, the amino acid requirements of fish were first estimated (Dupree and Halver 1970; Halver 1957). Other formulations for semipurified diets suitable for mineral studies, or other purposes, have been developed [National Research Council (NRC) 1993].

9.1.1.6. Microdiets: Larval Feeds

Several other types of diets are used in aquaculture, usually for larval fish. Examples are flaked and microencapsulated diets. Flaked diets, prepared by drying a slurry on a steam-heated, double-drum dryer, have been used for years as feed for ornamental fish in the aquarium industry. Micropulverized, flaked diets have been used experimentally as artificial food for larval fish. Microencapsulation remains a promising technique to produce feed for larval fish, due to the water stability of the capsule and the nutritional completeness of the feed particles. This method of diet preparation has not yet reached the practical stage, however.

9.2 Aims and Strategy of Fish Feed Production

Diet preparation is fundamentally a compromise between the ideal situation and practical considerations. It is not difficult to formulate a diet that contains all of the known required nutrients at levels that meet or exceed the known requirements of the fish. However, when practical matters are considered, the task becomes complicated. Practicalities such as the ingredient price and availability, diet acceptability, pelletability of the formulation, pellet storage and handling requirements, and levels of antinutritional factors in certain ingredients are all of critical importance. The

ultimate goal is to produce a feed that supports maximum production at the lowest possible cost. There are many possible formulations because aquaculture has many different aims, such as the production of food fish, fish for stocking public or private waters, viable egg and fry production and the propagation of ornamental fish. Each formulation is particularly suitable for a specific application. Even when one species of fish is being reared with one goal in mind, multiple formulations are used to fit various stages of growth or production. For example, a "finishing diet" might be fed during the final months before harvest to produce specific organoleptic properties, such as flesh pigmentation in salmonids, and to support continued growth.

Some of these practical considerations are quantifiable and thus subject to solution by mathematical means. Those that are not must be resolved by the experience gained from preparing specific diets and determining their qualities. Knowledge can also be gained by knowing the physical and chemical properties of both individual feed ingredients and prepared feeds.

9.3 Feed Ingredients

Animal and fish feed ingredients are, for the most part, by-products of food processing obtained when high-value food for humans is extracted from a raw material. Once the high-value products have been removed, the remaining material is further processed, usually by drying, to produce a material that itself becomes an article of commerce. These ingredients are normally available throughout the year, with prices depending on the forces of supply and demand.

Not all feed ingredients are by-products; some are produced directly from raw materials. Examples of these include anchovy meal, menhaden meal, and ground whole grains. Practical and economic factors determine the fate of these products. Feed ingredients for fish diets are chosen for a number of reasons already mentioned, including the nutrient content, cost, availability, and physical properties. Proximate composition is the primary means of evaluating feed ingredients. In industry, proximate composition is expressed on an as-is basis, which generally means a moisture content of 7–9%.

9.3.1. Feed Nomenclature

Feed ingredient nomenclature and classification began in Germany in the early 19th century, when methods of determining chemical composition were developed. Feed ingredients were first classified on the basis of nitrogen or digestible nutrient content. Nomenclature was originally based on common names, but as the number of by-products from a single, raw

material increased, the use of common names became confusing. Harris (1980) reported that a systematic investigation during the early development of today's nomenclature system revealed that more than 20% of the common names in use for feeds were simply different names for the same product. Today's system of nomenclature is called the International Feed Vocabulary (IFV) and it is accepted worldwide (Harris 1963, 1980). It has assigned a comprehensive name and number to each ingredient using descriptions from one or more of six categories. The categories are (1) origin, which includes the scientific and common names for specific plants and animals, poultry, fish, cereals, grass, minerals, chemical products, and drugs or other names for nonspecific materials; (2) part fed to animal as affected by processing; (3) process(es) and treatment(s) to which the feed ingredient was subjected; (4) stages of maturity and development; (5) cutting (for forage crops); and (6) grade. Using this system, herring meal is described as fish herring, *Clupea harengus,* meal, mechanically extracted, International Feed Number 5-02-000. Wheat mill run is described as wheat, *Triticum aestivum,* flour by-product, less than 9.5% fiber, International Feed Number 4-05-205. The last five numbers in the International Feed Number are assigned to each ingredient name, the first number being a code for the feed class. There are eight feed classes at present (Table 9.5). Over 18,000 feed ingredients have been assigned numbers using this system (Harris 1980).

An organization called the International Network of Feed Information Centers (INFIC) was formed to catalog information on feed ingredients and disseminate this information throughout the world. One of the participating centers, the Feed Composition Data Bank, National Agriculture Library, Beltsville, Maryland, provides the data used in feed composition tables, such as those found in the United States–Canadian Tables of Feed Composition (NRC 1971) and the National Research Council's series entitled

Table 9.5

Classes of Feed Ingredients

1. Dry forages and roughages
2. Pasture, range plants, and forages fed green
3. Silages (ensiled forages only)
4. Energy feeds
   Less than 20% protein (dry basis)
   Less than 18% crude fiber (dry basis)
5. Protein supplements: more than 20% protein (dry basis)
6. Mineral supplements
7. Vitamin supplements
8. Additives: antibiotics, coloring materials, flavors, hormones, and medications

Nutrient Requirements of Domestic Animals, which includes the *Nutrient Requirements of Fish* (NRC 1993).

The American Feed Industry Association (AFIA) publishes an electronic guide to feed ingredients, available to members on disk. This guide contains standard descriptions and definitions of feed ingredients, including limits on various constituents and components, plus standards for final products. The feed ingredient descriptions provided below are shortened versions of AFIA standards.

9.3.2. Protein Supplements

As indicated in Table 9.5, protein supplements are feed ingredients having a protein content above 20%, on an as-fed or wet weight basis. There are three general groups. The first group is made up of ingredients having a protein content of 20–30% which contain materials of plant origin that are by-products of the brewing and distilling industries, wheat germ meal and corn gluten feed. The second group is composed of ingredients having a protein content of 30–50% and includes the oilseed meals, crab meal, and dried milk products. The third group contains ingredients of over 50% protein and includes fish meals, blood meal, feather meal, tankage, meat and bone meal, yeast products, shrimp meal, poultry by-product meal, soy protein concentrate, wheat gluten, corn gluten meal, and casein.

9.3.2.1. Animal By-products

Animal by-products are derived from the meat-packing, poultry processing, and rendering industries. The protein content of these products after drying ranges from 50 to over 85%, and there are established standards for the quality of the proteins, generally a minimum pepsin digestibility level. The essential amino acid composition of animal by-products meal is similar to that of whole-egg protein, which is the standard by which protein quality is judged. These meals are good sources of lysine but poor sources of methionine and cystine, which are usually found to be limiting in diet formulations.

9.3.2.1.1. Meat Meal and Meat and Bone Meal. These products are dried mammalian tissues, exclusive of hair, hooves, horn, hide trimmings, manure, and stomach contents. The protein content of meat meal is about 51%, while that of meat and bone meal is closer to 50%. Fat levels in these products average 9.1–9.7%. The principal difference between the two products is the phosphorus level, with meat meal having, by definition, less than 4.4% phosphorus. The calcium content of meat and bone meal ranges from 8.8 to 12%, while meat meal generally contains about 3% less calcium. Both

ingredients have relatively high ash contents, about 27 and 31% for meat meal and meat and bone meal, respectively.

9.3.2.1.2. Blood Meal. Blood meal is a dry product made from clean, fresh animal blood, exclusive of all extraneous materials. The most common blood meal is produced by spray-drying after an initial low-temperature vacuum evaporation has reduced the moisture content to about 70%. Other processes of drying blood include flash-drying and conventional drying in a cooker. Blood meal has a minimum protein content of 85% and a lysine content of 9–11%, with a lysine availability of over 80%.

9.3.2.1.3. Feather Meal. Feather meal is made from poultry feathers, which have been hydrolyzed under pressure in the presence of $Ca(OH)_2$ and dried. It has a protein content of 80–85%, and not less than 75% of the protein must be digestible by the pepsin digestibility method. Its use in fish feeds is restricted, however, due to its low protein digestibility by fish [52.4–70.5%, (NRC 1981)]. Recent investigations have shown that the protein digestibility of feather meal may be higher for rainbow trout (Bureau *et al.* 2000).

9.3.2.1.4. Poultry By-product Meal. Poultry by-product meal is made from waste generated from poultry processing plants, exclusive of feathers and the contents of the gizzards and intestines. The material remaining after chickens are dressed is rendered and dried. The ash content cannot be higher than 16%, of which not more than 4% can be acid-insoluble ash. Regular poultry by-product meal contains about 58% protein and 13% fat on an as-is basis. Pet-food grade and low-ash poultry by-product meals contain higher protein and lower ash levels than regular poultry by-product meal.

9.3.2.1.5. Milk By-products. Several milk by-products are useful in the formulation of fish feeds, including dried whey, dried whey product, casein, and dried skim milk. Dried whey product is the residue obtained when a portion of the lactose has been removed, while dried whey contains a minimum of 65% lactose. The protein content of whey products is relatively low (13–17%), yet these products are classified as protein supplements. Dried skim milk is sometime used in starter diets, due primarily to its excellent digestibility and amino acid balance. Its protein content is about 34%. Casein is the residue obtained by acid or rennet coagulation of defatted milk. By definition, it contains at least 80% protein. Vitamin-free casein is the standard protein supplement used in semipurified diets.

9.3.2.1.6. Gelatin. Gelatin is an important ingredient in semipurified feed formulations (NRC 1993). It is a glutinous material obtained by the partial hydrolysis of collagen from animal skin, tendons, and ligaments. It is hard and brittle when dried but dissolves in hot water and forms a jelly when cooled. It is 88–92% protein and contains almost no tryptophan. It is used in semipurified diet formulations as both a protein source and a binding agent.

9.3.2.2. Fish Products and By-products

Fish meal is the most important protein ingredient used in diets of salmon, trout, shrimp, and marine fish. The major fish meals include anchovy, capelin, menhaden, sand eel, herring, whitefish, and salmon. They may be made of whole fish, as is the case with anchovy, capelin, or menhaden, or from processing residue, as is the case with whiting, pollock, herring, and salmon. Fish meal contains high levels of essential amino acids. The fat content ranges from 4 to 20%, and the ash content is highly variable, ranging from about 11–12% in anchovy meal to over 23% in whitefish meals made from filleting wastes. Typical specifications for fish meal for use in salmonid diets are listed in Table 9.6.

The waste products of shrimp and crab processing can be dried to produce shrimp and crab meal. These products contain about 40 and 32% protein, respectively. Their ash content is high because of the quantity of shell that remains in the waste after processing. These wastes are good sources of trace elements and carotenoid pigments.

9.3.2.2.1. Wet Fish Products. Fish waste may be preserved by means other than drying, such as acid preservation. When it is acid-preserved before

Table 9.6

Quality Standards for Fish Meal Required for Salmonid Diets[a]

Component	Level
Crude protein (N × 6.25)	>68%
Lipid	<10%
Ash	<13%
Salt (NaCl)	<3%
Moisture	<10%
Ammonia–nitrogen	<0.2%
Antioxidant	<200μg/g

[a] Ontario Ministry of Natural Resources (1998).

hydrolysis, it is called fish silage. When it is hydrolyzed and then acidified for preservation, it is called fish hydrolyzate. Either mineral or organic acids can be used as the acidulent as long as the pH of the material remains low (<4.0) and antifungal agents are added. During acid preservation, the proteolytic enzymes present in the waste material hydrolyze the fish protein, releasing water from peptide bonds, resulting in a liquid product. Short-time, high-temperature exposure will denature the enzymes and stop hydrolysis. Fish silage or hydrolyzate can be used as is, concentrated, or dried with other ingredients. Wet fish can also be pasteurized by heating to >85°C for 15 min and used directly as an ingredient in semimoist diets.

9.3.2.3. Plant Protein Sources

The most important protein supplements of plant origin are the oilseed meals, produced from the cake remaining after oil has been extracted from soybeans, cottonseed, canola, peanuts, sunflower seeds, coconuts, and so forth. Oils may be mechanically expelled or extracted by solvents. In North America, soybean meal is the most commonly used oilseed protein. Dehulled soybean meal contains 48% protein, while defatted soybean meal contains 44% protein. Soybeans contain several antinutritional factors that are important considerations in fish nutrition. Some, such as the trypsin inhibitors, can be inactivated by heat, while others, such as phytic acid, are unaffected by heat. Phytic acid can reduce zinc availability and reduce protein digestibility in salmonid diets (Richardson *et al.* 1985; Spinelli *et al.* 1983). Full-fat soybean meal is obtained by processing raw soybeans from which no oil has been removed and is sometimes used in trout and grow-out salmon feeds (Reinitz *et al.* 1978; Smith 1977). Its use is restricted in catfish feeds because of its oil content. It is not suitable in juvenile salmon diets (Fowler 1980).

Cottonseed meal has been used in the OMP formulation at 15% due to its protein content (48%) and its value as a binder. Cottonseeds, like most oil seeds, contain antinutritional factors that must be taken into account when considering their use in fish diet formulations (see Chapter 10).

Wheat gluten and corn gluten meals are protein supplements of plant origin that are derived from grains. Both corn and wheat gluten meals are by-products of starch production and are high-protein ingredients (>60 and >75% protein, respectively). Wheat germ meal is a by-product of wheat milling and consists primarily of the germ of the wheat seed, with some wheat middlings and bran added. It has a variable proximate composition, depending on the milling processes used. The protein content varies from 25 to 30%, while the fat level varies from 7 to 12%.

Brewery and distiller's by-products are another major category of protein supplements of plant origin. These products are made from the residue remaining after beer and liquor production. Since most of the starch in

the original material is fermented to alcohol and removed, the protein and fiber content of the by-products is increased on a relative basis. Distillers' products are enriched with vitamins and nucleotides as a result of synthesis by yeast.

Brewer's dried grains and brewer's yeast are the major by-products of brewing that are sometimes used in fish feed formulation. The protein and fiber contents of brewer's dried grains are 27 and 13%, respectively. Brewer's dried yeast contains about 44% protein and only 3% fiber. Brewer's grains have limited use in fish diets because of their bulk and the difficulty in grinding them to a consistent particle size. Brewer's yeast acts as a binder in some formulations, but its use is limited because of its relatively high cost. Three main distilling by-products are available: distillers' dried solubles, which is the dried stillage fraction; distillers' dried grains, which is the fraction remaining after removal of the stillage; and distillers' dried grains with solubles, which is a combination of stillage and grain sediments. The protein content of these products ranges from 26 to 28%, while the crude fiber level is higher in the dried grains (9–11%) than in the dried solubles (4–5%).

A fourth group of plant protein supplements includes the pulses, e.g., peas, beans, and lentils. These products contain 20–30% protein, but their methionine levels are low, limiting their use in fish diet formulation. The protein fraction can be concentrated by air classification, resulting in protein concentrates having 50–60% protein.

9.3.2.4. Other Protein Sources

Another category of protein supplements is sometimes referred to as unconventional feedstuffs. These products have not yet reached the level of availability or acceptance that allows them to be used routinely in fish feed formulations, but someday they may. They include single-cell proteins derived from yeast or bacteria grown on waste material such as paper mill sludge, sewage, crop processing wastes, and methane. The microorganisms are then harvested, purified, and dried into a product suitable for animal feeding. Products with 60–70% protein have been developed, and feeding trials with salmonids in which single-cell protein derived from yeast has replaced fish meal have been promising (Mahnken *et al.* 1980; Spinelli *et al.* 1979). Some concern has been raised about the digestibility of yeast products by salmonids and about the possibility of uptake of heavy metals and other undesirable compounds by single-cell organisms grown on industrial waste.

Krill, *Euphausia superba* and *E. pacifica,* is a major marine biomass that is increasingly being harvested and processed into supplements for fish feeds. Krill meal is 33–55% protein, 15–20% fat, and 15–28% ash. Besides having a well-balanced amino acid profile, it contains the carotenoid pigment, astaxanthin, which is the natural pigment that colors the flesh of salmonids

and the skin of many species of fish. In feeding trials, fish fed diets containing krill meals have grown nearly as well as those fed conventional diets (Gulbrandsen 1979; Hilge 1979; Ibraham *et al.* 1984; Koops *et al.* 1979; von Lukowicz 1979). Krill meal and krill hydrolyzates are added to salmon fry and fingerling diets to increase diet palatability.

A final unconventional ingredient category is dried insect meal. Most fish nutritionists, at some time in their careers, will be asked by a fish farmer if it is feasible to grow fly larvae or worms with which to feed fish. After all, are these not natural feeds for many fish? Studies of insect meals suggest that they have relatively high nutritional values (Spinelli *et al.* 1979; Tacon and De Silva 1983). In general, it is simply not cost-effective to produce them, but in some instances, ingredients such as silkworm pupae and maggot meal can be economical ingredients to include in fish diets.

9.3.3. Energy Sources

9.3.3.1. Carbohydrates (Starch) from Grains

Basal feeds, or energy feeds, are low-protein, high-energy feed ingredients. The upper limit for protein content of basal feeds is 20%, although most are in the 10–17% range. Grains are generally 68–72% starch, with the exception of oats, and about 10–12% protein. Domestic animals can digest about 95% of the starch in grains, hence their classification as energy feeds. The digestibility of the carbohydrates in grains is highly variable among fish. Carnivorous species, such as salmonids, derive very little energy from unprocessed plant starch. Omnivorous species, such as catfish, and herbivorous species, such as some carp, derive a high amount of energy from grain starch, providing that it is cooked.

By convention in animal nutrition, energy feeds cannot contain over 18% fiber (Crampton and Harris 1969). Otherwise, they are classified as roughages. Fiber is indigestible by carnivorous fish, while omnivorous and herbivorous fish are able to digest fiber to varying, limited degrees. The fiber content of grains is about 6%, although oats and barley contain higher levels, at least hulled varieties.

Numerous by-products of grain processing are used in animal feeds and are also classified as basal feeds. Wheat milling, which produces flour as its main product, also produces wheat shorts, wheat bran, wheat mill-run, feed flour (second-class flour), and wheat middlings, all of which can be used in fish feeds. By-products of oats, corn, and other grains are also potential fish feed ingredients. Due to the removal of starch (flour), these products contain higher protein, fiber, and fat levels than the grain from which they were derived.

Factors other than nutrient content influence the use of basal feeds in animal diets. The most important factor is cost, which is usually relatively low. Thus, after the nutrient requirements have been met, basal feeds can be used to fill out the energy needs in animal feed formulation. Basal feeds also have excellent binding properties and can help to hold feed pellets together in both dry and semimoist diets. Finally, they are relatively indigestible, and this quality is useful in animal production when a reduced rate of growth at a normal feeding level is desired. Rarely does a similar situation arise in fish culture.

A large number of feed ingredients other than those listed here may be suitable for use in fish feed formulation. As mentioned earlier, over 18,000 individual feed ingredients have been identified and classified throughout the world. Although most of these ingredients will never be used in fish feeds, some are important feed constituents in developing countries. Fish nutritionists continue to identify potential fish feed ingredients and characterize their chemical composition and nutritional value to fish.

9.3.3.2. Fats and Oils

Fats and oils are lipid sources, fats being the term for lipids that are solid at room temperature and oils being the term for lipids that are liquid at room temperature. The melting point of a lipid depends on its fatty acid composition. Lipids containing a high proportion of saturated fatty acids have a lower melting point than those containing a high proportion of unsaturated fatty acids. Thus, tallow is termed a fat, while plant or fish lipids are called oils. In either case, they are concentrated energy sources for fish and animals, having 2.25 times as many calories per gram as carbohydrates, a result of their structure.

It used to be thought that fish could not digest fats, because they were solid at the water temperature at which most fish are cultured. However, research has shown that fats are digestible to fish, although slightly less so than oils (Austreng and Refstie 1979; Ellis and Smith 1984). The main factors determining which lipid source to use in fish feed formulations are the fatty acid composition of the lipid source and its physical characteristics at ambient temperatures, which dictate how it must be stored and handled at the feed mill.

A variety of commercially available fats and oils is suitable as ingredients for fish diets. Salmonid diets normally include fish oils, such as herring, pollock, menhaden, anchovy, and capelin. Plant oils, such as soybean, corn, and cottonseed, and animal fats, such as tallow, lard, and poultry fat, may also be used as long as the diet contains sufficient levels of essential fatty acids (Takeuchi and Watanabe 1982). Fats and oils are chosen using the same criteria as for other ingredients: price, availability, nutritional value,

Table 9.7

Quality Standards for Fish Oil Required for Salmonid Diets[a]

Component	Level
Iodine value	Report value
Peroxide value	<5 mEq/kg
Anisidine value	<10
Pesticides (total)	<0.4 μg/g
PCBs	<0.6 μg/g
Nitrogen	<1.0%
Moisture	<1.0%
Antioxidant	<500 μg/g

[a] Ontario Ministry of Natural Resources (1998).

and quality, as defined by chemical tests. Since fats and oils are susceptible to oxidative and hydrolytic degradation, specifications on the percentage of free fatty acids and products of oxidation, such as malonaldehyde and peroxides, are generally made. Limits on water content, unsaponifiable matter content, and insoluble matter are also often specified. Occasionally, refined fish oils are available on the market; oils are refined to reduce free fatty acid values and nonfat material. Even though these oils are within specifications for fish feed use, they may be low in naturally present antioxidants, which can result in a shortened induction period prior to the onset of oxidation. This can be prevented by adding antioxidants. Antioxidants are usually added to fish oils during production to prevent oxidation, but buyers of oil for fish feed use should make certain this had been done. Ethoxyquin is the most common antioxidant used in fish oils. In the United States feeds cannot contain over 150 ppm ethoxyquin, from all sources. Typical specifications for fish oil for fish feed use are listed in Table 9.7.

Another concern with fats and oils is the possible presence of organic contaminants, many of which are lipophillic. Several published values for polychlorinated biphenyls (PCBs) and 1,1-dichloro-2, 2-bis(*p*-chlorophenyl) ethylene (DDE) in fish feeds are available (Crockett *et al.* 1975; Gruger *et al.* 1976). While none of the published values approach toxic levels, the levels may be of practical significance to scientists conducting contaminant-exposure studies (Mac *et al.* 1979). More recently, concerns about dioxin contamination of feed ingredients have been raised. Specifically, certain clays used in animal feeds were found to contain dioxin. Rising concerns about the purity and safety of food coupled with improvements in analytical testing for organic contaminants of feeds and foods will likely result in

more rigorous testing in the future. Since feed ingredients are the principal source of potential contamination of feeds and foods, we can expect increasing surveillance of feed ingredients and changes in ingredient processing and use patterns to result.

9.3.4. Other Nutritive Ingredients

Practical mixtures of protein supplements and basal feeds contain all of the nutrients needed by fish but not at levels sufficient to meet their total dietary requirements. Thus, additional vitamin and mineral premixes are added to feeds to ensure adequate intake of these nutrients. Fats and oils are also added at low levels to reduce dustiness in catfish feeds and at high levels to increase the dietary energy levels in high-energy feeds for salmonids and some marine fish, such as yellowtail.

9.3.4.1. Vitamin Premixes

Vitamin premixes are concentrates in which stable forms of essential vitamins are mixed with a carrier, usually a basal feed such as a wheat by-product. Choline chloride is not included in vitamin premixes, although it is a stable product, because it has been shown to reduce the stability of other vitamins (Gabaudan and Hardy 2000). Thus, it is added to feeds separately. Ascorbic acid is included in some vitamin premixes but is added separately to others. In the past, the concentration of vitamins in the premix was sufficiently high that the finished feed exceeded the recommended vitamin levels (NRC 1993). In recent years, the level of vitamin supplementation has been lowered to match the recommended levels more closely. Vitamin premixes are added to practical diets at levels ranging from 0.5 to 4% of the diet, depending on the formulation of the vitamin premix. The specifications for several vitamin premixes used in open-formula salmonid diets are listed in Table 9.8.

9.3.4.2. Mineral Premixes

Mineral premixes are concentrates of essential elements that are fortified in practical fish diets to make up for low levels in the formulation or to overcome antagonistic interactions among feed ingredients. Although the necessity for elemental fortification of practical diets is uncertain in most instances, trace element fortification is a wise precaution when diets contain high levels of protein supplements of plant origin, which are low in some trace elements and which also may contain phytic acid, which reduces the bioavailability of divalent cations. High dietary ash levels can result in antagonistic interactions between calcium phosphate and other minerals, i.e., phosphorus and zinc, which lower intestinal absorption (Sugiura *et al.* 2000b). The cost of trace element premixes is low, so including them in practical

Table 9.8

Vitamin Premix Specifications for Several Open-Formula Salmonid Diets[a]

Ingredient (form)	NRC dietary recommended level	Abernathy salmon diet premix[b]	Ontario Ministry of Natural Resources vitamin concentration per kg premix[c]
Vitamin A (palmitate or acetate)	2,500	440,000 (6,600)	6,000,000 IU
Vitamin D_3 (as cholecalcipherol)	2,400	29,398 (441)	400,050 IU
Vitamin E (as *dl*-α-tocopheryl acetate)	30	33,510 (502)	15,200 IU
Vitamin K (as menadione sodium bisulfate)	10	1,858 (28)	278 mg
Vitamin B_{12} (as cyanocobalamin)	0.02	3.97 (0.06)	1.8 mg
Ascorbic acid (as ascorbyl polyphosphate)	100	(1,000)	15,000 mg
d-Biotin	1	39.7 (0.6)	18 mg
Choline (as chloride)	400	8,818 (132)	30,0000 mg
Folic acid	5	849 (12.7)	500 mg
Niacin (nicotinic acid)	150	14,704 (220)	6,700 mg
Pantothenic acid	40	7,668 (115)	3,200 mg
Pyridoxine	10	2,504 (38)	1,500 mg
Riboflavin	20	3,527 (53)	1,600 mg
Thiamin	10	3,117 (47)	1,300 mg
Carrier (wheat middlings or starch)	−	+	+
Total premix		1000 g	1000 g

[a] All ingredients and premixes must be ground finer than 0.15 mm prior to mixing.
[b] Values in parenthesis are levels in the final diet after premix is added to the feed mixture.
[c] All quantities are expressed on the basis of active vitamins.

diets is inexpensive insurance against possible deficiencies. Several mineral premixes used in open-formula salmonid diets are listed in Table 9.9.

Additional mineral fortification is required in semipurified, experimental fish diets since their ingredients are highly refined and do not contain sufficient amounts of minerals to meet the nutritional requirements of fish. Several such diet formulations along with the mineral premix specifications are reported in National Research Council (1993) publications on the nutrient requirements of fish.

9.3.5. Nonnutritive Feed Additives

Nonnutritive feed ingredients are additives that are included in diets for reasons other than to provide nutrients. For the most part, these compounds have little or no nutritional value, yet they are important constituents of fish feeds, increasing pellet stability, diet safety, diet flavor, and animal and fish performance and health status and influencing the quality of the final

Table 9.9

Mineral Premix Specifications for Open-Formula Salmonid Diets

Element (form)	g element/kg premix	
	U.S. Fish and Wild life Service trace mineral premix No. 3	Ontario Ministry of Natural Resources mineral premix MIN-9504
Zn (as $ZnSO_4 \cdot 7H_2O$)	75	12.0
Mn (as $MnSO_4$)	20	17.64
Cu (as $CuSO_4 \cdot 5H_2O$)	1.54	1.5
I (as KIO_3)	10	1.5
or		
I ($C_2H_8N_22HI$)	—	—
Fe (as $FeSO_4 \cdot 7H_2O$)	—	2.6
NaCl	—	240

product. Nonnutritive feed ingredients include feed binders, carotenoid supplements, drugs and antibiotics, hormones, antifungals, antioxidants, fiber, flavorings, and water.

9.3.5.1. Pellet Binders

Fish feeds must be formed into particles or pellets that are strong enough to withstand normal handling and shipping without disintegrating. Moreover, fish feeds must be somewhat water-stable. These requirements make it necessary for feeds to contain binders. There are numerous materials that act as binders in fish feed, including regular feed ingredients and ingredients added solely for their binding properties. Some binders are by-products of cereal grains or plants and provide nutrients to the diet. For example, 20% pregelatinized potato starch is added to eel diets to increase the water stability of the dough and to provide energy. Other commonly used binders include bentonite, lignin sulfonate, and hemicellulose extract, none of which provides nutrients to the diet. Bentonite is a naturally occurring clay consisting mainly of trilayered aluminum silicate. It is available as either sodium bentonite or calcium bentonite. Sodium bentonite has, by definition, more than 1% and less than 2% available ion content, or sodium exchange. It swells when added to water, while calcium bentonite does not. Both sodium and calcium bentonite may be added to dry, compressed fish feeds at no more than 2% to act as a binding agent and also as a lubricant, increasing pellet mill production rates and pellet mill die life (Reinitz 1983). Some bentonites also bind aflatoxin, carrying it through the gut without harming the fish (Ellis *et al.* 2000).

Lignin sulfonate is a product of the wood pulping industry. It aids in pellet binding, reduces fines, and permits the addition of more steam during the manufacture of compressed pellets. Lignin sulfonate is added at up to 4% as a pelleting aid in dry, compressed (steam-pelleted) feeds. Hemicellulose extract is a product made by spray-drying the concentrated, soluble by-product of pressed wood manufacture. It is less commonly used than lignin sulfonate.

Moist and semimoist fish food production requires the use of both nutritive and nonnutritive binder materials. Nutritive binders include oat groats, vital wheat gluten, finely milled wheat bran, cottonseed meal, gelatin, fish hydrolyzates, and pregelatinized starches. Nonnutritive binders include tapioca, carboxymethylcellose, alginates, agar, and various gums. Chitosan, carageenan, and collagen have been evaluated as binders but are not commonly used (Heinen 1981).

Semimoist feeds, containing 25–35% moisture, can often be made into satisfactory pellets by careful selection of feed ingredients that possess binding properties. However, when feed formulations contain ingredients that do not possess suitable binding properties, it is necessary to add ingredients specifically to bind the diet.

Moist feeds, having moisture contents of 35 to 70%, always require the addition of a binder. For example, semipurified test diets, such as H440, the Oregon Test Diet, and the Guelph semipurified diet (Ensminger and Olentine 1978; NRC 1993), include gelatin and carboxymethylcellulose as binders. Moist diets, which are combinations of wet fish ingredients and dry meal, may contain 0.5–2.0% alginates as binders. Heinen (1981) found that alginates were better binders than gum, carageenan, chitosan, collagen, carboxymethylcellulose, and corn starch in a 41% moisture diet. Agar was an effective binder, but expensive. Calcium ions and a sequestrant, such as sodium hexametaphosphate, must be present in diets containing alginates as binders to control alginate activation.

9.3.5.2. Carotenoid Supplements

A great deal has been written about the addition of carotenoid pigments to fish diets to color flesh and/or eggs (Ellis 1979; Torrissen *et al.* 1989; Yamada *et al.* 1981). Over 300 pigments are found in various plants and animals, with xanthophylls and carotenoids being the most important classes of carotenoid pigments that add color to fish. For the most part, xanthophylls are found in plants, such as corn, and carotenoid pigments in crustaceans and fish.

Some finfish and shellfish possess the ability to convert certain xanthophyll pigments to carotenoid pigments. Goldfish and common carp can convert the yellow xanthophyll pigment, zeaxanthin, to the red carotenoid

pigment, astaxanthin (Hata and Hata 1972). Similarly, *Penaeus japonicus,* a shrimp, can convert both β-carotene and zeaxanthin to astaxanthin (Tanaka *et al.* 1976). Salmon, trout, and red sea bream, which normally have pigmented flesh and skin, do not convert xanthophyll pigments to the carotenoids, canthaxanthin, and astaxanthin. In nature, they receive these pigments in their diet. Fish raised in hatcheries and farms must receive canthaxanthin and/or astaxanthin in their diets to become pigmented; in addition, carotenoid supplementation is necessary for salmonid offspring to produce viable offspring (Christiansen *et al.* 1995). In nature, carotenoid pigments are synthesized by algae and bioconcentrated in the food chain, ultimately ending up in fish.

Carotenoid supplementation of fish diets is accomplished by adding natural materials containing the desired carotenoid pigments, carotenoid extracts of natural products, or chemically synthesized pigments. Natural materials that pigment fish include herring gull eggs, salmon eggs, paprika, zooplankton, krill products, *Haematococcus* algae, and processing waste from shrimp, crab, and crayfish processing. Dietary levels of 20% or more of wet crustacean processing waste are required to get the desired pigment in trout and salmon. Concentrated carotenoid extracts of red crab and crayfish are effective dietary supplements for salmonids (Chen and Meyers 1982; Mahnken *et al.* 1980). The amount added to the diet depends on the concentration of carotenoid pigments in the extract, but dietary levels normally range from 3 to 7%, replacing added fats and oils. Synthetic canthaxanthin [Carophyll-red (Hoffman–LaRoche Basel, Switzerland) or Lucanthin-red (BASF Ludwigshafen, Germany)] is a commercial product containing a minimum of 10% canthaxanthin and is added to commercial feeds at 0.05% to produce a dietary canthaxanthin level of about 50 mg/kg feed. Astaxanthin [Carophyll-pink (Hoffman–LaRoche) or Lucanthin-pink (BASF)] is the most widely used, manufactured carotenoid pigment. It contains 8% astaxanthin, by weight, encapsulated in gelatin, and is added to fish feeds at approximately 0.065% to produce a dietary astaxanthin level of 45 mg/kg feed.

Astaxanthin is produced by several microorganisms, including *Phyaffia* yeast and *Haematococcus* algae meal, and products are being produced from these microorganisms specifically for use in fish feeds. Because they are produced naturally, they are desirable for use in salmon production for markets demanding a natural food product. Krill products fill the same market niche and are also effective feed palatability enhancers.

9.3.5.3. Therapeutants and Nonspecific Immune Stimulants

Therapeutants are added to fish feeds to treat, cure, mitigate, or prevent disease. A number of drugs are effective against fish diseases, although in the United States, the only ones approved for use with fish feed are

sulfamethazine, terramycin (oxytetracycline), and furox. Erythromycin and azithromycin have been used to treat bacterial kidney disease in captive broodstock of endangered salmon stocks, but they are not allowed in normal production. In Europe, oxalinic acid is used in feeds as an antimicrobial drug. As with livestock feeds, medicated fish feeds have specific labeling requirements, including a warning to withdraw for a proscribed length of time before the fish are marketed. Antibiotics have been supplemented at subtherapeutic levels for decades in poultry and swine feeds to stimulate growth. Their benefit is derived through control of intestinal microflora, preventing toxin-producing microorganisms, such as *Clostridium perfringens,* from becoming established in large numbers and lowering the growth rate of the animal. This practice has never been used in aquaculture, in part because it is not effective, due to differences between aquatic and terrestrial animals with respect to intestinal microflora. Given the serious concerns about antibiotic resistance and human health, it is likely that antibiotic supplementation in terrestrial animal production for growth promotion will be limited or possibly eliminated in the future.

Nonspecific immune stimulants, sometimes referred to as neutriceuticals, are another story. They are unregulated feed additives that are intended to enhance the health and well-being of farm and companion animals. In fish, the focus on neutriceuticals lies in making the fish less susceptible to infectious disease. The most common supplements are β-glucans, which are fragments of the cell walls of yeast and mycelial fungi. The rationale behind their use is that β-glucans supposedly come into contact with leukocytes in the intestinal mucosa. Glucans supposedly possess the same chemical signals as infectious disease agents and, therefore, activate the leukocytes. Glucans are also hypothesized to physically attach to pathogens and thus render them inactive. In fact, although glucans have been shown to reduce fish disease, and also to stimulate the nonspecific immune response of fish, exactly how they work is not known. Other theories of their mode of action have been presented, but none, as yet, has been proven. Glucans are sometimes effective, and other times not. Questions remain concerning the effective dose, route of administration, and chemical form. There are many forms of β-glucans, and other materials that stimulate the immune system of fish, all of which have been reviewed by Raa (1996).

9.3.5.4. Probiotics

Probiotics are live, microbial feed supplements that are thought to stimulate animal and, possibly, fish growth by affecting the microbial flora population in the gut of the animal. Probiotics may be a single species of microorganisms or a mixture of species. The concept behind their use is that the species of microorganisms present in the supplement colonizes the gut

and outcompetes detrimental species of microorganisms, thus limiting their numbers and allowing the animal (fish) to avoid wasting metabolic energy fighting the effects of detrimental microorganisms. Obviously, probiotics must be added to feeds after pelleting.

9.3.5.5. Enzyme Supplements

Enzyme supplements are either single, purified enzymes or crude enzyme preparations containing multiple enzymes that are added to feeds to enhance the digestion of feed components that the fish either cannot digest or cannot digest efficiently. Phytase is an example of a single enzyme supplement used in poultry and swine feeds and likely to be used in fish feeds in the near-future. Phytase hydrolyzes phytate, the storage form of phosphorus in seeds, i.e., grains and oilseeds. Phytase liberates phosphorus from phytate, thus making it available to the animal or fish. Enzyme supplements are available to assist in the digestion of complex carbohydrates, collagen in skin and bones, and other feed constituents. Enzymes are typically denatured at temperatures above 65°C, so adding them to feed mixtures before compression or extrusion pelleting destroys their activity. Thus, enzyme supplements are typically sprayed on feeds after pelleting.

9.3.5.6. Hormones

The use of anabolic steroids in domestic animal feeds is no longer permitted in many parts of the world due to concern about hormone residues in food products. The same concerns exist for fish products, and the addition of steroids and other hormones to the diets of fish raised for market will almost certainly never be approved. However, there are some aquaculture situations in which the addition of hormones to fish diets for a short period may pose no human health risk and may prove useful to fish culturists. Hormones fall into three categories: (1) those that affect growth and feed conversion, (2) those that affect sexual development, and (3) those that affect osmoregulation.

In public salmon culture in the Pacific Northwest, salmon fingerlings are reared in freshwater hatcheries until the optimum time of release. After release, the fingerlings migrate to the ocean, spend 2–4 years growing, and return as adults to the near-shore areas where they enter the fishery. For some species and stocks, the size at hatchery release is positively correlated with the percentage of returning adults. However, many hatcheries cannot rear fish to the optimum size for high ocean survival by the required time of release.

Fish growth rates can be accelerated by supplementing diets with anabolic steroids and thyroid hormones, thereby increasing feed intake and metabolic efficiency (Donaldson 2000). An alternative is to add compounds or feed components that stimulate hormone production or that overcome

hormone inhibition associated with certain feed ingredients, notably canola (rapeseed) meal (Plisetskaya *et al.* 1991; Riley *et al.* 1996). Pituitary growth hormones and insulin may also have growth-promoting effects when added to fish feed (Donaldson 2000). For example, weight gain increases of over 90% have been observed in salmon fed diets containing low levels (1 mg/kg) of 17 α-methyltestosterone. Some species, such as chinook salmon, do not respond as dramatically to dietary supplementation with hormones. However, a 26% increase in growth was achieved with fall chinook salmon when testosterone was fed in the diet (Fowler 1982).

Thyroid hormones have been shown to promote growth of teleost fish in numerous studies (McBride *et al.* 1982). However, growth-promoting effects are dependent on their synergism with endogenous pituitary hormones, especially growth hormone (Gorbman 1969). Dietary administration is the most practical form of delivery of thyroid hormones, but triiodothyronine (T_3) is the only thyroid hormone that can be used this way. Oral administration of thyroxine (T_4) is ineffective, probably because it is poorly absorbed from the gut. Levels of 4–20 mg T_3/kg feed effectively promote growth and also can overcome negative effects of feeding diets containing thyroid-inhibiting compounds contained in canola meal (Teskeredzic *et al.* 1995).

Hardy (1985) found physiological levels of testosterone in commercial salmon feeds, which is not surprising since fish feeds contain fish meals made from spawning fish, such as herring. Borghetti *et al.* (1989) showed that fish meals made by low-temperature drying of spawned salmon carcasses contained androgens that stimulate growth in coho and chinook salmon fry. The use of naturally derived products containing anabolic hormones remains an unexplored avenue of increasing fish growth rates.

Recently, Schelling *et al.* (1999) demonstrated that injections of recombinant bovine somatatropin (bST) greatly accelerated the growth rate of sturgeon. Maturation in sturgeon is size related and can take up to 15 years in captivity. If injections of bST in juvenile sturgeon can accelerate their growth by four to five times, it is conceivable that fish could mature in 6 to 8 years simply by giving six injections per year to juveniles for several years or, possibly, just through the winter months. In fish culture situations involving restoration of threatened stocks, this may be a viable approach to accelerate maturation and provide juveniles for restocking depleted populations.

Hormones are added directly to fish diets to cause sterility or sex reversal. The use of sterile fish in aquaculture is advantageous because it eliminates precocity and the cessation of somatic growth that accompanies sexual maturity. Sex reversal is currently practiced in species of fish, such as chinook salmon, when one sex has a desirable product or qualities, or when control of reproduction is desired, as is the case with tilapia. For a review of these subjects, see Donaldson (2000).

9.3.5.7. Antimicrobial Agents

Microorganisms require unbound water to grow in foods and feeds. Feeds containing more than 12% moisture can support bacterial, mold, and yeast growth unless they are frozen. Microbial growth occurs very rapidly in semimoist feeds, and many molds produce compounds that are toxic to fish. In semimoist feeds at room temperature (22°C), mold growth is visible within 3 days, while at refrigerated temperatures (1–3°C), it may not be visible for 10–20 days. During frozen storage, microbial growth is completely stopped. Thus, if semimoist diets are fed to fish shortly after manufacture, there is no need to use antimicrobial additives in the feed. If longer storage is required, additives and other strategies must be used to prevent feed spoilage.

Over 20 compounds are used in the feed industry to inhibit fungal or microbial growth (Table 9.10). Some have general and some specific applications in food products. The benzoates and parabens are wide-spectrum antimicrobials, which are effective against bacteria, fungi, and yeast. Propionates are used primarily to inhibit yeasts and molds but are also effective against bacteria, fungi, and yeast. Choosing an appropriate antimicrobial

Table 9.10

Antimicrobial Agents Used in Feeds[a]

Name	Limit or restriction
Benzoic acid	0.1%
Calcium propionate	None
Calcium sorbate	None
Distearyl thiodipropionate	0.005%
Formic acid	2.5%
Methylparaben	0.1%
Potassium bisulfite	Not for use in B_1 sources
Potassium metabisulfite	Not for use in B_1 sources
Potassium sorbate	None
Propionic acid	None
Propylparaben	0.1%
Sodium benzoate	0.1%
Sodium bisulfite	Not for use in B_1 sources
Sodium metabisulfite	Not for use in B_1 sources
Sodium nitrite	0.002%
Sodium propionate	None
Sodium sorbate	None
Sodium sulfite	Not for use in B_1 sources
Sorbic acid	None

[a] 1981 Food Industry Red Book.

agent for fish food involves more than picking the least expensive one. An intelligent choice includes a complete study of the specific microbial problem, solubility and ease of application of the agent, pH requirements, empirical information, and effect of the antimicrobial agent on the palatability of the feed.

Control of spoilage in intermediate moisture products (18–36% moisture), such as semimoist fish feeds, is not simply a matter of adding antimicrobial compounds to the product. The best results are obtained when a combination of approaches is used. The first factor is the microbial load of the product. It is essential that the feed has a low plate count at the start, with no more than 500,000 colony-forming units per g of material. If the microbial load of the starting material is higher than this, it is difficult to control spoilage with antimicrobial compounds alone. A second means of controlling microbial spoilage in feeds is by the use of special packaging that permits the maintenance of a controlled atmosphere after manufacture until feed is used. The aim of this process is to reduce O_2 tension in the feed package during storage and thereby restrict the growth of aerobic microorganisms. The feeds are stored in hermetically sealed pouches or bags that are impermeable to water vapor or atmospheric gases. An inert gas, such as N_2 or CO_2, is introduced into the bag before it is sealed.

A third approach to controlling spoilage in intermediate moisture products is by controlling the water activity (A_w). Microorganisms require an abundant supply of water to grow. Reducing the supply of water in a feed is therefore an effective way to limit their growth. In intermediate moisture products, sufficient water to permit microbial growth is present. However, water exists in feeds in three forms, as a liquid, as a vapor, and as chemically bound water. Control of water activity in intermediate moisture products involves increasing the proportion of total moisture in the product that is chemically bound, thereby decreasing the proportion present as a liquid. This principle is used to preserve many human foods such as jelly. Water activity is defined as the ratio of the water vapor in a feed to the vapor pressure of pure water at the same temperature. The water activity of pure water is 1.0. Water in feeds has an A_w value of less than 1.0. Small differences in A_w may result in relatively large differences in the resistance of a product to microbial spoilage. The approximate lower limits of A_w for microorganism growth are 0.91 for bateria, 0.88 for yeasts, and 0.80 for molds. It is not difficult to achieve A_w values in this range in intermediate-moisture products by the addition of compounds such as sugar (sucrose), glycerol, NaCl, and propylene glycol.

Semimoist fish feeds not requiring frozen storage are commercially available in North America. These feeds contain agents to inhibit microbial growth, and often manufacturers use other means to restrict microbial

spoilage. The feeds are highly palatable to salmonids and other species of fish. Once a bag of feed is opened, it should be used within days, because the protective atmosphere is replaced with air.

9.3.5.8. Antioxidants

Antioxidants are chemical compounds that are added to feed ingredients to control oxidation of lipids. Other food components, such as carotenoid pigments and tocopherols, can also undergo oxidation. The mechanism of highest concern in feed manufacturing is autoxidation, also known as atmospheric oxidation, which is the oxidation of moderately unsaturated fatty acids, resulting in products that produce off-flavors and off-odors. The rate of autoxidation of lipids can be accelerated by an increased radiation level, divalent cation concentration, temperature, and oxygen concentration.

Autoxidation of lipids is a process involving three steps (Fig. 9.1). The first step involves the formation of free radicals and is called initiation. Initiation is enhanced by a number of factors, including light, heat, UV radiation, and the presence of divalent cations, such as copper and iron, known as pro-oxidants. The second step in autoxidation is called propagation and involves the reaction of free radicals formed in the initiation step with more free double bonds on fatty acids, forming a number of secondary products and radicals. The final step is termination, in which free radical production slows and, finally, stops; various secondary products of fatty acid oxidation react in various ways to form stable end products. Because the propagation step itself forms more free radicals than it uses, autoxidation reactions are autocatalytic, meaning that once oxidation starts, it continues at an accelerating rate until substrates (double bonds) are used up. The number of free radicals formed from oxidation of individual fatty acids is related to the number of its double bonds, making oxidation of the fatty acids in fish oils (very unsaturated) a much more rapid process than oxidation of less unsaturated lipids.

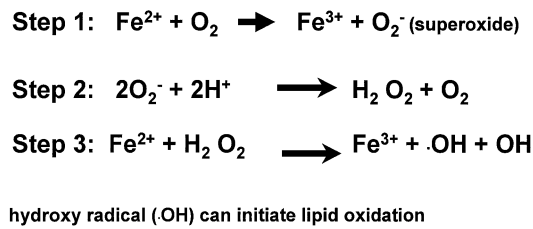

FIG. 9.1

Free radical formation in oxidizing lipids.

Antioxidants work by chelating pro-oxidant divalent cations, by acting as free radical acceptors, or by donating hydrogen. The latter two functions are considered sacrificial because once an antioxidant molecule reacts, it no longer possesses antioxidant properties and is therefore "destroyed" in the process. Thus, antioxidant concentrations fall during the initiation phase, and once they are used up, oxidation reactions proceed very rapidly.

Antioxidants added to lipids and feeds to prevent oxidation by reacting with free radicals are phenolics, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and amines, such as ethoxyquin (Thorisson *et al.* 1992). BHA and BHT are added to feeds at a level of 0.1%, while ethoxyquin is added at 0.015%. Other antioxidants in use include dilauryl thiodipropionate, propyl gallate, and thiodipropionate.

Antioxidants that prevent oxidation by chelating metallic pro-oxidants include ascorbic acid, phytic acid, tartaric acid, oxalic acid, and ethylenediaminetetraacetic acid (EDTA). There is a synergistic effect when phenolic or amine antioxidants are combined with an antioxidant that chelates pro-oxidants.

Many lipid sources contain naturally occurring antioxidants, mainly tocopherols. These compounds inhibit autoxidation of lipids until they are used up, at which time the rate of oxidation reactions increases very rapidly. The period of time during which antioxidants prevent oxidation is called the induction time. Chemical tests to detect lipid oxidation, such as peroxide values and TBARS, cannot measure induction time, and low values from these tests can give a false sense of security to a feed company. By testing a lipid source or feed before and after an accelerated oxidation test, such as the Schall oven test (described later in this chapter), the induction time can be estimated, and appropriate precautions taken to avoid oxidation.

9.3.5.9. Fiber

Fiber is the nonnutritive portion of feed ingredients that is measured as crude fiber in proximate analysis. It is indigestible by salmonids and other carnivorous fish, but channel catfish have intestinal microflora capable of digesting a small portion of dietary fiber (Stickney and Shumway 1974). Some herbivorous fish, such as grass carp, derive nutrients from fiber but some, such as *Tilapia aurea,* do not (Stickney 1975). Fiber is added to semipurified diets to facilitate binding as well as to increase digestion efficiency (Buhler and Halver 1961). Generally, fiber is not added to practical diets; rather it is avoided because it passes through the fish and adds fecal solids to rearing water and farm effluents. This point is critical in aquaculture systems employing water recirculation and in rainbow trout farming, where high volumes of water are discharged into rivers and lakes. Upper limits for fiber in feed formulations are generally specified, thus eliminating many potential

fish feed ingredients and restricting the levels of others. In diets for fish that do not possess the ability to digest fiber, levels of fiber above 3–5% are not recommended (NRC 1993). Fiber levels as high as 8–12% are tolerated by most fish, but such levels often result in growth depression (Edwards *et al.* 1977; Leary and Lovell 1975). Fish fed diets high in indigestible fiber increase their feed intake and gastric evacuation time, but the extent to which fish can compensate in this manner is limited (Hilton and Slinger 1983).

9.3.5.10. Water

The water content of feeds ranges from 6–10% for dry-compressed or extruded pellets to 65–70% for high-moisture, wet pellets. The moisture content of feeds is important because of the potential for microbial growth in high-moisture feeds, and the moisture content is critical in the pelleting process, where it is added to the mixture as live steam just prior to pelleting. Steam pelleting and cooking extrusion increase the moisture content of the feed mixture to approximately 18 and 23%, respectively, but the pellets are dried to <11% immediately after pelleting.

Some fish species accept moist feeds more readily than dry feeds, particularly Pacific salmon fry (Crawford *et al.* 1973; Higgs *et al.* 1985) and large-mouth bass (Snow and Maxwell 1970). However, brown trout and turbot grow equally well on moist or dry diets (Bromley 1980; Poston 1974). In the past, researchers reported that chinook salmon reared in marine netpens grew more rapidly when fed diets containing 15–30% water than when fed dry diets, but improvements in feed formulation and manufacture have eliminated this effect .

9.3.5.11. Flavorings and Palatability Enhancers

Fish are very sensitive to certain tastes in their feed, a trait that can be both harmful and beneficial in diet formulation and manufacture. For example, chinook salmon fry are extremely sensitive to the presence of low levels of dietary soybean meal (Fowler 1980; Spinelli *et al.* 1979) and respond to its presence by reducing their intake. Trout are less sensitive to dietary soybean meal, although in semipurified diets, the addition of a "fishy" component to the diet to mask the taste of soybean meal must sometimes be made to induce trout to consume feed (Wekell *et al.* 1986).

Flavorings are common feed additives in the pet food industry but their use in aquaculture diets is only beginning to be investigated. Generally, feed acceptance is not a major problem among cultured species of fish, with the exception of fry and certain species of cold-water fish. Extracts of crustaceans, such as krill, and certain amino acids may increase appetite in fry and crustaceans, respectively.

9.4 Diet Formulation

Diet formulation is a complicated process, providing it is done well. By various procedures, feed formulators select dietary ingredients establishing upper and lower limits for each, to create a mixture that is pelletable, palatable, nutritious, inexpensive, and easy to store, ship, and use. Ingredients are chosen on the basis of cost, availability, chemical composition, and nutritional value. Protein supplements are often chosen on the basis of price per unit protein. The process of diet formulation involves a difficult choice between two approaches that can be represented on the spectrum of feed cost. At one end of the spectrum is the approach of basing the formulation solely on nutritional value, thereby producing a more expensive feed that is more productive. The other end of the spectrum is the approach of basing the formulation solely on the total feed cost and attempting to minimize that cost. The vast majority of feed formulations falls somewhere in the middle of the spectrum, but the type of feed being formulated will dictate which determinant, nutritional value or feed cost, is more important. Larval or starter feeds are very important to the long-term health of fish and account for a very small proportion of the total feed cost to raise a fish for market or release. Thus, selecting a formulation based more on nutritional value than feed cost is warranted for starter or larval feeds. In contrast, grow-out feeds account for nearly 90% of the total feed used in a production cycle. Small differences in feed costs can have a profound effect on the cost of production and, thus, profit and loss of a fish farm. Obviously, feed formulations for grow-out feeds are developed with feed cost as an important determinant.

Regardless of what type of feed is being formulated, feed formulation involves selecting a combination of ingredients which will produce a mixture containing levels of essential nutrients at or above the minimum requirements of the fish. To do this, one has to know the nutritional requirements of the fish and the available nutrient content of the ingredients. The gross nutrient content is determined by chemical analysis. Data on the nutrient content of all common fish feed ingredients are readily available in feed composition tables. However, the values listed in such tables are averages and represent the gross nutrient content, not the available nutrient content. Individual lots of ingredients sometimes vary in nutrient content depending on the season or stage of maturity at harvest, the procedures used to process the raw material into the feed commodity, and many other factors. Chemical analysis methods are also subject to experimental error. Thus, it is difficult to formulate a feed that contains the exact amount of each critical nutrient at the desired level. To prevent possible problems of this nature, feeds are usually formulated to contain slightly higher levels of protein, energy, and individual nutrients than actual required or recommended levels.

The availability of nutrients in feed ingredients is another aspect that must be considered in feed formulation. Incorporating information on nutrient availability into the feed formulation process is easy, but obtaining such information is a much more difficult process. Factors such as the procedures used to manufacture the ingredient and interactions with other dietary ingredients can reduce the availability of a nutrient to a fish, even though the results of chemical analysis show that adequate dietary levels are present. An example of a reduction in nutrient availability as a result of processing is the reduction that occurs when protein supplements are dried in the presence of reducing sugars. Amino acid analysis will detect a certain level of lysine, but the level of available lysine will be much lower. An example of a reduction in nutrient availability due to interaction with other dietary ingredients is that of zinc availability in fish fed diets containing high-ash meals, elevated levels of calcium and phosphorus, or elevated levels of calcium, phosphorus, and phytic acid (Ketola 1979; Hardy and Shearer 1985; Richardson *et al.* 1985). A dietary zinc level in the range of the requirement for trout (15–30 ppm) will be adequate in a semipurified diet but insufficient in a practical diet containing high-ash fish meal or in a semipurified diet containing elevated levels of calcium, phosphorus, and/or phytic acid. Determining dietary availability involves feeding experimental diets to fish and measuring parameters such as digestibility, enzyme activity, and tissue nutrient saturation. It is essential that nutrient availability values are considered in the feed formulation process. For nutrients for which the availability can vary and for which no reliable availability values exist, levels significantly above the minimum requirements must be used.

One assumption commonly made in feed formulation is that a nutrient in one ingredient is interchangeable with the same nutrient in another ingredient. This assumption must be made to allow the feed formula to be manipulated by changing the levels of ingredients and by substituting ingredients as price and availability change. The assumption is not always justified or even recognized. For the most part, however, and despite the potential pitfalls in feed formulation arising from inadequate information on the nutrient availability in ingredients, fish feeds are formulated, manufactured, and used successfully to rear fish every day. Enough information is available to avoid the major problems, and, as mentioned, feeds are normally formuated to contain a slight excess of critical, limiting nutrients.

9.4.1. Feed Categories

As mentioned earlier, feed formulation is a balance between nutrient levels or ingredient sources and cost. The general goal is to support the

highest performance at the lowest cost. In some cases this involves setting nutrient levels above the estimated requirements of the fish and using highly palatable ingredients, which may be more expensive than equivalent ingredients but are necessary in the diet to stimulate feed intake and support high growth. First-feeding fish benefit from being fed diets with higher nutrient levels and/or palatability enhancers such as liver meal than fish in the grow-out, postjuvenile stage of production. Fish generally dislike the flavor of medicated feed, but the addition of highly palatable ingredients, such as liver meal, to the diet helps maintain feed intake. In feed formulation, it is important to first determine the function of a feed, such as supporting maximum growth, feed intake, feed efficiency, or reproductive performance. Once the function or use of the feed has been defined, the type of feed, its formulation, and the appropriate manufacturing technique can be determined.

Phase feeding is a term used to describe the selection of feed formulation based upon the life stage of an animal. Feed formulations can be categorized by the intended function or phase of production in which the feed will be used. This simplifies communication between feed manufactures and fish producers. The purpose of phase feeding is to minimize input (feed costs) and maximize output (fish performance). Specific feeds can be formulated to increase survival, growth, fish health (nonspecific immunostimulation), fish quality, and body composition or to minimize the amount of nutrients in hatchery or farm effluents (pollution reduction). The characteristics desired in feed for a particular phase are discussed in detail below.

9.4.1.1. First Feed for Fry and Larvae

Many terms have been used to describe the first feed that is offered to young fish. The terms "starter feeds" and "larval feeds" are sometimes used interchangeably, but there are distinct differences between them. Starter feeds are larger than larval feeds, typically larger than 400 μm in diameter compared to larval feeds, which are less than 400 μm. Larval feeds are developed for species of fish which, at first feeding, may not be aggressive feeders and, also, may not possess fully developed digestive systems. Starter feeds are intended for species of fish which are aggressive feeders and do have fully functional digestive tracts at first feeding, e.g., salmon or trout fry. The manufacture of larval feeds is more involved than that of starter feeds, and the techniques used are more sophisticated. Good growth and survival of catfish, salmon, trout, and tilapia fry can be obtained by feeding starter feeds. In contrast, performance advantages are seen when sea bass, sea bream, walleye, and striped bass fry are fed larval feeds. Although larval feeds are more expensive than starter feeds, they are more than worth the extra cost for first feeding of many fish species.

Although larval feeds and starter feeds have somewhat different applications, they do have several characteristics in common. First-feeding fry and larvae have nearly used up their yolk material when the search for exogenous feed begins. At this developmental stage, fry and larvae have virtually no nutrient reserves. Thus, it is critical that they consume the feed or they will rapidly starve to death. Feeds must possess several characteristics for optimum fish performance. The most important of these are as follows: (1) the feed must be readily consumed by the fish; (2) the feed must be highly digestible to accommodate a developing, and often not fully functional, digestive tract; and (3) the feed must be highly water-stable to maintain water quality and, thereby, limit bacterial contamination of the culture system. Both the feed formulation and the feed manufacturing method determine the critical characteristics of feed particles. The feed formulation affects consumption by influencing the color, texture, taste, and smell of the feed. Manufacturing affects consumption by influencing the feed particle size, shape, texture, density, and buoyancy. A high-quality feed results from feed formulation and manufacturing methods working in harmony to optimize fish performance. Poor fish performance results when the interaction of formulation and manufacturing is not optimized. For example, expensive, highly palatable ingredients are useless in a diet if the method of feed manufacturing results in a feed texture that is unacceptable to larvae and thus reduces feed consumption.

During this phase of production the most important consideration is maximum survival and fish health. The cost of the starter or larval feed should not be a factor that determines the feed ingredient selection, formulation, and particle manufacturing method. Only a small quantity of larval feed is used relative to the total quantity of feed used during the production cycle, making economic outcomes dependent on the successful production of healthy fry, not on the cost of the feed.

9.4.1.2. Fry Feeds

Once fry or larvae are actively feeding and reach a weight of 0.50–0.75 g, they can be switched from a starter feed to a fry feed. Dry fry feeds have traditionally been produced by crumbled compressed particles and screening the particles to appropriate sizes. New processing techniques and modifications of older methods now permit the production of small particles and pellets without crumbling. These small particles have several advantages over crumbles, which are covered in Section 9.5, below. Fry feeds for most species are formulated to contain relatively high levels of protein, since nearly all species of fish require high levels of dietary protein as fry, regardless of their protein requirements at later life history stages. At the fry stage, the greatest relative gains in growth can be made for the least total feed investment.

In other words, a 25% increase in weight gain between groups of fry fed different diet formulations may be a small difference in actual weight, but the 25% advantage will persist between the groups up to harvest, all other things being equal. Thus, the gains obtained with fry will increase later production. Fry feeds should be formulated and used with this in mind. Cost savings at the expense of nutritional quality in fry feed is false economy due to advantages gained in growth rate and to the fact that fry feed is a very small percentage of the total feed consumed during a fish production cycle and, like starter feed, not a major element of feed cost per weight of fish harvested.

9.4.1.3. Conversion and Transition Feeds

The formulations and processing methods used for conversion feeds and transition feeds are slightly different from those used to produce larval feeds. Conversion and transition feeds are larger than larval feeds but utilize similar formulations and manufacturing technologies. Conversion and transition feeds are fed to fish that have been transferred to a hatchery after first being reared in ponds or tanks, where they consumed live, natural foods. The term conversion is usually applied to feed offered when fish are "converting" from live food to formulated feed. Fish fed conversion feeds after hatchery transfer exhibit higher survival and initial growth rates than those fed grower feeds. A key factor in this difference is the fact that conversion feeds are formulated to be highly palatability, a characteristic that enhances feed acceptance by fish when they are first given formulated feed. Although conversion feeds enhance fish performance, they cannot perform miracles. It is critical that the fish coming from a pond are in good condition. If the fish are in poor condition as a result of food scarcity in a pond, survival will be low no matter what feed is offered after transfer to the hatchery.

Transition feeds are fed when fish have been fed highly palatable starter feed, but are ready to move to a lower palatability fingerling or grower feed. Transition feeds are intermediate in many characteristics and ease the "transition" period. This feed type is most useful for undomesticated species of fish, such as those being cultured for reintroduction by conservation hatcheries, or for new species that are being reared for aquaculture production.

While conversion and transition feeds have different applications, their formulation and manufacture are similar. In fact, the primary considerations in selection of a conversion/transition feed are similar to those for larval feeds: (1) a high palatability, (2) a low contribution of the feed to water pollution (water stability), and (3) a high energy content to compensate for interrupted feeding. Since conversion and transition feeds are fed to a larger fish than are larval feeds, ingredient selection can be less restrictive.

Conversion feeds may contain ingredients with relatively low digestibility coefficients due to the mature stage of development of the gastrointestinal tract in fingerling fish and the shorter period of feeding these feeds. Palatability, however, should not be compromised.

9.4.1.4. Fingerling Feeds

The general practice in fish feed formulation is to reduce the amount of dietary protein in feeds as fish grow. When fish are in the 10- to 100-g weight range, they are fed fingerling diets. Semimoist pellet sizes of $\frac{3}{64}$–$\frac{3}{32}$ inch (1.2–2.4 mm) cover this range, and dry, compressed feeds are fed as crumbles to fish at the lower end of their weight range and as $\frac{3}{32}$ to $\frac{1}{8}$ inch (2.4- to 3.0 mm) pellets to larger fish. Growth rates are higher during this stage than in later stages of the life cycle. Thus, it is essential to ensure that the growth potential of the fish is realized by feeding a diet that is slightly overfortified with limiting amino acids (protein) and with vitamins. Nearly all of the information on fish nutritional requirements has been obtained from studies with fish in this size range. This information allows fingerling feeds to be formulated quite precisely.

9.4.1.5. Grower Feeds

Grower feeds are formulated to promote efficient and economical growth of fish from the fingerling stage up to market size. Formulations for these fish contain less protein and more energy than fingerling formulations. The energy content and proportion of the total dietary energy derived from protein must be carefully formulated to ensure that protein retention is high, that is, a high proportion of dietary protein is used to synthesize tissue protein rather than metabolized to yield metabolic energy. The majority of feed fed during a production cycle, often more than 90%, is fed during the grower stage. Most fish nutrition research has also focused on this type of feed because of the massive quantities required during production. Grower feeds are formulated for maximized growth of fish that are past early development, have a fully developed digestive system, and readily consume manufactured diets. Feed cost and feed conversion ratios (FCR) are very important factors to consider when selecting grower feeds. Small changes in feed costs or FCR can have a major impact on the profitability of a fish farm.

9.4.1.6. Broodstock Feeds

Somatic growth continues uninterrupted in most fish species until a combination of external and internal factors initiates sexual maturation. The temporal aspects of the sexual maturation process in fish are variable, proceeding for weeks or months, depending upon the species. During

sexual maturation, somatic growth slows and gonadal growth accelerates until the fish spawns. In many species of cultured fish, feeding ceases during part or all of the maturation process. If the fish is a species that survives spawning, feeding then begins again sometime after spawning. Research on the nutritional requirements of broodstock is both time-consuming and expensive, but some information is available to guide feed formulation (Luquet and Watanabe 1986; Hardy 1985). Relative to other life stages the knowledge base on the specific dietary nutrient requirements of maturing fish is limited. Salmonid broodstock perform relatively well when fed slightly modified grower diets. Marine species, such as sea bass, require omega-3 (ω-3) fatty acids during ovarian development. Generally, formulations contain higher levels of protein, energy, and certain vitamins associated with the conversion of maternal tissue nutrients to egg nutrients. Ascorbic acid levels are often increased in broodstock feeds, given the fact that, in some species, egg levels are relatively high. For salmonids, it is necessary to supplement diets with carotenoid pigments, such as astaxanthin, to ensure viable offspring (Christiansen *et al.* 1995).

Long-term studies on the nutritional requirements of maturing Pacific salmon are currently being conducted in connection with restoration efforts of threatened and endangered stocks. The information being compiled in these studies may be applicable to related species, such as trout and char. At present, salmon are fed grower diets supplemented with extra vitamins, carotenoid pigments, and selected trace minerals. Maturing captive salmon fed grower diets tend to accumulate fat, likely in anticipation of a spawning migration that, for captive fish, never happens. Thus, dietary caloric intake should be reduced at selected times to avoid this problem.

Fecundity is associated with fish size in most fish species, so the faster fish grow prior to the onset of maturation, the more eggs they will spawn. However, this may not be the most economical strategy to produce eggs, as calculation of the relative fecundity illustrates. Relative fecundity is the number of eggs per kilogram body weight. Small trout, for example, produce more eggs per kilogram body weight than do large trout. Thus, from a strictly economic perspective, one should limit the size of broodstock females.

9.4.1.7. Low-Pollution Feeds

In recent years salmon and trout aquaculture has been under increased scrutiny by regulatory agencies for the level of nutrients leaving hatcheries in the effluent water. Since hatchery effluents are a point source of pollution that is easy to sample compared to nonpoint sources, pressure has grown to reduce the concentration of several nutrients in hatchery effluents. The primary nutrient or materials subject to regulation so far are phosphorus

and fecal solids. Nitrogen may be the next nutrient to be considered by regulatory agencies.

For years, phosphorus levels in salmonid feeds were unintentionally high for economic reasons; fish meal was the main constituent of salmon and trout feeds. Regular fish meal contains high levels of phosphorus as a result of its fish bone content. Fish meal was such a high proportion of salmon and trout feeds because it was usually relatively inexpensive, was very palatable, and supported high growth rates because of its favorable profile of essential amino acids. Reducing phosphorus levels in salmonid feeds required feed formulators to reduce the proportion of regular fish meal, and this has proved difficult without increasing feed ingredient costs.

Several approaches have been taken to reduce phosphorus levels in effluents. The first involved more precise estimations of phosphorus requirements for all life stages of salmon and trout (Rodehutscord 1996; Ruohonen *et al.* 1999; Sugiura *et al.* 2000b). In addition, low levels of phosphorus could be fed during the final month or two of the grow-out stage in trout farming without decreasing growth rates (Lellis, Barrows, and Hardy, unpublished data, 2001). The fish utilized their stored phosphorus reserves from their skeleton and other hard tissues to supply their needs for growth. Additional research showed that large trout have a lower dietary phosphorus requirement than do small trout, making it possible to lower the phosphorus content of grow-out trout feeds (Sugiura *et al.* 2000b).

Suboptimal levels of phosphorus in broodstock diets decreased the growth rate but did not affect the fertility, hatchability, or overall egg viability. The relative fecundity increased when the fish were fed very low phosphorus feeds. The same effect has been observed in broodstock fed low-protein feeds (Smith *et al.* 1979).

The second approach to phosphorus reduction has been to increase the level of available phosphorus in the feed so that a higher percentage can be utilized by the fish. Increased digestibility, coupled with reduced dietary levels, results in less phosphorus being excreted in the feces and urine. Plant protein sources, such as wheat and corn gluten, contain lower levels of phosphorus than protein sources of fish or animal origin. The apparent phosphorus digestibility is similar to that in fish meal (Sugiura and Hardy 2000). Modification of fish meals has also been an area of active investigation (Babbitt *et al.* 1994). Mechanically deboning the fish meal prior to drying, or employing air classification to remove bone particles in dry meals, appears to offer two benefits. First, the total level of bone (ash fraction) and, thus, phosphorus is decreased. This allows for the processing of previously unusable meals such as seafood processing waste, which is mostly heads and frames after filleting, into usable fish meal. Second, the

phosphorus availability increases as the bone component is removed, as a result of lower levels of calcium in the product (Sugiura *et al.* 2000a).

Phosphorus availability can be increased using feed additives. Phytate is the storage form of phosphorus in seeds, e.g., corn, wheat, barley, and soybeans. Phytate phosphorus is unavailable to fish and all other simple-stomached (monogastric) animals, including humans. The shift from animal protein to plant protein to lower total feed phosphorus levels has resulted in an increase in the level of Phytate phosphorus in feeds. Phytate phosphorus passes through the gut of the fish and is excreted, thus contributing to phosphorus levels in hatchery effluents or ponds. The addition of phytase, a natural enzyme in seeds that releases phosphorus from the Phytate complex as seeds sprout, increases the phosphorus availability in fish. Phytase is commercially produced by several companies and is added to poultry and swine feeds. Phytase activity increases with temperature; its maximum activity is at 50–55°C. Thus, its activity in fish varies with water temperature. It is more effective in warmwater fish, such as catfish and tilapia, than in coldwater fish. Phytase can also be added to feed ingredients to treat them prior to their being used in feeds (Cain and Garling 1995).

Preliminary studies have suggested that the addition of citric acid to trout feeds increases phosphorus digestibility in grow-out-size rainbow trout (Sugiura *et al.* 1998b; Vielma *et al.* 1999). However, feeding diets containing citric acid to first-feeding rainbow trout lowers fish performance. This approach to increasing the phosphorus digestibility in trout feeds, even though promising, needs further research before it can be implemented.

Another approach to reducing solids production is to increase the overall digestibility and energy content of the diet and thus decrease the total amount of feed fed. As mentioned, high-energy diets, over 25% total lipid, are being fed to Atlantic salmon and rainbow trout. Fecal solids production can be further reduced by pelleting feeds using extruders or expanders that cook the carbohydrate portion of the feeds, eliminating the need for indigestible binders. These feeds yield lower feed conversion ratios compared to traditional steam-pelleted feeds. Extruded and expanded feeds are comprising an increasing portion of the aquaculture feed market in the United States. Very high energy feeds are not fed as widely in the United States as in Europe, perhaps due to differences in the consumer acceptance of high-lipid products produced by feeding very high energy diets and, also, due to differences in the size of fish (trout) at harvest.

Modification of feeds to produce intact fish feces is another area of current research. Culture systems using quiescent zones where particles can settle and off-line settling basins for further settling are being used at many farms and hatcheries to capture insoluble nutrients and total solids. However, some of the feces break up into particles too small to settle from the

water column and escape in farm effluents. If dietary changes that produce intact, high-density fecal pellets can be identified, further reduction in total solids and perhaps dissolved nutrients in hatchery effluents could be achieved.

Each of the approaches to pollution reduction is best applied to fish in the grow-out phase or with broodstock because of the large quantities of feed used during these stages. The amount of starter feed fed during a production cycle is so small that even complete elimination of phosphorus excretion would not significantly affect the total phosphorus discharges in effluents during the production cycle of the fish. The small gains in phosphorus reduction are not worth the risk of lowering fish performance in the early life stage.

9.4.1.8. Feeds to Increase Immunocompetence or Seawater Transfer

In some culture situations, disease outbreaks occur on a seasonal basis and special feeds are fed slightly before an outbreak period to help the fish fight disease. Supplements include vitamins, especially vitamin C, glucans, and other compounds reported to stimulate the immune system of the fish. In salmon farming, small changes in formulation are made for feeds that are supplemented before, during, and after fish are transferred from freshwater hatcheries to seawater farms. Betaine, which is reported to help some species of salmon make a successful transfer to seawater, especially during out-of-season transfers, is a supplement sometimes added to seawater transfer diets.

9.4.1.9. Feeds for Recirculation Systems

Interest in using recirculating systems for aquaculture has increased rapidly in the last few years. Fish farms in certain areas of the United States, such as the upper Midwest, use these systems, which employ filtration equipment to remove both particulate materials and dissolved nutrients from the water. These substances originate from the feed and thus a feed formulation or manufacturing technique that lowers the workload of the filtration system should increase production and/or lower production costs. Specifications for feeds designed for recirculating systems are similar to those for pollution reduction feeds, but even more restrictive, especially with respect to the dietary mineral content. Research and production of feeds formulated specifically for use in recirculation systems will be a growing sector of fish nutrition in the next decade.

9.4.1.10. Product Quality Feeds

Product quality feeds are those that are fed to fish to increase the quality of the product in the market. The development of product quality feeds is an area in which there is more potential than accomplishment in fish feed

formulation, but the development of finishing feeds for domestic livestock illustrates the potential for development of product quality feeds for fish. At present, product quality in salmonids and red sea bream is enhanced by dietary carotenoid supplementation. Other potential changes in product quality feeds include formulation adjustments to manipulate the lipid content and fatty acid composition, and percentage dress-out, and dietary supplementation with α-tocopheryl acetate to reduce oxidation of lipids during frozen storage (O'keefe and Noble 1978; Boggio *et al.* 1985). The flavor (odor) of fish can be modified by diet, especially the dietary lipid source. Texture has been reported to change slightly when the dietary lipid source is changed, but more research is needed to confirm this.

9.4.2. Practical Considerations in Diet Formulation and Manufacture

9.4.2.1. Ingredient Limits

In all fish feed formulations, limits are placed on the levels of certain ingredients, irrespective of cost. These limits may be upper limits, lower limits, or fixed limits, meaning that the level of an ingredient is set at a fixed percentage. Upper limits are often placed on ingredients which may contain antinutritional factors or toxicants or on ingredients which affect the palatability or pelletability. Lower limits are placed on ingredients which are desirable in the formulation despite their cost. For example, fish meal may be an expensive source of protein in feed formulations, and replacing fish meal in the formulations with rendered animal proteins may reduce the feed costs. Nevertheless, a lower limit of fish meal is generally made in trout feed formulations to guarantee performance. Fixed levels in fish feed formulations are used for vitamin and trace element premixes and for carotenoid pigments and binders. In practical fish feed manufacture, feed mills sometimes have ingredients in inventory that they wish to remove because they need space or because the ingredient is getting old. This would call for a lower limit to be placed on the level of the ingredient to force it into the formulation, even though the ingredient might not be the least expensive or best choice at that level.

9.4.2.2. Type of Diet

The type of feed particle being produced determines to a large extent the limits placed on the levels of many ingredients in a feeds formulation. Dry, compressed pellets, for example, must contain adequate levels of wheat by-products to allow the exterior surface of the pellet to gelatinize during pelleting. If pellets are to be crumbled and screened, additional considerations must be given during feed formulation to ensure pellet hardness.

In moist pellet or semimoist pellet formulations, the ingredients must be chosen with the water content of the pellet in mind. Ingredients that act as pellet binders, such as pregelatinized potato starch, must be included at levels that provide a sufficient binding capacity to produce a high-quality pellet. In formulations containing moisture levels higher than 30–35%, such as those containing liquefied fish products or ground fishery waste, additional binders must be used.

In extruded, dry pellet formulations, ingredients that can expand and thus produce low-density pellets must be included. Finally, in semipurified diets, highly refined ingredients must be used to produce diets suitable for experimental use.

9.4.2.3. Pellet Stability

In practical fish farming, pellet hardness and the absence of fines are qualities that are highly valued. Any fish farmer who has tossed a scoop of feed into the wind and gotten a faceful of dust understands this and will base his next feed purchase on this experience. Fish feed manufacturers usually screen pellets to remove fines before the pellets are sacked, but pellet disintegration can occur in bagged feeds during shipping and handling at the hatchery or fish farm. Pellet durability can be improved by including certain ingredients in the formulation, such as blood meal in compressed pellet formulations, that might not otherwise be included if least-cost formulation procedures are followed.

Pellets produced for slow-eating fish and crustacea must remain in water for hours without disintegrating. This requires special formulations and/or manufacturing processes. In eel diets, for example, pregelatinized potato starch is included in the formulation to enhance the water stability of the dough-like feed. Pregelatinized potato starch would not be included in these diets based on price and nutrient content alone.

9.4.3. Feed Formulation Calculations

9.4.3.1. Best-Buy Ingredients

Aside from the limitations discussed in previous sections, ingredients should be selected in a formulation on the basis of value. If several fish meals are available and suitable for use in a formulation, and the fish meals are equal in protein content and protein availability, the decision about which to select is simple: choose the least expensive. If, however, the meals differ in protein content or protein availability and price, calculations are required to determine which is the best value. For example, suppose that one wants to choose between white fish meal having a protein content of 64% and costing

$425 per metric ton (mt) and anchovy meal having a protein content of 69% and costing $450 per mt. To compare the two meals, compute the cost per kilogram protein as follows.

(1) White fish meal: $\dfrac{\$425/\text{mt}}{640 \text{ kg protein/mt}} = \$0.664/\text{kg protein.}$

(2) Anchovy meal: $\dfrac{\$450/\text{mt}}{690 \text{ kg protein/mt}} = \$0.652/\text{kg protein.}$

The results of this example show that anchovy meal, although it is more expensive per metric ton, is the best value, since the cost per kilogram protein is lower than that of white fish meal.

Often protein supplements are compared on the basis of cost per unit protein, or "protein unit." This simple calculation is made by dividing the price per metric ton by the percentage protein in the product. Some examples follow:

(1) White fish meal: $\dfrac{\$425/\text{mt}}{64} = \$6.64$ per protein unit.

(2) Anchovy meal: $\dfrac{\$450/\text{mt}}{69} = \$6.52$ per protein unit.

(3) Herring meal: $\dfrac{\$500/\text{mt}}{72} = \$6.94$ per protein unit.

(4) Meat and bone meal: $\dfrac{\$204/\text{mt}}{50} = \$4.08$ per protein unit.

Anchovy meal is the best value among the fish meals listed in the examples above, at least based on the price per protein unit. Meat and bone meal is the best value on a protein unit basis. Comparing ingredients in this fashion is valuable, but it does not take into consideration the quality of the protein, which is determined by its amino acid content and the apparent digestibility of protein and amino acids.

This process of comparing feed ingredients on the basis of cost per unit nutrient can be applied to any nutrient and to the energy content. For example, suppose that one wishes to determine if it is less expensive to add DL-lysine or increase the level of fish meal in a feed formulation to increase the lysine content. One approach is to calculate the cost of lysine in each product as follows.

(1) DL-Lysine costs U.S. $3.90 per kg and contains 98% DL-lysine, or 49% L-lysine. The cost per unit is

$$\frac{\$3900/\text{mt}}{49} = \text{U.S. } \$79.59 \text{ per unit lysine}$$

(2) Herring meal costs U.S. $500/mt and contains 5.36% lysine. The cost per unit is

$$\frac{\$500/\text{mt}}{5.36} = \text{U.S. } \$93.28 \text{ per unit lysine}$$

At these prices, it would be less expensive to add DL-lysine than to increase the level of herring meal in the formulation to satisfy the dietary lysine requirement of the fish.

The same calculation can be used to compare the costs of adding energy to a fish feed from various ingredients or to compare the costs of obtaining energy from dietary protein or lipid. For example, if herring meal costs U.S. $500/mt and contains 4717 kcal of digestible energy (DE)/kg, the cost per 1000 kcal is U.S. $0.106. Although fish oil contains more calories per gram than protein, the exact amount that is available to fish is uncertain. A close estimate based on the fat digestibility values of 90% given by Halver and Shanks (1969) is 8000–8100 kcal DE/kg. Using a value of 8000 kcal/kg, one can calculate the price below which fish oil must sell to warrant replacing herring meal with fish oil as an energy source.

(1) The price per 1000 kcal DE for herring meal is U.S. $0.106.
(2) The DE content of fish oil is 8000 kcal/kg, so $(0.106) × (8000) = 0.848/kg or $848/mt. This is the price one could afford to pay for fish oil to provide energy to the fish diet equivalent to the cost of energy from herring meal. If the price of fish meal increases, the cost advantage of providing dietary energy from fish oil increases.

9.4.3.2. Simultaneous Equations for Solving Least-Cost Analysis

Simultaneous equations can be used to solve simple feed formulations once the ingredients have been chosen. This method is preferable to trial-and-error substitutions, although an experienced formulator can produce a very good formulation in a short time by substitution and recalculation. The process of formulating a fish feed is divided into several steps (Cho *et al.* 1985). The first step is to define the nutrient levels desired in the feed (Table 9.11). In the example, we wish to formulate a feed containing 45% protein and 4000 kcal of digestible energy per kilogram of feed.

Table 9.11

Nutrient Levels Desired in Feed

Feed (kg)	Protein (kg)	Digestible energy (kcal)
100	45.0	400,000 (4000/kg feed)

Table 9.12

Composition of Dietary Ingredients on a Dry Matter Basis

Ingredient	Protein (%)	Digestible energy (kcal/kg)
Fish meal (herring)	70.0	4490
Poultry by-product meal	58.0	3320
Soybean meal	48.0	3224
Wheat middlings	17.0	1672
Fish oil	—	9000
Vitamin premix	—	—
Mineral premix	—	—
Choline chloride	—	—
Ascorbic acid	—	—

We will ignore, at this point, the essential amino acid levels of the formulation, although they can be included if we desired.

The second step in the formulation process is to choose ingredients and to list the protein and digestible energy content of each (Table 9.12). The third step is to list the ingredients which will be in the feed formulation at fixed levels and to calculate the contribution of each ingredient to the total desired levels of protein and digestible energy in the feed (Table 9.13). These values are then added, and the totals subtracted from the desired levels in the finished feed. In the example, the levels of soybean meal, poultry by-product meal, and vitamin and mineral supplements are fixed, while the levels of

Table 9.13

Contribution of Fixed Ingredients to Nutrient Content of Final Formulation

Ingredient	Percentage	Protein (kg)	Digestible energy (kcal/kg)
Poultry by-product meal	10	5.8	33,200
Soybean meal	10	4.8	32,240
Vitamin premix	2.0	—	—
Mineral premix	0.1	—	—
Choline chloride	0.5	—	—
Ascorbic acid	0.1	—	—
Total of fixed ingredients	22.7	10.6	65,440
Amount needed from ingredients	77.3	34.4	334,560

Table 9.14

Simultaneous Equations to Determine Levels of Wheat Middlings (WM), Fish Meal (FM), and Fish Oil (FO) Needed in Formulation

(1) WM + FM + FO = 77.3 kg feed
(2) 1672 WM + 4490 FM + 9000 FO = 334,560 kcal DE
(3) 0.17 WM + 0.70 FM + 0 FO = 34.4 kg protein
(4) From Eq. (1); let WM = 77.3 − FM − FO
Substitute Eq. (4) into Eqs. (2) and (3) as follows:
(a) 1672 (77.3 − FM − FO) + 4490 FM + 9000 FO = 334,560
(b) 0.17 (77.3 − FM − FO) + 0.70 FM + 0 FO = 34.4
Solve (a) and (b) to make Eqs. (5) and (6)
(5) 2818 FM + 7328 FO = 205,314.4
(6) 0.53 FM − 0.17 FO = 21.259
From Eq. (5); rearrange to make Eq. (7)
(7) FM = 205,314.4 − 7328 FO/2818 or 72.858 − 2.6 FO
Substitute Eq. (7) into Eq. (6)
(8) 0.53 (72.858 − 2.6 FO) − 0.17 FO = 21.259
Solve Eq. (8) for FO to obtain
FO = 11.2
Substitute 11.2 for FO in Eq. (5)
(9) 2818 FM + 7328 (11.2) = 205,314.4
Solve Eq. (9) for FM to obtain
FM = 43.7
Solve for WM using Eq. (4):
WM = 77.3 − 43.7 − 11.22 = 22.4

Solution:
FM = 43.7 kg/100 kg diet
WM = 22.4 kg/100 kg diet
FO = 11.2 kg/100 kg diet

fish meal, wheat middlings, and fish oil in the formulation are variable. The fourth step is to determine the appropriate levels of the various ingredients in the formulation using simultaneous equations (Table 9.14).

The solution of the simultaneous equations yields the following: fish meal, 43.7 kg/100 kg feed; wheat middlings, 22.4 kg/100 kg feed; and fish oil, 11.22 kg/100 kg feed. The last step is to check the final feed levels of protein and digestible energy to ensure that the desired levels are present (Table 9.15). The levels of individual essential amino acids can also be calculated at this point to make certain that the levels in the feed meet or exceed the dietary requirements of the fish.

9.4.3.3. Linear Programming

Computers are used in the feed industry to calculate least-cost formulations. The process by which this is done is called linear programming, which

Table 9.15

Recalculation of Feed Formulation to Check Nutrient Levels

Ingredient	Amount (kg)	Nutrient contribution	
		Protein (kg)	Digestible energy (kcal)
Fish meal	43.7	30.6	196,200
Poultry by-product meal	10	5.8	33,200
Soybean meal	10	4.8	32,240
Wheat middlings	22.4	3.8	37,500
Fish oil	11.2	—	100,800
Vitamin premix	2.0	—	—
Mineral premix	0.1	—	—
Choline chloride	0.5	—	—
Ascorbic acid	0.1	—	—
Total	100	—	—

involves the simultaneous solution of a series of linear equations. Linear programming has been used in animal feed formulation for over 40 years and is used almost exclusively in modern feed formulation in agriculture today. In the past, linear programming required access to a mainframe computer and specialized knowledge in mainframe use, i.e., programming ability. While the actual computer time required to arrive at a least-cost formulation was short, the time required for data input and setting up was lengthy. For simple formulations or for small feed manufacturers, hand calculations were often more practical. Today, personal computers can be used to "least-cost" feed formulations.

Linear programming, applied to least-cost feed analysis, is used to calculate the combination and levels of ingredients that provide the desired nutrient content of the diet at the least cost. To do this, the computer must have access to data on the nutrient content of each potential feed ingredient, which are obtained from feed composition tables or from analyses at the mill itself. The cost of each ingredient must be provided, along with the nutrient specifications of the formulation desired. Ingredient limits, i.e., maximum and minimum levels allowed in the formulation, must be imposed. At this point, the computer is told to begin, and the answer (output) rapidly appears. Outputs vary with different computer software but generally include, in addition to the least-cost formula in which ingredients and levels to add to the mixture are listed, listings of calculated levels of nutrients in the formulation and information on the effects of the restrictions placed on the levels of individual ingredients or desired nutrient levels on the cost of the

diet. Information on the effects of restrictions placed on ingredient levels or nutrient levels is useful to the feed formulator. It shows the cost of maintaining the level of a nutritional parameter, such as protein, and the savings that would result from relaxing the restriction, say from 45 to 44%, in a salmonid diet. This output is sometimes called dual activity. Information on the effects of relaxing upper and lower restrictions on the levels of individual ingredients is shown in another output sometimes called reduced costs. If, for example, the upper limit on soybean meal was set at 10% in a trout diet formulation, and the feed formulation obtained from least-cost formulation contained the maximum, i.e., 10%, in the reduced-cost output, there would be a value showing the savings that would result by relaxing this restriction to allow higher levels of soybean meal to be used. Of course, the decision to limit the soybean meal level to 10% of the formulation may have been made for other reasons, such as availability, pellet texture, and palatability concerns. The nutritionist must decide what to do in such a situation, but the reduced cost output clearly shows what changes could be made in the formulations to arrive at a less expensive feed formulation. It is common for feed formulations to make slight adjustments in nutrient specifications or in upper or lower limints on ingredients to arrive at a final formualtion.

To illustrate how the output from linear programming can aid the nutritionist, two cases are presented in which the restrictions placed on the minimum specification for dietary protein and lipid are changed in a salmon feed formulation. In Diet 1 (Table 9.16), the minimum level of protein is set at 45%, with a maximum of 47%, while only a minimum dietary lipid level is set (23%). The least-cost solution for this diet formulation is shown in Table 9.16. The computer must select the best combination of ingredients, chosing among corn gluten meal, fish meal (anchovy), meat and bone meal, soybean meal, wheat middlings, ground whole wheat, poultry by-product meal, and fish oil. Each of these ingredients has some restriction placed on its minimum level, its maximum level, or both. Five additional ingredients, e.g., vitamin premix, trace mineral premix, choline chloride, ascorbic acid, and astaxanthin, are fixed in the formulation, meaning that the minimum and maximum level are the same. In other words, the computer cannot change the levels of these ingredients. Restrictions on each ingredient are also shown in Table 9.16; the operator places these restrictions.

The feed formulation chosen by the computer costs $776.79/mt, using prices entered by the operator into the database used by the computer. Table 9.16 also shows the constraints of the formulation, simplified in this example to show only constraints associated with the dietary nutrient specifications placed on the formulation by the operator. This shows that the computer was constrained by dietary protein and lipid levels, and the output suggests that the price of the feed would decrease if these restraints

Table 9.16

Diet 1: Least-Cost Formulation and Nutrient Analysis for Salmon Feed[a]

Ingredient	Least-cost formulation[b]		
	Minimum	Actual	Maximum
Fish meal	32.00	32.00	40.00
Corn gluten meal	3.00	3.00	10.00
Meat and bone meal		10.00	10.00
Soybean meal (48 CP)		5.137	20.00
Wheat middlings	5.00	5.00	15.00
Wheat, ground grain	10.00	10.00	
Poultry by-product meal	5.00	17.73	20.00
Fish oil	12.00	15.98	
Ascorbate-2-phosphate	0.10	0.10	0.10
Vitamin premix	0.50	0.50	0.50
Trace mineral premix	0.10	0.01	0.10
Choline chloride	0.35	0.35	0.35
Astaxanthin	0.10	0.10	0.10
	Nutrient analysis		
	Minimum	Actual	Constraint (decrease)
Crude protein	45.00	45.00	3.024
Crude fat	23.00	23.00	5.477
Crude fiber		2.073	
Phosphorus (total)		1.963	
Phosphorus (available)	0.600	1.332	
Lysine		2.927	
Methionine		1.089	

[a] With minimum dietary protein and lipid set at 45 and 23%, respectively.
[b] Cost is $776.79 per metric ton.

were relaxed. The larger number shown under the heading "Constraint (decrease)" for lipid level indicates that relaxing the minimum lipid specification for the diet by 1% would result in a larger savings than relaxing the dietary protein by 1%. Another output, not shown, shows the operator which ingredients were not chosen by the computer because they were too expensive. This output also shows the what the price of the omitted ingredients would have to be for them to be selected for the formulation.

A second formulation was run, this time with the restrictions on the minimum levels of protein and lipid relaxed to 44 and 20%, respectively. The computer selected a slightly different formulation, keeping the same

Table 9.17

Diet 2: Least-Cost Formulation and Nutrient Analysis for Salmon Feed[a]

Ingredient	Least-cost formulation		
	Minimum	Actual	Maximum
Fish meal	32.00	32.00	40.00
Corn gluten meal	3.00	3.00	10.00
Meat and bone meal		10.00	10.00
Soybean meal (48 CP)		**16.78**	20.00
Wheat middlings	5.00	5.00	15.00
Wheat, ground grain	10.00	10.00	
Poultry by-product meal	5.00	**7.39**	20.00
Fish oil	12.00	**14.68**	
Ascorbate-2-phosphate	0.10	0.10	0.10
Vitamin premix	0.50	0.50	0.50
Trace mineral premix	0.10	0.01	0.10
Choline chloride	0.35	0.35	0.35
Astaxanthin	0.10	0.10	0.10
	Nutrient Analysis		
	Minimum	Actual	Constraint (decrease)
Crude protein	44.00	44.00	0.568
Crude fat	20.00	20.00	1.171
Crude fiber		2.210	
Phosphorus (total)		2.391	
Phosphorus (available)	0.600	1.405	
Lysine		2.871	
Methionine		1.019	

[a] With minimum dietary protein and lipid changed to 44 and 20%, respectively. Changes are in boldface type.

[b] Cost is $735.24 per metric ton.

ingredients but changing their percentage in the formulation, at least for those not fixed or already selected at the minimum restricted level (Table 9.17). The cost of this formulation was $735.24/mt, a savings of $41.55/mt over the first formulation. Again fish meal, corn gluten meal, wheat middlings, and ground whole wheat were included at the minimum percentage allowed by the operator. The computer lowered the percentage of poultry by-product meal, increasing soybean meal. Fish oil was reduced by a little over 1%.

Similar changes can be made in the dietary specifications for nutrients and in the limits (lower and upper) for each ingredient. Through subtle

manipulation of these specifications, and by using experience, skillful operators can take full advantage of least-cost feed formulations, keeping in mind the need for the final formulation to be palatable, pelletable, and so on.

9.5 Diet Manufacture and Storage

Diet manufacture and storage is a complex process and it is beyond the scope of this chapter to cover all phases of the subject in comprehensive detail. The information presented here is intended to provide the reader with an overview of the steps and processes in diet manufacture. This will help the biologist and fish farmer appreciate the complexity and limitations of commercial diet manufacture, as well as understand the manufacturing differences between types of fish feed pellets. Hopefully, the information will show the reader how best to utilize the products available. Those interested in more detail on feed manufacture should consult Barrows and Hardy (2000) and McEllhiney (1994).

The basic steps in diet manufacture are grinding, mixing, conditioning, pelleting, cooling (or freezing), top-dressing, sacking, storing, and shipping (Fig. 9.2). These steps are discussed in sequence, and where different steps are required to produce different types of pellets, these are noted. Ingredients arrive at feed mills either in sacks or in bulk, and in both dry and liquid form. Ingredients should be examined for condition on arrival, and samples taken for analysis and dated. Drugs and medications must be segregated. Ingredients must be stored to protect against contamination, moisture, and pest damage. Each ingredient should be used in the order of batch delivery to prevent deterioration associated with long storage. In other words, feed ingredient inventory should be rotated, similar to food in a grocery store. In most modern plants, bulk ingredients are cleaned using a large rotating cylindrical screen before being conveyed to storage bins.

9.5.1. Grinding

Many ingredients arrive at feed mills in a coarse form and need to be ground before being used. When ingredient particle size is not uniform and sufficiently small, mixing inefficiencies and blockage of dies may occur during pelleting. Grinding increases the surface area of ingredient particles, thus facilitating mixing and pelleting. Various kinds of grinders reduce particle size by impact [hammer mills, pulverizers, and attrition mills (Fig. 9.3)], cutting (rotary cutters, roller mills, and attrition mills), and crushing (roller mills). Particles are reduced to various sizes, depending upon the process by which the feed will be pelleted.

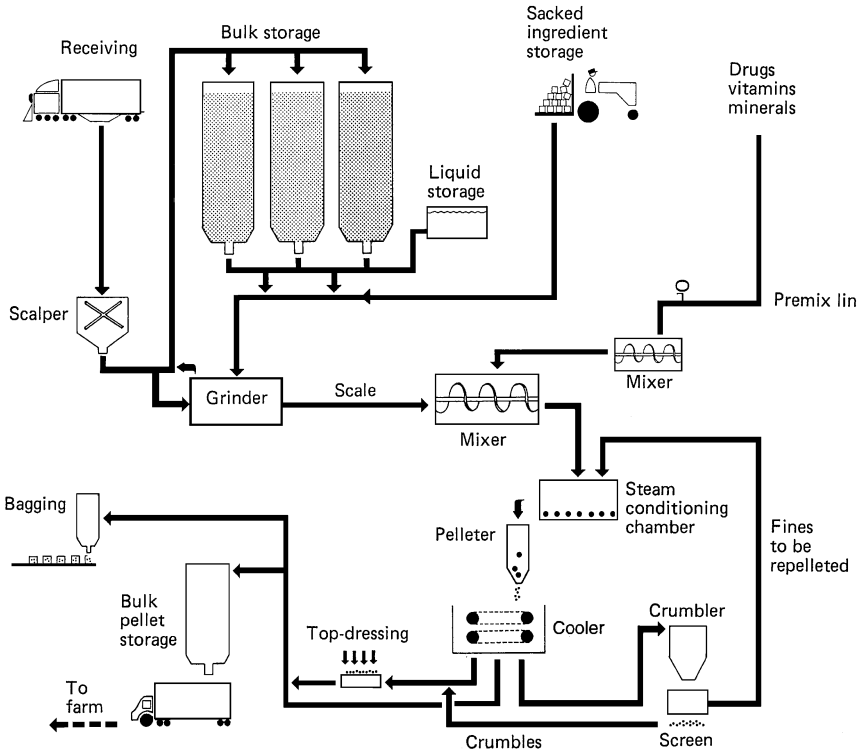

FIG. 9.2

Simplified flow diagram of compressed pellet manufacturing.

Particles that will pass through a 40–45 U.S. standard sieve are small enough to manufacture pellets as small as $\frac{3}{64}$ inch (1.2 mm), although further reduction may be necessary for smaller pellets and for larval feed production. The general rule is to have ingredients ground to one-third the diameter of the die holes, and some feed mill operators prefer that feed mixtures are ground to one-fourth the diameter of the die holes.

Grinding is an expensive part of the feed manufacturing process, and overgrinding to produce very small particles adds significantly to the energy cost of manufacturing feeds. Different types of particle size reduction equipment vary considerably in initial cost and in operating cost, but the primary factor affecting the choice of equipment should be the range of ingredients that the equipment will be required to grind. Ingredients with high lipid content are more difficult to grind than low lipid ingredients.

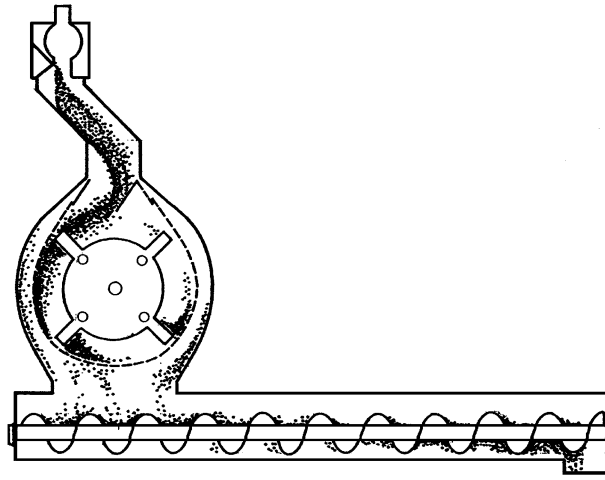

FIG. 9.3

Hammer mill.

Fish meal and other animal protein meals usually contain 8–12% lipid and, thus, tend to plug the screens of hammer mills. Other factors, such as the feed rate of the ingredient into the grinding machinery, affect the tendency of the grinder to plug, but the primary factor causing plugging is the lipid content. Air-swept pulverizers are desirable for fine grinding of high-lipid ingredients, because air pressure is used to control grinding, rather than a screen. Air-swept pulverizers can be identified by the lack of retaining screens and the high volume of air that passes through the grinding chamber. In general, plant-derived feed ingredients, such as soybean meal, corn gluten meal, and wheat by-products, are low-lipid ingredients that are relatively easy to grind. High-lipid ingredients can be combined with low-lipid ingredients and ground together.

9.5.2. Mixing

The second step in diet manufacture is mixing the ingredients to produce a homogeneous blend. If feed ingredients are not mixed for a sufficient period of time, or if they are mixed for too long, particle segregation can occur, resulting in a nonhomogeneous blend. Microingredients (vitamins, minerals, drugs, carotenoid pigments) are premixed separately with a diluent, such as wheat flour, before being mixed with other dietary ingredients. The mixing procedure should result in a blend from which all of the pellets

contain the same proportion of ingredients as the formulation. Ingredients are generally added to the mixer in a predetermined sequence to ensure adequate mixing. Dry ingredients are first combined, with dry premixes added in the middle of the sequence. If particle size is about the same for each ingredient, particle segregation will be minimized. After sufficient mixing of dry ingredients, liquid ingredients are added and mixing continues.

The amount of time that a feed is mixed is important to obtain a uniform blend of ingredients. Mixing and segregation of particles of different sizes or densities are cyclical. This means that in the initial period of mixing, particles combine, but with further mixing, they segregate. If mixing continues, particles combine again. It is therefore important to know the proper amount of time for mixing with each type of mixer and type of feed blend. Manufacturers of high-quality fish feeds will determine the efficiency of their mixer and mixing procedures. This is done by collecting samples at different times from the mixer and analyzing the samples for the concentration of a marker, such as salt, silica, or an inert tracer. In this manner, a manufacturer can assure that a complete mix of ingredients has occurred.

In the past, mixing was done with shovels on the floor with a set number of turns per batch. Most mixing today is done mechanically with batch or continuous mixers. Batch mixers are generally cylinders or cones in which the feed ingredients are mixed with moving paddles, augers, or ribbons. Continuous mixers are similar, except that the design of the moving parts is such that material moves through the mixer as it is being mixed. Types of mixers include horizontal ribbon mixers, vertical mixers, Nanta mixers, and turbine mixers (Figs. 9.4 and 9.5). For laboratory use, dough mixers are

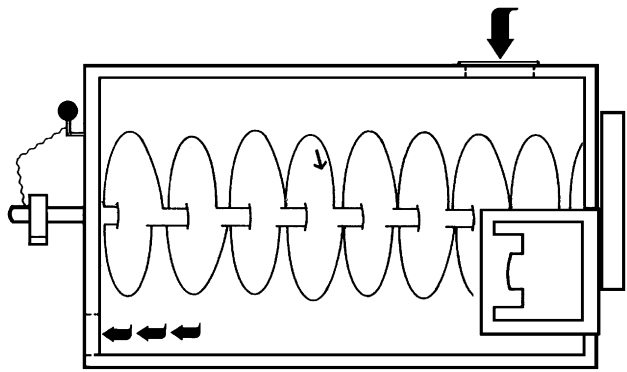

FIG. 9.4

Horizontal mixer.

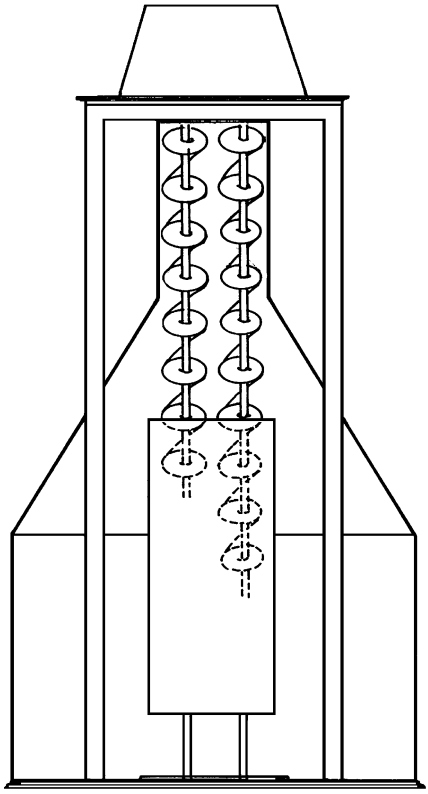

FIG. 9.5

Vertical mixer.

appropriate. Mixing microingredients may require the use of specially designed mixers, such as twin-shell blenders, to disperse the microingredients in a carrier, such as α-cellulose or wheat flour.

9.5.3. Conditioning and Expansion

Conditioning is a term used to describe processes used to prepare a feed mixture for pelleting and includes thermal and physical processing. Thermal processing is generally accomplished by the addition of steam in a conditioning chamber. The chamber may be pressurized or it may be open to the atmosphere. Conditioning chambers contain agitators that mix and work the feed mixture as steam is added. Besides ensuring that

added moisture is thoroughly mixed with the feed mixture, agitators activate some ingredients, such as wheat gluten. Feed mixtures remain in conditioning chambers for a short time (~30 sec) if the mixture will be steam (compression) pelleted, but may remain for 2–3 min or longer prior to extrusion pelleting. The longer period results in a higher degree of starch gelatinization.

Expansion is a conditioning step that occurs after mixing but before compression pelleting. The process involves steam injection and mixing in a preconditioning chamber, followed by pressure (shear) being applied along a barrel. The mixture is then forced through a narrow gap created by the presence of a cone in a tapered outlet of a chamber. Heat from steam, pressure (shear), and frictional energy generated as the feed mixture squeezes through the gap causes starch gelatinization. As the pressure is lost when the mixture exits the gap, moisture is lost. The mixture is then conveyed through a steam (compression) pelleting system, and the resulting pellets are identical to steam pellets, except for the degree of gelatinization, which also affects the pellet density and the amount of oil that can be top-dressed. Pellets made by this process can be top-dressed to achieve up to 22% total fat.

9.5.4. Pelleting

The objective in pelleting is to convert the homogeneous blend of ingredients into durable particles having physical characteristics that make them suitable for feeding. There are many ways to produce feed pellets. Each feed manufacturer has developed particular methods to increase the quality or decrease the cost of their feed. We present a generalization of each processing method, but the reader should be aware that there are many small modifications in the processes described below that feed manufacters utilize.

9.5.4.1. Compressed Pelleting

Compressed pelleting is a process which forces a feed mixture, which has been exposed to dry steam for about 5–25 sec to increase the temperature to about 85°C and the moisture to about 16%, through holes in a metal die by the action of a roller located inside the die (Fig. 9.6). This process is also known as steam pelleting, due to the use of steam to precondition the mix prior to compression. The combination of heat, moisture, and pressure forms the mixture into a compressed pellet (bulk density, 0.5–0.6 g/cm^3) in which gelatinization of the starch occurs. As the pellets emerge from the outside surface of the die, they are cut off by a stationary, adjustable knife to the desired length.

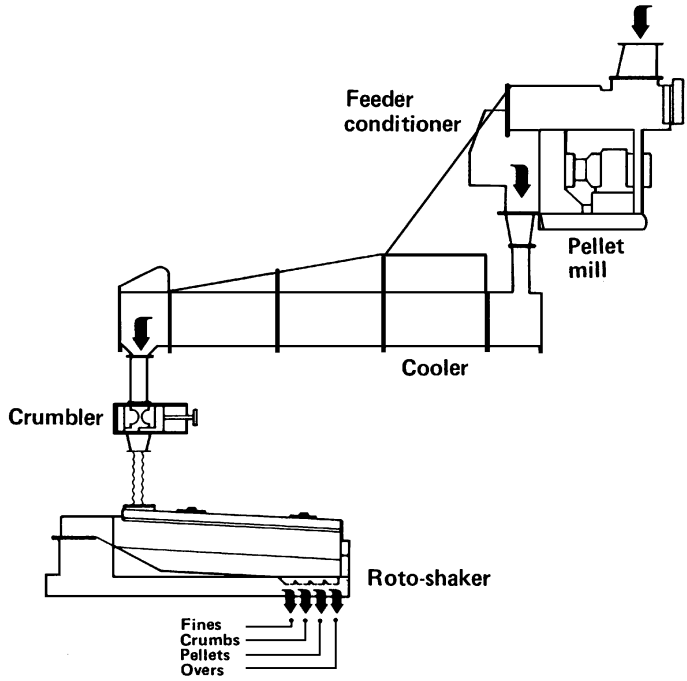

FIG. 9.6

Equipment used to make pellets and crumbles.

Pellet quality is influence by several factors, including fat level, moisture, and humidity. The fat level of the mixture should be no lower than 2–3% to lubricate the holes in the die and to reduce dustiness and no higher than 8–10% to avoid excessive die lubrication causing insufficient compression of the feed mixture. The moisture level is critical in that if affects the pellet quality. Either insufficient or excessive moisture in the feed mixture reduces the pellet hardness. Pellets made with insufficient moisture are dry and crumbly, while excessive moisture results in soft pellets due to insufficient compression. Changes in atmospheric humdity can be enough to change the pellet quality, and the pellet machine operator must make adjustments in the amount of steam added to the feed and/or the rate at which the feeds mixture is introduced into the steam chamber to compensate for changes in the atmospheric humidity. The addition of steam improves pellet quality by wetting and softening the feed particles, thereby improving compression, and by increasing the gelatinization of raw starch, which helps bind the ingredients together in the pellet. The lubricating effects of fat and moisture together

lower the electrical power required to operate a pellet mill and extend the operating life of the die. An experienced pellet mill operator becomes familiar with various feed mixtures and manufacturing conditions and makes the adjustments necessary to make high-quality compressed pellets.

9.5.4.2. Extruded Dry Pelleting

Extruded pellets are made in the same general way as compressed pellets, but the use of different dies and physical conditions results in a very different product. This process is more versatile than compression pelleting, thereby allowing for the control of pellet density, which affects bouyancy in water, and for the addition of very high lipid levels. However, the versatility of the extrusion process also makes it more expensive than steam pelleting. Extrusion technology is used to produce snack foods, such as crackers, breakfast cereals, and pet foods for dogs, cats, and caged birds.

The equipment functions by increasing the temperature of the feed mixture to 125–150°C in a pressurized conditioning chamber and increasing the moisture content to 20–24%. The combination of these two steps gelatinizes the starch. The preconditioned mixture is then worked into a dough-like consistency in a long barrel by an auger that has tapered flights, which increases the pressure on the mixture as it moves down the barrel toward the die. Pressure is sufficient to convert water vapor (steam) back into a liquid. As the pellet leaves the die, the reduction in pressure results in a sudden expansion of water vapor in the pellet, causing an air pocket to form. Altering the formulation and the processing conditions changes the bulk density of the feed so that it floats on, sinks slowly in, or sinks quickly in water. Bonds are formed within the gelatinized starch, which results in a durable, water-stable pellet. After cooling and drying, the pellet density is typically 0.25–0.3 g/cm^3. Many fish farmers prefer to use floating feed to allow them to observe the feeding activity of their fish.

Production of floating pellets requires higher temperatures and pressures than production of compressed (steam) pellets. The additional cost of producing floating feeds must be carefully considered. Pond production systems benefit from the use of floating feeds, because, as mentioned above, the feeding activity of the fish can be observed. This has benefits with respect to pond management and reduces feed waste due to overfeeding.

Cooking extruders are available with both single- and twin-screw configurations. The self-wiping design of screws used in the twin-screw configuration allows for greater flexibility in manufacturing, resulting in pellets that will absorb higher amounts of lipid compared to pellets produced with single-screw extruders. In European fish feed production, mainly twin-screw extruders are used, while in the United States, single-screw extruders are more common.

An important advantage of the cooking-extrusion process is that it increases the digestibility of carbohydrates in the feed mixture as a result of the exposure of the feed mixture to high temperatures and pressures. The total energy available to fish is increased by the extrusion process, although for some species of fish, such as salmonids, limits must be placed on the proportion of dietary energy coming from carbohydrates. Cooked carbohydrate also serves as an effective binder in extruded and expanded pellets, thus eliminating the need to include undigestible binders in the feed formulation. Gelatinized starch resists disintegration in water, so extruded pellets are more water-stable than steam pellets, making extruded pellets better suited for use with slow-feeding species and in water recirculation systems. Extruded pellets are very hard, unlike steam pellets, and this reduces the proportion of fines produced from extruded pellets during shipping and handling. Spraying oil onto the surface of pellets, e.g., top-dressing, is better suited to extruded pellets than to steam pellets, making this another example of the advantages of extruded pellets for many aquaculture situations.

9.5.4.3. UPC Pelleting

A new system of feed pelleting has been introduced which combines some aspects of compressed pelleting and cooking extrusion. This system is known as the universal pellet cooker (UPC), and the pelleting equipment resembles cooking-extrusion equipment. Basically, the UPC process involves enhanced preconditioning for 2–3 min, during which the addition of steam results in 40 to 50% starch gelatinization. Less steam and water are added during preconditioning than in cooking extrusion, resulting in a feed mixture with 16 to 18% moisture. The other major difference with the UPC system is the modified action in the barrel of the pelleter, where the auger, or rotor, turns two to three times faster than in a conventional cooker extruder, which increases production levels to 18–20 mt/hr, nearly the same as with steam pelleting. The fast-turning rotor adds much more energy to the feed mixture, and the frictional energy associated with the faster-turning rotor and lower moisture in the feed mixture further gelatinizes the starch, to 60 to 80%. In addition, the combination of frictional energy and steam pasteurizes the feed mixture, unlike compressed pelleting, even when annular gap expansion is used. Because the moisture content of the feed mixture is lower than in cooking extrusion, there is less water entrapped in the feed mixture and, thus, less expansion when pellets exit the die. This results in a higher bulk density for UPC pellets than for cooking-extrusion pellets, but the UPC system can produce pellets from 400 to 600 g/liter, depending on how it is operated (Fig. 9.7). Pellets can be dried in a cooler, rather than a

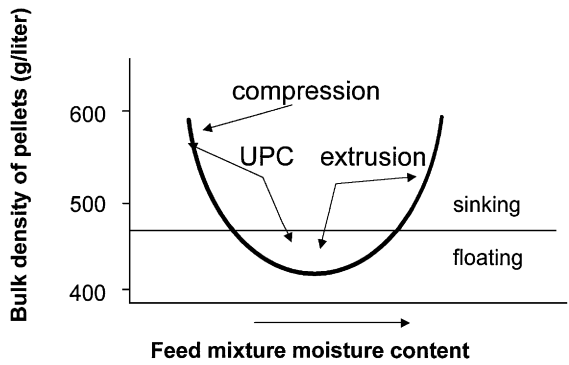

Fig. 9.7

Schematic relationship among feed pelleting methods, moisture content, and bulk density of pellets.

dryer, and this reduces the amount of equipment and lowers the cost of production. The UPC produces higher-density pellets than cooking extrusion, but without the glazed surface associated with compressed pelleting. Thus, high fat levels, up to 30%, can be reached in the dried pellets by top-dressing. An additional benefit of the UPC system is that dense particles for starter feeds can be produced simply by turning the external knife faster. This eliminates conventional crumbling by rollers and the inevitable production of fines. As is the case with cooking extrusion, gelatinized starch acts as a pellet binder.

9.5.4.4. Cold Pellet Extrusion

Modified noodle extruders are used to manufacture semimoist pellets. By varying the moisture content and by carefully selecting ingredients with binding activity, a stable, durable moist pellet can be produced. In contrast to other methods of pellet formation, no thermal activity is involved in cold extrusion. The mixture is not heated by steam before extrusion, but rather the wet mixture is forced through a plate with holes drilled into it. The resulting noodles are cut into appropriate lengths by an external cutting blade as they leave the die. After extrusion, the pellets are screened, "quick-frozen," sacked, and stored. Soft moist pellets not requiring frozen storage due to the presence of antimicrobial agents are made either by extrusion through a noodle machine or by compressed or extruded pelleting without subsequent drying. The overall process is depicted in Fig. 9.8.

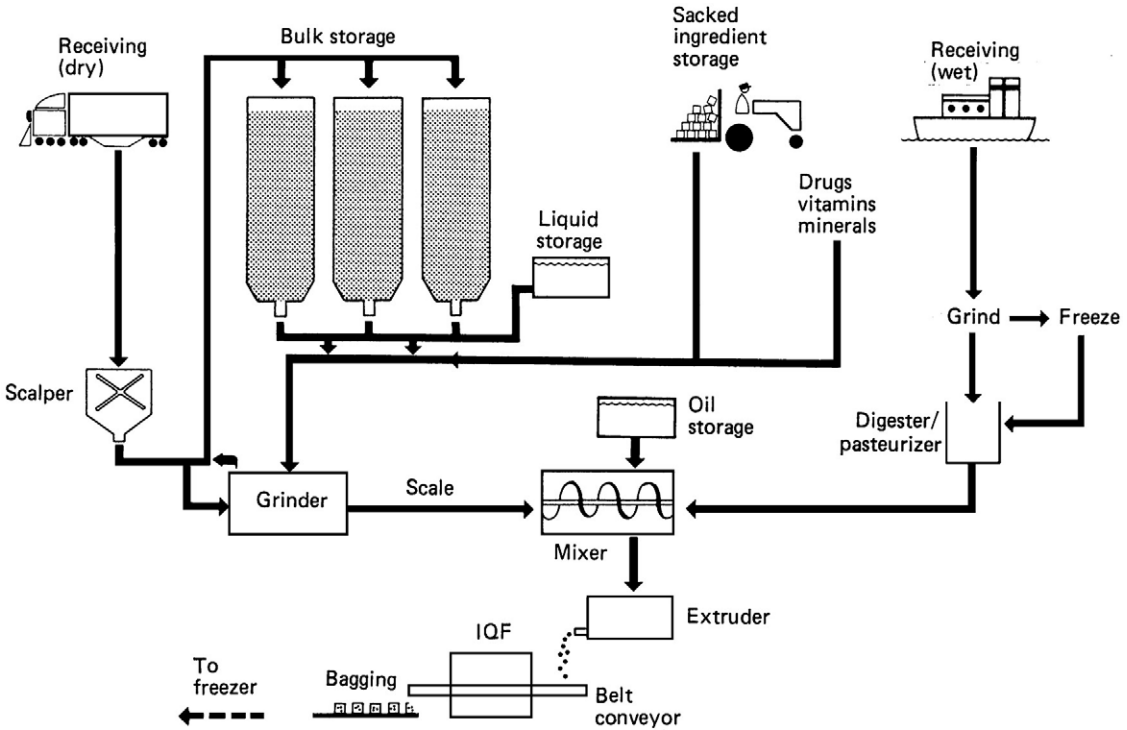

FIG. 9.8

Simplified flow diagram of moist pellet manufacturing.

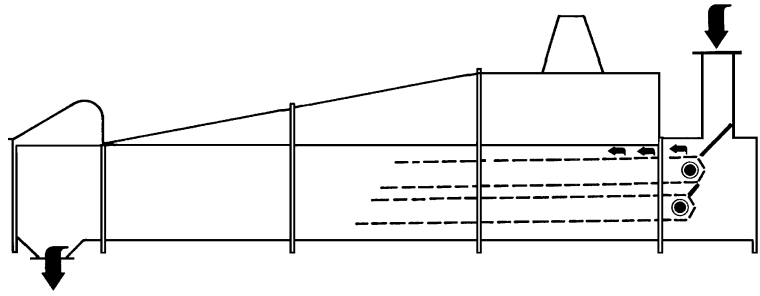

FIG. 9.9

Horizontal cooler.

9.5.5. Cooling and Drying

Immediately after manufacturing, the pellets made by compression pelleting are cooled and dried by passing them through a cooler–dryer (Fig. 9.9). Cool air is blown through the pellets, which are spread thinly on a moving belt in a horizontal cooler. Vertical coolers drop hot pellets through a cooling tower. The pellets are hot (approximately 90°C) when leaving the pellet mill and this heat facilitates drying. Passage through the cooler–dryer takes 5–15 min, and when the pellets leave the cooler–dryer, they are at ambient temperature and about 10% moisture. For laboratory-scale pelleting, cooling and drying can be accomplished by spreading the pellets and blowing air over them with a fan.

Extruded pellets contain more moisture than compressed pellets and, therefore, need to be heated to reduce moisture to 10% or less. Extruded pellet dryers are generally large pieces of equipment through which pellets are conveyed. Hot air is blown over and through the pellets in a crosscurrent fashion. Drying takes approximately 30 min.

To summarize pelleting methods, each manufacturing process results in pellets having specific physical properties (or, more accurately, a range of properties). The properties important to feed producers and fish farmers are listed in Tables 9.18 and 9.19.

9.5.6. Crumbling and Screening

Dry pellets are crumbled by passing the cooled, dried pellets through the corrugated rollers of a crumbler. In a crumbler, one roller is driven by a motor and the other spins freely (Fig. 9.10). Generally, particles smaller than $\frac{1}{8}$ inch (3–4 mm) in diameter are produced by crumbling $\frac{5}{32}$- or $\frac{3}{16}$-inch pellets. The objective in crumbling is to maintain a high rate of production

Table 9.18

Physical Qualities of Various Types of Fish Feed Pellets

Physical quality	Compressed	Annular gap	Extruded	UPC
Density (g/liter)[a]	590	680	400–550	400–600
Maximum temperature (°C)	95	135	150	150
Time exposed to steam (min)	<1	<1	2–5	2–3
Starch gelatinization (%)	<40	65–70	>80	60–80
Maximum fines (%)	2–3	<1	<1	<1
Maximum fat level (%)	18	25	38	30

[a] 480 g/liter is the breakpoint for pellets to float; higher-density pellets sink, and lower-density pellets float in fresh water.

of smaller particles without producing excessive amounts of fines. This is controlled by adjusting the speed of the rollers and the distance between them. The crumbles are screened to appropriate feed sizes using a shaking, vibrating screen. Even when conditions are optimized, a significant percentage of the starting weight of pellets is reduced to fines, as high as 40–50%. Fines produced during crumbling of steam-pelleted feeds are usually added back to feed mixtures for incorporation into pellets. Fines produced from pellets made by extrusion, expansion, or UPC can also be added back to feed mixtures, but at a lower percentage than fines from steam pellets, because the carbohydrate fraction has already been cooked (gelatinized). Incorporation of a high percentage of fines from pellets containing cooked carbohydrate lowers pellet strength and water stability.

Table 9.19

Attributes of Pellets Produced by Various Manufacturing Processes

Attribute	Compressed	Expanded	Extruded	UPC
Starch digestibility[a]	Low	High	High	Medium to high
Pellet buoyancy	Sinking	Sinking	Floating/sinking	Floating/sinking
Water stability	Low	Low	High	High
Durability	Low	Medium	High	High
Nutrient destruction[b]	Low	Low	Medium	Medium/low
Cost of pelleting	Lowest	Low	Highest	Medium

[a] Starch digestibility is a function of the degree of gelatinization.

[b] Nutrient destruction during pelleting is caused by high temperature, high pressure, and the length of time that the feed mixture is exposed to a high temperature and is most important for certain vitamins and the carotenoid pigment, astaxanthin.

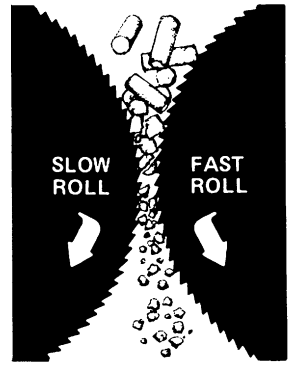

FIG. 9.10

Action of rollers in crumbler.

9.5.7. Coating (Top-Dressing)

The total amount of lipid required in feed formulations cannot usually be included in the mixture being pelleted, due to processing limitations, except in the case of twin-screw extrusion. High-lipid mixtures simply do not pellet; the lipid lubricates the mixture, limiting compression. Hence, lipid is added after pelleting, either by conveying the pellets through a continuous oil spraying chamber or in batch systems. Very high pellet lipid levels (>30%) require that the oil is added to pellets in a vacuum chamber. Top-dressing provides the following benefits: (1) high lipid levels can be achieved; (2) heat-sensitive substances, such as enzymes, pigments, and vitamins, can be added after pelleting; and (3) feed palatability can be increased by coating with palatability-enhancing substances.

9.5.8. Shipping and Storage

After pellets have been cooled, dried, screened, and top-dressed, they can be transferred to bins for storage and bagging. Feed can be delivered to farms in bulk by truck, transferred by auger to storage bins at the farm, and fed. Bagged, or sacked, feed is generally placed on pallets, wrapped in plastic, and shipped. This prevents excessive feed handling, which can break pellets and tear bags. Pellets containing high lipid levels are less durable than low-lipid pellets and, therefore, must be handled with care. Sacked feed should not be thrown or walked on. Sacks should not be stored more than 10 sacks high. It is good practice to color-code the tags used to identify feed sizes and formulas.

The maximum storage time for pelleted feeds after manufacture depends on many factors, including the type of manufacturing, formulation, use of antimicrobial compounds, storage conditions, and source and quality of dietary fat (O'Keefe 2000). Generally, dry feeds should be used within 90 days, while moist feeds should be used within 60 days. Longer storage times may result in nutrient loss and/or rancidity problems. Some specialty feeds are manufactured for longer storage times and are correspondingly more expensive. The storage life of feeds can be reduced by abusive storage conditions. For maximum storage life, dry feeds should be kept in areas where temperatures do not exceed 20–22°C and the relative humidity remains below 75%. Semimoist feeds requiring frozen storage should be kept at −20°C and thawed just before use. Dry feeds (<12% moisture) should not be stored in a freezer. This may seem counterintuitive, but freezing can actually accelerate the deterioration of dry feeds. Any sign of mold in a sack of feed is grounds to discard the entire sack. If a feed smells like drying paint, the lipids are oxidizing and the feed should be discarded. If feed has been kept past its recommended storage time, it should be discarded.

9.5.9. Semipurified Diets

Semipurified diets are used by researchers to determine nutrient requirements, and the cost of such feeds is much too high to consider other uses. The ingredients in semipurified diets are highly refined, allowing the researcher to formulate deficient diets by eliminating an essential nutrient from the formulation. The other ingredients generally contain only trace amounts of vitamins, minerals, and, in some cases, specific amino acids. Semipurified diets for fish have all evolved from diet H440 (Table 9.4). Various researchers have improved upon this formulation, taking into account new information on the nutrient requirements of fish, and modified it to make it suitable for new fish species. Semipurified diets usually contain vitamin-free casein, gelatin, dextrin, fish oil, carbohydrate binders, a vitamin premix, a mineral premix, and water.

Several methods can be used to manufacture semipurified diets. An early method involvs dissolving gelatin in hot water, adding liquid choline chloride and fish oil, and then combining this wet mixture with the dry ingredients. After further mixing, the blend is transferred to pans, cooled, and pelleting using a cold extruder. The pellets contain over 20% water and must be stored frozen. Laboratory-sized compression pellet mills can also be used to pellet semipurified diets, but care must be taken to avoid heat generation, which causes the dies to plug. Diets suitable for conducting requirement studies with crustacea are based on vitamin-free casein (Conklin *et al.* 1980) or crab protein concentrate (Castell *et al.* 1989).Further information on

the use of semipurified diets in crustacean studies is given by D'Abramo and Castell (1997).

Other protein sources have been used in place of casein and gelatin in semipurified diet formulations, specifically to produce amino acid-deficient diets. Whole-egg protein, wheat gluten, fish protein concentrate, and semipurified fish protein have specific applications in experiments in which casein and gelatin are not appropriate protein sources.

9.5.10. Microdiets: Larval Feeds

The manufacture of larval feeds is a rapidly changing field. Many good feeds exist, but much improvement is still needed. Manufacturing larval feeds is much more difficult and time-consuming than manufacturing production feeds. This is due to the extremely small size of the feed particles, the relatively undeveloped digestive system of the larvae, and the needs for high palatability, water stability, and nutrient stability of the particles. The size of larval feeds should be in the range of 5–150 μm for filter feeders and 40–700 μm for particle feeders. These very small particles have very high surface area-to-volume ratios; hence the rate at which water-soluble nutrients leach from the particles is rapid. A high nutrient stability, which means a slow leaching rate, is difficult to achieve with microdiets and is the focus of several manufacturing methods that are discussed later in this section. The production of high yields of particles of the correct size with the correct physical qualities (i.e., buoyancy) is also the focus of several production methods. There are three main categories of larval diets based on manufacturing method: (1) microbound feeds, (2) microencapsulated feeds, and (3) complex or combined feeds (Barrows and Hardy 2000).

9.5.10.1. Microbound Feeds

Microbound feeds can be conveniently separated into two major classes: crumbled and on-size feeds. These classes refer to the process used in the production of the feeds. Crumbled feeds are produced by making a pellet, particle, or "cake" and then fracturing the feed into smaller pieces that are sifted to the proper size. The on-size class of feeds does not require breaking of feed to achieve the proper size; the particles of the correct size are produced directly. Not only does this eliminate a production step, but also it creates different physical shapes of on-size feeds compared to crumbled feeds.

9.5.10.1.1. Crumbled Feeds. Two types of crumbled feeds have been produced and fed for many years. These are steam-pelleted and extruded crumbles. These pellet types have been used effectively for many years in the

salmonid and catfish industries. However, these processing methods have not been effective for producing larval feeds. However, research is under way evaluating possible ways to utilize cooking extrusion to prevent leaching of water-soluble nutrients from larval feeds.

9.5.10.1.2. Flake Feeds. Flake feeds, another of the crumbled class of microbound feeds, are the most common type of feed fed to aquarium fish. Effective binders used in flaked feeds include agar, gelatin, carageenan, and alginates (Teshima *et al.* 1982; Levine *et al.* 1983), but protein hydrolyzates are also being investigated as binders. Although a variety of methods can be used to produce a flake, the most common method is the double-drum drier. This machine consists of two parallel drums rotating in opposite directions. A feed slurry coats the drums, and as the drums rotate the slurry is spread to a uniform thickness. Steam is used as a heat source for the drum, which quickly dries the feed. As the drum rotates, the dried feed is flaked off in less than one rotation. The thickness of the flake can be adjusted by altering the distance between the drums. Drying conditions may influence the nutritional value of the product (Gabaudan *et al.* 1980; Teshima and Kanazawa 1983), so that this method is often criticized because of the high temperature required to dry the flakes. Proteins can be burned and lipids oxidized by high temperatures, however, the feed is exposed to the heat for only a short time (~30 sec). There are few studies evaluating the effect of flake processing compared to other larval feed production methods that have not been confounded by differences in feed formulation. Some data indicate that a high temperature is not a problem if the time of heat exposure is short.

Flake feeds come off the drum drier as sheets, which must be ground, and sometimes sifted to produce appropriately sized, thin flakes. This process results in a particle with a high surface area-to-volume ratio. Because of this high ratio, particles float on the surface for a long time before becoming saturated with water and sinking. Long floating times are beneficial, since the fish have more time to consume the feed. The high surface area-to-volume ratio of flaked feeds, coupled with the relatively long period of water exposure, can lower the particle and nutrient stability if stabilization measures have not been taken. Carbohydrates are often used as a binder in flake feeds, but other binders can be used.

9.5.10.1.3. Crumbled Cake Feeds. Many types of binders can be used to produce crumbled-cake feeds. Alginate, zein, cold-water gelling starches, carageenan, gelatin, and egg albumin are a few examples of binders used to make a large matrix of feed material which is crumbled to the appropriate sizes (Teshima *et al.* 1983; Kanazawa, 1982). Each binding system is activated

differently. For example, zein is a protein found in corn and is soluble in alcohol but not water. Zein was used effectively in preparing diets for prawn larvae (Kaushik and Inquet 1979). Egg albumin can also form a matrix, but must be activated by heat. Combining different binders into a binding system is sometimes the best way to produce particles with the desired physical characteristics. The cake can be dried by freeze-drying, oven-drying, or drum-drying. Nutritional quality has been shown to be affected by the type of drying (Teshima and Kanazawa, 1983). Crumbled cake feeds are currently produced commercially and can be very effective larval feeds.

9.5.10.2. On-Size Feeds

9.5.10.2.1. Microextruded Marumerization. One on-size method that is applicable to fish feeds and has been used in the pharmaceutical industry for many years is microextrusion marumerization (MEM). This is a two-step process that results in a shaped, extruded product (Barrows *et al.* 1993). These cold extruders can produce noodles down to 500 and even 300 μm in diameter with specialized extruder configurations.

Once the wet mash is extruded into noodles, the second step of the process begins. The noodles are placed into a marumerizer, which consists of a cylindrical shaped device with a high-speed rotating plate at the bottom. Plates are grooved and are available with different depths of grooves. The depth of the groove affects the amount of energy that is transferred to the feed during marumerization. A very strong noodle will require a deeply grooved plate and a soft noodle will be processed most effectively with a shallowly grooved plate. The marumerizer imparts two effects to the feed. The first effect is to reshape the particle into a spheroid shape. Noodles are broken into particles with lengths equivalent to the diameter of the noodle. The consistency of particle length and the amount of shaping are affected by the feed formulation, the moisture level, and other factors. The second effect of marumerization is to impart a surface densification to the particle. As the feed spins in the marumerizer, centrifugal forces cause migration of water and small ingredient particles to the surface of the feed particle. The surface, therefore, becomes denser than the interior of the feed particle. Because the feed is extruded, the particle size range within a given production run is very narrow, and sifting is not as important as with other manufacturing methods.

Many types of binders can be used with MEM particles, as long as they are moisture and pressure activated. No heat is added to the process, but some is generated at the extrusion screen due to friction. The amount of heat generated is affect by the formula and moisture level but is normally quite low. Binding systems based on gums have been used, but protein hydrolysates are also effective. The hydrolysates have the added advantage of

contributing nutrients as well as binding the particle. Care must be taken when using hydrolysates, as high levels can result in agglomeration of feed particles in the marumerizer into very large particles.

Particles produced by MEM can be characterized as smooth and spheroid, with a high density. The smooth shape may decrease nutrient leaching by decreasing the surface area-to-volume ratio. The high density of the feed results in a faster-sinking particle, which is a negative property for fish species that feed in the water column.

9.5.10.2.2. Particle-Assisted Rotational Agglomeration Feeds. A second on-size, microbound feed production method also utilizes the marumerizer. This method, particle-assisted rotational agglomeration (PARA), does not use an extruder. A quantity of wet mash is placed directly into the marumerizer, along with a charge of inert particles. The rotation of the marumerizer imparts energy to the inert particle, which transfers energy to the mash, and spheroid feed particles in a wide size range are produced. Feed formulation and moisture content are very important in this method. Five other process variables can be controlled to affect the size distribution and the density of the particles. Advantages of this process compared to MEM are the lower capital expenditure required and lower operating costs, since the extruder has been eliminated from the process. The same binders used with MEM particles are effective in PARA particles, with minor modifications. The PARA particles are not as uniform in shape as particles produced by MEM. The size distribution of PARA particles is also much greater than that of MEM particles, thus necessitating sizing of the particles. The PARA process is a low-pressure agglomeration method that results in a low-density feed particle. The low density results in a slower sinking rate of the feed, benefiting species that feed in the water column.

9.5.10.2.3. Spray Beadlets. A third on-size, micro-bound feed production method is spray beadlets. These are particles produced by spraying a slurry of material into a liquid that assists in the formation of particles (Villamar and Langdon 1993). Binders such as alginate and gelatin can be used effectively. This process produces a wide range of sizes so particle sieving is often necessary.

9.5.10.3. Microencapsulated Feeds

Microencapsulation is a process in which a material (the wall) surrounds and coats another substance (the payload). This technology has a variety of other applications (e.g., carbon paper, pharmaceuticals) and has been adapted and developed for larval feeds. Microcapsules can be separated into two major categories: (1) impermeable and (2) controlled release. The

composition of the capsule material determines the permeability of the wall, and the composition can be varied to control the release of the payload material. Because of the wide application of microencapsulation technology, many materials have been used that are not appropriate for aquatic feeds. The most appropriate types for aquaculture are cross-linked proteins (Langdon *et al.* 1985) and lipid-walled microcapsules (Langdon 2000; Langdon and Sergfried 1984). Physical characteristics important when evaluating microcapsules include (1) nutrient retention, (2) digestibility of capsule, (3) payload capacity, and (4) survivability/durability. A primary goal of microencapsulation is to reduce or eliminate nutrient leaching (high nutrient retention). Varying the fatty acid composition of lipid-wall microcapsules can change the rate at which certain nutrient leach from the particle. The molecular weight of the nutrient being carried by the microcapsule will affect the type of wall best suited for that nutrient (Langdon 2000). The digestibility of the wall material of a microcapsule to a particular species is important to assure nutrient availability (Hardy 1989). The wall may also be a nutrient source as well. Some types of microcapsules, such as lipid-walled, have a very low payload capacity, making them most valuable as micronutrient carriers (Villamar and Langdon 1993). The survivability or durability of a microcapsule will determine which feed delivery systems can be used and also how capsules can be used in different types of complex particles.

The double-emulsion method can also be used to produce lipid-walled microcapsules (Langdon *et al.* 1985). This technique involves emulsifying the aqueous payload in lipid wall material. A secondary emulsion is then formed by placing the primary emulsion into an aqueous solution. Lipid particles that are formed are removed and sized (Villamar and Langdon 1993). Different lipids used in the primary emulsion will impart different characteristics to the final particles, such as nutrient stability and particle durability.

9.5.10.4. Complex Feeds

Complex particles are an exciting development in larval feeds and allow greater flexibility for development of feeds to more closely fit specific needs. Each larval feed production method has advantages and disadvantages. Many of the disadvantages can be overcome by combining different particle types to produce a complex particle. Complex particles are feeds that are made of particles produced by two or more processing methods (Villamar and Langdon 1993). For example, a water-stable microencapsulated particle may be embedded in a PARA, a spray beadlet, or a MEM particle. The complex particle combines the advantages of several types of feeds and, in some cases, eliminates the disadvantages. Microcoated diets

are considered by some investigators to be a separate class of larval feeds. However, these feeds might be better classified as complex particles, since there are two processes involved in the production of the particle. Microcoated feeds are made by coating microbound diets with cholesterol, lecithin (Teshima *et al.* 1982) or modified corn gluten or zein (Kanazawa *et al.* 1982). Thus, it is a two-step process, the first to form the particle and the second to coat the particle to decrease nutrient leaching. Commercial diets are currently being manufactured by this method and are being used successfully in larval feeding.

9.6 Ingredient and Diet Evaluation

Ingredients and finished diets can be evaluated by a variety of chemical and biological tests. These tests are used to check on the accuracy of the manufacturing process arriving at a finished feed of the desired composition (quality control), to measure nutrient loss during manufacture and storage, to predict the nutritional value of a particular formulation to detect oxidative rancidity, and to measure the nutritional or feeding value of a formulation. The best method to evaluate a diet formulation is to feed it to a group of fish and compare their growth to that of fish fed a standardized diet for which the nutritional value is known. This is not always practical, particularly when the feed formulation may change frequently in response to changing ingredient prices. The value of chemical methods of feed evaluation is that they do not require feeding trials to be conducted. The strength of biological methods of feed evaluation is that the data obtained can be used to estimte the nutritional value of new diet formulations by summing up the nutritional value of the ingredients. The weakness of such tests is that they cannot exactly predict the nutritional value of the performance of fish fed a given formultion. Understanding the limits of each test at predicting nutritional value of feeds will reduce the likelihood of inappropriate use of the evaluation methods, i.e., overinterpreting evaluation results.

9.6.1. Proximate Analysis

Proximate analysis involves the a partitioning of compounds in a feed into six categories based upon the chemical properties of the various constituents of feeds. This analysis was developed in Germany during the 1870s to evaluate forages and is the most common method of evaluating feed ingredients and finished feeds. The six categories are moisture (water), crude protein, ether extract, crude fiber, ash, and nitrogen-free extract. It is important to

Table 9.20

Composition of Categories in Proximate Analysis

Category	Composition
Water	Water
Crude protein	Essential amino acids
	Nonessential amino acids
	Free amino acids, amines, nucleic acids
Ether extract	Triglycerides
	Phospholipids
	Sterols
	Fat-soluble vitamins
	Miscellaneous lipids (waxes, spinglomyelins, etc.)
Crude fiber	Insoluble polusaccharides (cellulose, hemicellulose, chitin)
Nitrogen-free extract	Monosaccharides
	Oligosaccharides
	Soluble saccharides
	Water-soluble vitamins
Ash	Essential elements
	Nonessential elements
	Toxic elements

remember that proximate analysis, like many chemical analyses, is not a nutrient analysis; rather it is a partitioning of both nutrients and nonnutrients into categories based on common chemical properties of the nutrients and nonnutrients (Table 9.20). Accepted methods for conducting proximate analysis are described in detail in *Official Methods of Analysis,* published by the Association of Official Analytical Chemists (AOAC) (1995). The procedures are described briefly below.

9.6.1.1. Water

The water content of a feed is determined by drying a sample in an oven until a constant weight is reached. The difference between the initial weight of the sample and that after drying is the weight of water in the sample. The official method of analysis calls for 5 hr of drying in a vacuum oven at 95–100°C or 2 hr of drying at 135°C. The latter procedure should not be used if ether extract (fat) determinations will be conducted on the same sample. At these temperatures, volatile fatty acids and other substances can evaporate, resulting in an undestimation of the actual percentage of crude lipid in a sample. Using a lower temperature for a longer drying period is a necessity for a sample containing appreciable amounts of such substances.

9.6.1.2. Crude Protein

Crude protein in feeds is typically determined by measuring the nitrogen content of a sample, either by wet chemistry (Kjeldahl) or by thermal expansion or other means. The protein content is calculated by multiplying the nitrogen content by a conversion factor. On average, proteins contain 16% nitrogen, so the nitrogen content is multiplied by 6.25 (100/16) to calculate the protein content. The actual nitrogen content of proteins used in feeds ranges from 15 to 19%. Nevertheless, the use of 6.25 as a conversion factor gives a result that is accurate enough for most feed applications. One potential pitfall in this procedure is that nonprotein nitrogen, such as urea in the tissues of elasmobranch fish and in chitin in crutacea, is counted as protein and will inflate the true protein content of certain feed ingredients. A correction for nonprotein nitrogen in such ingredients must be made.

9.6.1.3. Crude Lipid

Crude lipid refers to the lipid-soluble materials in a sample that can be extracted with a nonpolar solvent, such as petroleum ether, chloroform, or, alternatively, supercritical CO_2. Using either a Soxhlet or a Goldfisch extraction apparatus, the dry sample is extracted with a hot solvent. Other less explosive solvents, such as methylene chloride, can be used in place of ether. After extraction, the solvent is evaporated and the extracted material is weighed. This procedure is the least accurate of the proximate analysis methods. Solvents typically do not remove all of the lipid from a sample; some phospholipids and lipoproteins remain. Other nonlipid compounds, such as fat-soluble vitamins and sterols, are extracted and are counted as crude lipid. To obtain the total lipid content of extruded pellets, acid hydrolysis must precede solvent extraction, because a portion of the lipid in pellets is bound (Limsuwan and Lovell 1984).

9.6.1.4. Crude Fiber

Crude fiber measures the material remaining in a sample after it has been boiled in a weak acid, followed by boiling in a weak base, minus the inorganic residue (ash). Carbohydrates that are removed from the sample by this procedure are considered those that would also be digested by an animal and those that are left are considered complex carbohydrates that are indigestible. In view of the wide range of digestibility of carbohydrates of various species of fish, the usefulness of this procedure for fish varies. Nevertheless, the results do provide a measure of feed composition and a basis for comparison of feed ingredients and finished feeds. Fish tissue does not contain crude fiber, so this analysis is omitted from proximate analysis of fish. One exception is chitin in crustacea. Chitin, a polymer of

glucosamine, is partitioned into the crude fiber fraction in the proximate analysis procedure.

9.6.1.5. Ash

Inorganic materials that remain after a sample is heated at 600°C are counted as ash. This temperature is sufficient to burn the organic material in a sample. If the sample is to be used for mineral analysis, a slightly lower combustion temperature (550°C) is sometimes used to prevent loss of certain volatile compounds. The ash category contains essential elements, nonessential elements, and toxic elements.

9.6.1.6. Nitrogen-Free Extract (NFE)

This category of compounds includes simple sugars, compound sugars, and soluble polysaccharides, such as starch. NFE is not determined directly but, rather, is the amount or percentage remaining after the other categories are subtracted from 100%.

9.6.1.7. Other Procedures

Other procedures are sometimes used to measure crude protein and lipid levels in feeds and tissues. For measuring protein levels, the Lowry and biuret methods are used. In addition, nitrogen can be measured directly using a nitrogen analyzer. These and other methods are covered in detail in the AOAC (1995) publication. For measuring lipid levels in fish products, other methods are sometimes used (Folch *et al.* 1957; Bligh and Dyer 1959). These methods are relatively gentle and produce lipid extracts that can be used in further analysis, such as gas chromatography (GC).

9.6.2. Nutrient Analysis

Individual essential nutrients can be measured directly in feeds by chemical analysis. These include amino acids, fatty acids, minerals, and vitamins. The levels of essential nutrients determined by chemical means are not necessarily equal to the levels that are biologically available to a fish, so such values must be interpreted and used with caution. Amino acids and many vitamins can be analyzed by high-performance liquid chromatography (HPLC). Fatty acids are analyzed using GC. Lipid classes, i.e., triglycercides and phospholipids, are measured using thin-layer chromatography or, more recently, supercritical CO_2 separations. Minerals are measured using atomic absorption spectrophotometry or inductively coupled plasma spectrophotometry (ICP).

9.6.3. Chemical Tests

To manufacture high-quality diets, one must use high-quality ingredients. The trick is to be abled to identify high-quality ingredients The chemical tests for diet quality discussed below are commonly used to measure ingredient quality, although they may also be used to test diet quality.

9.6.3.1. Protein Quality

The following chemical tests for protein quality are used to measure the effects of processing on protein quality.

9.6.3.1.1. Pepsin Digestibility. Exposure to high temperatures for long periods during the drying process can reduce the nutritional quality of fish meal by causing linkages to form between amino acids that make them indigestible by fish and other animals (McCallum and Higgs 1989). For years, the animal and fish feed industry has relied upon the pepsin digestibility test, performed in the laboratory, to detect fish meals that have been subject to thermal abuse during their manufacture. This test relies upon the enzyme pepsin, usually obtained from pig stomachs, to digest the protein in the fish meal. If the protein has been damaged by thermal abuse during drying, the pepsin digestibility value will be lower than the values typically obtained with high quality fish meal. The original pepsin digestibility test (AOAC, 1998, method 971.09) was modified by diluting the concentration of pepsin to increase the accuracy of the test with respect to discriminating between fish meals of average and those of high quality (Olley and Pirie 1966). This modification is known as the Torry method. The original method could reveal only differences between poor- and high-quality fish meals, as illustrated by the results of Anderson *et al.* (1993), who reported pepsin digestibility values of 97.7, 96.8, and 98.5% for menhaden, anchovy, and Norse LT-94 fish meals, respectively. Using the Torry method, the pepsin digestibility values for the same fish meals were 84.0, 87.4, and 96.8%. Anderson *et al.* (1993) found no correlation between the AOAC pepsin digestibility test and biological tests of fish meal quality but did find a significant positive correlation between the results of the Torry pepsin digestibility method and the protein quality of the fish meals determined by biological tests.

A similar method for measuring the *in vitro* digestibility of fish meals and other protein sources is the multienzyme pH-stat method. This method uses a combination of proteolytic enzymes rather than a single enzyme (pepsin) to digest the sample and maintains a constant pH in the test solution during digestion. This method has been applied to aquaculture feed ingredients by substituting proteolytic enzymes extracted from the pyloric cacao of rainbow trout for enzymes from terrestrial animals (Dimes

et al. 1994). Several studies suggest that multienzyme tests accurately predict the biological value of fish meals (Anderson *et al.* 1993; Dimes *et al.* 1994).

9.6.3.1.2. Tests Used to Determine the Nutritional Quality of Soybean Meal. Vohra and Kratzer (1991) summarized the various chemical tests used to determine the adequacy of heat treatment of soybean meal. They divided the chemical tests into two groups: those that detect underheated soybean meal and those that detect overheated meal. Chemical tests to detect underheated soybean meal were urease activity, trypsin activity, and protein solubility. Urease is an enzyme naturally present in soybeans that does not have any significant nutritional relevance except that it is heat-sensitive and its activity correlates well with residual trypsin activity in dried soybean meal. It is also relatively easy to measure (AOAC 1995). Urease activity in commercial soybean meal ranges from a 0.02 to a 0.1 increase in pH (Vohra and Kratzer 1991). Values of over a 0.5 increase in pH indicate insufficient heat treatment of the soybean meal. If no increase in pH is detected with the urease test, this may mean that the soybean meal has been overheated, so some residual urease activity in the meal is preferred, at least for soybean meal intended for use in poultry feeds. Unheated soybean meal has a urease activity of a >2.25 pH rise (Waldroup *et al.* 1985).

As mentioned, trypsin activity in soybean meal decreases with heat treatment in proportion to urease activity. Unheated, solvent-extracted soybean meal can contain over 21 trypsin inhibitor units/mg sample (Araba and Dale 1990), but commercial soybean meal subjected to normal heating during the presscake drying process generally contains about half the trypsin inhibitor activity of unheated meal. Additional heating further reduces trypsin inhibitor activity, the amount of reduction depending on the temperature and the duration of heat treatment (Arndt *et al.* 1999). The heat generated by extrusion cooking is sufficient to lower trypsin inhibitor levels, at least in full-fat soybeans (Wilson 1992). Wilson (1992) extruded full-fat soybeans having an initial trypsin inhibitor activity of 46.5 trypsin units inhibited/mg sample, reducing the trypsin inhibitor activity to 8.1 trypsin units inhibited/mg sample.

A third method for measuring the extent of heat treatment of soybean meal is the water solubility test, which involves measuring Kjeldahl nitrogen levels in the soybean meal and in a water extract of the soybean meal (Vohra and Kratzer 1991). The method has been slightly modified by extracting the sample in 0.2% KOH (Araba and Dale 1990). Heating decreases the percentage of 0.2% KOH-extractable protein, from about 99% in raw soybean meal to about 72% after 20 min of autoclaving, corresponding to a decrease in trypsin inhibitor units from 21.1 to 1.0 (Araba and Dale 1990).

Excessive heat treatment of soybean meal is thought to reduce protein digestibility by fostering the creation of protein–carbohydrate linkages that are indigestible by most animals. Tests for detecting overheated soybean meal are based on the number of free functional groups in the protein fraction of the soybean meal, which are detected by several dye-binding tests, formaldehyde titration, or a fluorescent derivative (Vohra and Kratzer 1991). Cresol red dye binding is a relatively simple, rapid test requiring only a spectrophotometer. Olomucki and Bornstein (1960) reported that values in the range of 3.8–4.3 mg Cresol red absorbed per gram of meal indicate properly heated soybean meal, and values over 4.3 indicate that overheating has occurred. Orange G dye binding and Coomassie blue are other rapid, simple tests to detect overheated soybean meal, with the Coomassie blue test being preferred because it is rapid and sensitive (Kratzer *et al.* 1990).

9.6.3.1.3. Available Lysine. When proteins containing lysine are heated in the presence of reducing sugars, a reaction occurs between the ε nitrogen of lysine and the reducing sugar, producing a complex that is undigestible by animals. Amino acid analysis will show the lysine to be present but it is unavailable. The amount of available lysine can be determined and compared to the total lysine to judge the quality of the protein source (AFIA 1999).

9.6.3.1.4. Total Volatile Nitrogen (TVN) and Ammonia–Nitrogen. Fish meal quality is affected by the degree of spoilage of raw material used to produce the fish meal. Fish meal produced from freshly caught fish has a higher nutritional value than fish meal made from decomposing fish (Pike 1991). Both TVN and ammonia–nitrogen are values that are used to determine the freshness of raw material used to make fish meal. Because both are volatile, they are lost during fish meal drying. Nevertheless, these tests are sometimes used to estimate the quality of dried fish meal. Caution must be used in interpreting such values, as low values for fish meal may not accurately measure its nutritional quality. The TVN is sometimes called the total volatile base (TVB). Ammonia–nitrogen and TVN are measured using Kjeldahl distillation units (AFIA 1999).

9.6.3.1.5. Biogenic Amines. Biogenic amines are compounds that are formed during fish meal production and include histamine, cadaverine, putrescine, tyramine, and gizzerosine (Pike and Hardy 1997). Gizzerosine is highly toxic to poultry, causing gizzard erosion, bleeding, and death. This condition is known as "black vomit" because the birds vomit dark blood. Fish meals produced from scombroid fish (mackeral, tuna) are more likely to contain histamine, a decomposition product of histidine. Histamine combines with lysine, most likely during stickwater concentration, to form gizzerosine. The other biogenic amines are decomposition products from

lysine (cadaverine), arginine (putrescine), and tyrosine (tyramine). There is no evidence that these biogenic amines potentiate the effects of histamine or gizzerosine.

The toxicity of biogenic amines to fish and crustaceans is much lower than their toxicity to birds. Histamine is reported to cause stomach swelling and thinning of the stomach wall in rainbow trout but does not reduce feed intake or fish growth (Fairgrieve *et al.* 1994). The main concern associated with biogenic amines is that they indicate that fish meal may have been subject to abusive manufacturing conditions, and thus they correlate with fish meal quality, relating to the freshness of fish used to make fish meal. Because biogenic amines are heat-stable, they can be measured in fish meal, using HPLC techniques. Specifications for upper limits of biogenic amine in fish meals are widely available (Pike and Hardy 1997).

9.6.3.2. Lipid Quality

The two major concerns with lipids are hydrolytic and oxidative rancidity. Both are undesirable in dietary lipids and in finished feeds. The use of high levels of fish oils in salmonid feeds creates a high potential for oxidative rancidity to develop during prolonged feed storage. As mentioned earlier, the onset of lipid oxidation in a feed or lipid can be delayed by the presence of antioxidants, both those naturally present and those added. Antioxidants prevent oxidation from moving from the initiation stage to the propagation stage by capturing free radicals. Once they are expended, oxidation moves to the propagation stage and proceeds very rapidly.

9.6.3.2.1. Hydrolytic Rancidity. Hydrolytic rancidity is caused by enzymatic hydrolysis of fatty acids from triglycerides and phospholipids. Moisture is required for lipases, the enzymes involved in releasing fatty acids, to operate, and lipases can originate from the tissues of fish or from microorganisms living in feed or lipids. Free fatty acids are measured by a titration method (AOAC 1995) and are expressed as a percentage. In fish oils used in fish feeds, the usual upper limit for fatty acids is 3%.

9.6.3.2.2. Oxidative Rancidity. Oxidative rancidity is caused by the reaction of a free radical with double bonds of unsaturated fatty acids. Free radicals arise through various mechanisms, including enzymatic activity and radiation, and their production is enhanced by the presence of divalent cations, especially Cu and Fe. There are a nearly infinite number of intermediate products of oxidation of a complex lipid such as fish oil, but intermediate products include hydroperoxides and peroxides, which in turn produce aldehydes and ketones. Oxidative rancidity is measured by detecting and quantifying aldehydes and ketones or by measuring the final products of oxidation. The levels of intermediate products of lipid oxidation rise in the

early stages of oxidation, then fall in the late stages. This is important to remember when interpreting the results of the following tests.

9.6.3.2.2.1. Peroxide Value. The peroxide value measures products of the initial stages of lipid oxidation. There are several methods for measuring the peroxide value, but the most common are described in the AOAC (1995) and AFIA (1999) publications. Peroxide values are expressed as milliequivalents of peroxide per 1000-g sample. Since the test measures intermediate products of oxidation, values rise in the early stages and fall in the later stages. Fresh herring oil is reported to have a peroxide value of 6 (Hung *et al.* 1980).

9.6.3.2.2.2. Thiobarbituric Acid-Reactive Substances (TBARS) Test. The TBARS test measures intermediate products of lipid oxidation, mainly malonaldehyde. Malonaldehyde reacts with thiobarbituric acid to produce a red color. The TBARS test can be done on oils, fish flesh, or feeds, although a modification of the procedures of Lemon (1975) and Yu and Sinnhuber (1977) by Asakawa *et al.* (1975) should be used for feeds.. The TBARS number is expressed as milligrams of malonaldehyde per 1000-g sample. Hung *et al.* (1980) found TBARS numbers of about 50 in fresh herring oil, while oxidizing oil had numbers of over 500. As with the peroxide value, TBARS numbers in oils increase and then decrease with continuing oxidation. The heat and pressure of pelleting a fish feed destroy malonaldehde, making evaluation of freshly pelleted feed by TBARS a less valuable tool than evaluation of the ingredients or mixtures before pelleting (Hardy *et al.* 1983).

9.6.3.2.2.3. Anisidine Value. The anisidine value test measures the presence of aldehyde in a sample rather than an intermediate product of oxidation. The anisidine value is a useful method for measuring the oxidative rancidity in oils but is not useful for measuring oxidation in feeds due to color interference caused by chromogens in the diet (Hung *et al.* 1980). The anisidine value is expressed as 100 times the optical density of a solution resulting from a mixture of 1 g of oil and 100 ml of a mixture of solvent and *p*-anisidine, measured at 350 nm in a 10-mm cell (List *et al.* 1974).

9.6.3.2.2.4. Kries Test. The Kries test is a rapid test which indicates oxidative rancidity when a red color appears in a sample mixed with phloroglucinol. The test can be quantitative or qualitative. The appearance of a red color indicates the presence of an aldehyde (Rossell 1983).

9.6.3.2.2.5. Schall Oven Test. Frankel (1993) described a method used to determine the induction time remaining for a lipid before oxidation

moves from stage 1, initiation, to stage 2, propagation. This method overcomes the problem of trying to estimate the quality of a lipid source from a single measurement of the peroxide value or TBARs, in other words, the problem of not knowing whether the value is going up or going down. By measuring the PV in a lipid sample, then subjecting the sample to an elevated temperature in an oven for a day or more and retesting, one can determine if the sample is in the early or late stages of initiation and/or propagation, plus estimate the degree of protection provided by antioxidants in the sample.

9.6.3.3. Ash Components

Fish meals are often tested for ash and/or NaCl content to meet the specifications required by many fish food manufacturers. High-ash fish meals are those containing over 15% ash. In white fish meals high ash levels indicate that a high proportion of seafood processing waste was used in manufacturing the meal. High ash levels are usually associated with fish meals having protein contents below 65%. Sodium chloride levels above 5–6% are generally undesirable in fish meals, mainly because high levels dilute protein.

9.6.3.4. Antinutritional Factors and Toxins

Plants employ a variety of defenses to prevent their seeds and tubers from being eaten or, if they are eated, from being digested. Oilseed meals contain antinutritional factors, some of which can be destroyed by heat treatment. Those that cannot be destroyed include glossypol in cottonseed meal, glucosinolates in canola meal, and phytic acid in soybean meal, cottonseed meal, canola meal, other oilseed meals, and some grain by-products. Suitable methods for testing for these compounds are reported in the AOAC (1995) and AFIA (1999) publications.

9.6.4. Chemical Score and Indispensable Amino Acid Index (IAAI)

Two mathematical methods of predicting the nutritional value of feed ingredients have been used in the past and merit mention here. Both methods compare the amino acids in a protein with those in whole-egg protein, a complete protein. The chemical score is equal to 100 minus the percentage deficiency of the most limiting amino acid, calculated as follows:

$$\text{Chemical score} = \frac{\text{g limiting amino acid in test protein}}{\text{g amino acid in whole-egg protein}} \times 100$$

A more complex and accurate calculation is that used to compute the IAAI, which is the ratio of the indispensable amino acid in the test protein (TP) divided by the indispensable amino acids in whole-egg protein (WEP) as

follows:

$$\text{IAAI} = \frac{\text{ARG(TP)}}{\text{ARG(WEP)}} + \frac{\text{HIS(TP)}}{\text{HIS(WEP)}} + \frac{\text{ISO(TP)}}{\text{ISO(WEP)}} + \cdots + \frac{\text{VAL(TP)}}{\text{VAL(WEP)}} \times 100$$

9.6.5. Biological Evaluation

Biological evaluation of feed ingredients and finished feeds involves feeding fish and analyzing some aspect of fish performance and/or diet digestibility. Biological evaluation methods can be divided into three general categories: (1) retention studies, in which the deposition of a nutrient in the carcass over a short time is measured: (2) loss studies, in which the various losses of ingested food via the feces, urine, and gills are measured; and (3) performance studies, in which some measure of growth is used to evaluate and compare feeds. The value of biological evaluations of feeds is that they measure performance directly, whereas chemical tests results are correlated only with fish peformance. The weakness of biological methods is that values obtained on individual ingredients cannot always be used together to predict the value of a mixture of ingredients. This is particularly true of protein quality evaluations. Despite this weakness, biological evaluation of proteins is a useful process, particularly to evaluate various samples of a single ingredient that may have been subjected to different heat treatments, for example. The biological evaluation methods are organized into three groups: general methods used for various nutrients, methods used for proteins, and methods used for energy.

9.6.5.1. General Methods

9.6.5.1.1. Growth. Over a specific time period, the growth of groups of fish fed various experimental diets is calculated and compared. Growth can be either gain in weight or gain in length.

9.6.5.1.2. Growth Rate. Daily instantaneous growth rate, also called specific growth rate (SGR), can be used to compare growth on a daily basis.

$$G_w = \frac{1n\,W_1 - 1n\,W_0}{T}$$

where W_1 is the fish weight at the end of the study; W_0, the fish weight at the start of the study; and T, the time interval in days.

To convert the daily instantaneous growth rate to the percentage increase in weight per day (%W/day):

$$\%\,W/\text{day} = (e^{G_w} - 1)100$$

Knowing the percentage increase in weight per day is very useful for predicting the size of fish at a future date. For example, if 10-g fish are growing at a rate of 1.8% per day, one can predict their weight after 12 weeks (84 days) as follows:

$$1.018^{84} \times 10 \text{ g} = 44.7 \text{ g after 12 weeks}$$

For this equation to be accurate, the fish must be growing at a constant weight over the time period of the calculation. If growth rate is not constant due to changes in rearing water temperature or if comparisons are to be made over a long rearing period, thermal unit growth coefficient (TGC) is a more appropriate term to use for growth rate comparisons. TGC is calculated as follows:

$$\text{TGC} = 100 \times (\text{final weight}^{1/3} - \text{initial weight}^{1/3})/\text{sum degree days}(^{\circ}\text{C})$$

9.6.5.1.3. Feed Conversion Ratio. The feed conversion ratio is defined as the dry weight of feed per unit wet weight gain (feed fed/gain). This term is useful to compare the ability of feed formulations to support weight gains. The feed conversion ratio changes with the feeding level and the size of the fish. The reciprocal of the feed conversion ratio is the feed efficiency ratio (gain/feed fed). Some researchers use variations or combinations of these terms, such as "feed conversion efficiency." Because the values are ratios, the terms should be used as described above.

9.6.5.1.4. Apparent Digestibility Coefficient. This method is useful both with feed ingredients and with finished feeds. An inert material, such as chromic oxide or yttrium oxide, is added to the feed at a level of 0.5–1.0% (0.1% for yttrium oxide). The fish are fed for several days, feces are collected, and the chromic oxide (or yttrium) levels of both the feed and the feces are determined. The digestibility coefficient is then determined as follows:

$$\text{Dig coefficient} = 1 - \frac{\text{Cr}_2\text{O}_4 \text{ in feed}}{\text{Cr}_2\text{O}_4 \text{ in feces}}$$

or, for a nutrient,

$$\text{Dig coefficient} = \frac{\text{Cr}_2\text{O}_4 \text{ in feed}}{\text{Cr}_2\text{O}_4 \text{ in feces}} \times \frac{\text{nutrient level in feces}}{\text{nutrient level in feed}}$$

If the digestibility coefficient of a nutrient is being measured, the value obtained by the above procedure is called the "apparent" digestibility coefficient, because no correction has been made for endogenous fecal excretion. To obtain the "true" digestibility coefficient, one must feed a control diet that completely lacks the nutrient being tested, measure the amount of that

nutrient in the feces, and subutract it from the fecal level as follows:

$$\text{True dig coefficient} = 1 - \frac{Cr_2O_4 \text{ in feed}}{Cr_2O_4 \text{ in feces}} \times \frac{[\text{nutrient in feces of fish fed experimental diet}] - [\text{nutrient in feces of fish fed control diet}]}{\text{nutrient in feed}}$$

There are various ways to obtain feces, including stripping, dissection, and sedimentation (Cho and Slinger 1979; Cho *et al.* 1982; Choubert *et al.* 1979; de la Noüe and Choubert 1986; Lovell 1998; Windell *et al.* 1978). Indicators other than chromic oxide can also be used (Atkinson *et al.* 1984), but the one advantage of using chromic oxide is that it can be measured relatively easily in most laboratories, in contrast to yttrium (AFIA 1999).

When feed ingredients are being tested, they are usually combined with a semipurified diet at 25–35% of the combined diet. The semipurified (basal) diet is also supplemented with the inert indicator and fed to separate groups of fish. The apparent digestibility of protein in the test ingredient, comprising 30% of the test diet, is calculated as follows(Forster 1999; Sugiura 2000):

$$AD_{Ing} = (Nutr_{TD} \times AD_{TD} - 0.7 \times Nutr_{BD} \times AD_{BD})/(0.3 \times Nutr_{Ing})$$

where AD_{Ing} is the apparent digestibility (or availability) of nutrients in the test ingredient; $Nutr_{TD}$, the nutrient concentration in the test diet; AD_{TD}, the apparent digestibility (or availability) of nutrients in the test diet; $Nutr_{BD}$, the nutrient concentration in the basal diet AD_{BD}, the apparent digestibility (or availability) of nutrients in the basal diet, and $Nutr_{Ing}$, the nutrient concentration in the test ingredient.

9.6.5.1.5. Carcass Deposition or Nutrient Retention. The retention of specific nutrients or energy in the whole body of fish over a specific time period is a useful way of evaluating the availability and balance of amino acids and the availability of some essential elements and other nutrients as well. Carcass deposition, or apparent retention, is calculated as follows:

$$\text{Apparent retention} = \frac{[\text{carcass nutrient content at end of experiment}] - [\text{carcass nutrient content at start of experiment}]}{\text{nutrient intake during experiment}}$$

Nutrient retention is most frequently used for protein, energy, and phosphorus, although retention values can be calculated for any nutrient. A formula for calculating protein retention is given in the next section.

9.6.5.2. Evaluating Protein and Amino Acid Quality

9.6.5.2.1. Biological Value. Biological value measurements are used to determine the percentage of absorbed nitrogen retained by a fish by measuring the nitrogen excreted during a test period. The biological value is thus similar to carcass deposition or apparent retention. It is calculated as follows:

$$\text{Apparent biological value} = \frac{\text{food N} - (\text{fecal N} + \text{urinary N} + \text{branchial N})}{\text{food N}} \times 100$$

The true biological value may be calculated as follows:

$$\begin{aligned}\text{True biological value} = \text{food N} - \{&(\text{fecal N} - \text{metabolic fecal N}) \\ &+ (\text{urinary N} - \text{endogenous urinary N}) \\ &+ (\text{branchial N} - \text{endogenous branchial N})\} \times 100\end{aligned}$$

9.6.5.2.2. Protein Efficiency Ratio (PER). The PER is a measure of the weight gain per unit protein fed and is a useful method to compare protein sources in a single experiment. There is a standard PER method (AOAC 1995) involving rats. In this method, proteins are compared at a suboptimal dietary level. The PER is calculated as follows:

$$\text{PER} = \frac{\text{weight gain (g)}}{\text{protein fed (g; dry weight basis)}}$$

9.6.5.2.3. Net Protein Utlization (NPU). The net protein utilization is a measure of the protein gained by fish during an experimental period per unit protein absorped by the fish. To calculate the NPU, one must know the protein content of the fish at the start and at the end of the experiment and the digestibility coefficient of the dietary protein. The apparent NPU is calculated as follows:

$$\text{Apparent NPU} = \frac{\begin{array}{c}[\text{protein content (g) of fish} \\ \text{at end of experiment}]\end{array} - \begin{array}{c}[\text{protein content (g) of fish} \\ \text{at start of experiment}]\end{array}}{\text{dry protein fed (g)} \times \text{protein digestibility coefficient}}$$

To calculate the true NPU, the protein content of fish fed a protein-free diet for the duration of the experiment must be subtracted from the numerator of the above formula.

9.6.5.2.4. Protein Retention. Protein retention is similar to NPU, except for the omission of the protein digestibility coefficient. Protein retention is therefore the percentage of protein fed to a group of fish over an experimental period that was deposited in the bodies of the fish. Protein retention is expressed as a percentage.

9.6.5.3. Methods Used to Evaluate Dietary Energy

The question of how much energy is available to fish from ingredients and finished feeds is of critical importance in feed formulation. Energy values are calculated, measured, and expressed in many ways (Jobling 1983). As discussed in Chapter 1, dietary energy is expressed as kilocalories or kilojoules per kilogram and as gross energy, digestible energy, metabolizable energy, and net energy. Energy values can be determined by measurement energy losses at each stage of digestion and metabolism (NRC 1993), by comparative slaughter techniques (Cho and Kaushik 1985), and by estimation of metabolizable energy and net energy for production (Murai *et al.* 1984). The choice of which energy value to use in practical diet formulation depends on many factors, including the species for which a diet is being formulated and the ingredients being considered. For salmonids and catfish, digestible energy values are used. For many other species, insufficient data are available on the digestible energy in common ingredients, so salmonid or catfish values are used in diet formulation.

For ingredient and diet evaluation, determining the digestible energy, using comparative slaughter techniques, and estimating the metabolizable energy and net energy for production are methods that are suitable for use in most laboratories. Measuring metabolizable energy using metabolizable energy chambers is a much larger investment of resources, for which the possible benefits do not justify the effort.

Energy retention is sometimes of interest in fish nutrition studies. Energy retention is calculated in a manner similar to that for protein retention, with gross caloric values of fish and feed, measured by bomb calorimetry, substituted for protein in the equation.

9.6.5.4. Microbiological Standards

Very little work has been conducted on the prevalence of microorganisms in fish feeds or on their effects on fish. There are at present no regulations in the United States that limit the total bacterial counts in fish feeds, although limited research has shown that a number of species of bacteria are commonly found. The earliest published work in this area concerned the transmission of bacterial kidney disease to juvenile salmon fed ground adult salmon carcasses (Wood and Wallis 1955). Wood and Ordal (1958) and Ross *et al.* (1959) reported that mycobacterial infections found in juvenile Pacific

salmon reared in hatcheries and fed diets containing unprocessed adult salmon carcasses were caused by transmission of the bacteria from adults to juveniles via the feed.

Several researchers have measured total aerobic bacterial counts and the range of bacterial species present in commercial fish feeds. Trust (1971) and Trust and Money (1972) reported total colony-forming units (cfu) ranging from 10^3 to 10^9 cfu/g in commercial fish feeds. Kitao and Aoki (1976) found levels of 10^3 to 10^5 cfu/g in Japanese fish feeds. Moffitt-Westover (1986) reported levels of 4.8×10^7 cfu/g in the Oregon moist pellet (OMP). Improved sanitary practices in pellet manufacture, particularly in the hydrolyzate portion of the OMP, reduced the level to 1.9×10^6 cfu/g. Bacteria isolated from the OMP were *Aeromonas hydrophila, Clostridium perfringens, Pseudomonas, Staphylococcus, Streptococcus,* 4 *Salmonella* spp., and 13 members of Enterobacteriaceae.

Bacterial contamination of fish feeds results from the use of contaminated ingredients and from the inoculation of feed after pelleting within the feed production line. Fish meal and meals produced by rendering, e.g., meat and bone meal, blood meal, and poultry by-product meal, are the groups of feed ingredients most likely to be contaminated with microorganisms. Feed producers should set specifications for cfu per gram of ingredient and periodically test batches from various suppliers to ensure compliance with purchase specifications. Extrusion pelleting and UPC pelleting heat feed mixtures to sufficient temperatures and for sufficient periods to kill most bacterial species. Steam pelleting subjects feed mixtures to lower temperatures for shorter periods and likely does not kill all bacteria in feed mixtures. Thus, greater care must be taken to ensure that feed produced by steam pelleting is not contaminated by bacteria. In all feed manufacturing facilities, maintaining proper sanitation, especially in equipment and conveyors after the pelleter, is the most effective means of reducing the risk of bacterial contamination of feed. A program of periodic monitoring of ingredients and finished feeds for total bacterial counts, coupled with an aggressive program of in-plant sanitation and the use of commercial antimicrobials in the feed, is a prudent practice for all fish feed manufacturers.

A recent concern is the possibility of transmitting viral diseases to shrimp via the feed. White spot virus, taura virus, and possibly others can be present in shrimp processing by-product, which is used in some countries as an ingredient in shrimp feeds. Shrimp meal made from shrimp by-product is processed by wet reduction and drying in most areas, but in some countries, it is simply sun-dried before being sold. Viruses cannot survive conventional wet reduction and drying of shrimp by-product, but sun-drying is another issue, and shrimp feed producers would be wise to avoid such products.

9.6.5.5. Economic Evaluation

Diet formulation and manufacture integrate information on the nutrient requirements of the species of fish for which a diet is being produced, its life stage, any special requirements for feeding and culture, the goals of production, and other aspects such as feed quality and product quality. However, the reality of fish production is that feed accounts for over 50% of the operating costs of growing fish, and any savings on feed costs have a large impact on the profitability of an enterprise. This creates a temptation to focus on the cost of feed, the idea being to purchase the least expensive feed available. This approach is usually counterproductive. Feed costs must be considered, but combining biological evaluation with the economic aspects permits a farm to maximize production and minimize the costs of production. In other words, one must examine the cost of feed per unit of product sold to truly understand the contribution of feed to the overall cost of fish production. This must be done in the context of feed type and fish life history stage, taking into consideration the quantities of feed used at each stage and the benefits of using superior (expensive) feed in the early stages of rearing compared to the relative cost. Broodstock feeds can also represent a major proportion of total feed costs. Evaluation of the cost of feed per unit product produced, in this case viable eggs and healthy fry, is the sensible approach to evaluating the economies of feed selection.

State and federal agencies often use a bidding process to purchase fish feeds. The contract is awarded to the feed producer that can supply feed at the lowest cost per metric ton. The bidding process is intended to reduce feed costs through competition but can lead to unintended consequences. When open-formula feeds are specified, feed costs are sometimes elevated by the supplier to provide protection from losses that might occur as a result of the changing nature of the commodity market and the inflexibility of open-formula feeds. If the contracting agency modifies the formulation, yearly to adjust to changing markets, these problems can be minimized.

9.7 Glossary

Antimicrobials—Feed supplements that inhibit the growth of microorganisms, e.g., bacteria, yeast, and mold

Antioxidants—Feed supplements that prevent oxidation of feed components, primarily fatty acids

Binders—Feed additives that hold feed mixtures together as pellets

Bulk feed—Feed delivered to a user without being sacked

Carotenoid—A class of compounds that impart color to the skin and flesh of fish (plus animals, birds, and plants)

Closed-formula diet—A diet formulation that is the proprietary property of a feed company (not public knowledge)
Crumbles—Small particles produced by breaking pellets and screening particles
Diet—A prescribed or controlled mixture or combination of feed ingredients or foods
Dry diet—A diet containing less than 13% water
Expanded feed—Feed pelleted by compression after the feed mixture passes through an expander, which gelatinizes starch by heat and pressure
Extruded feed—Feed pelleted by cooking-extrusion technology
Feed—A mixture or preparation used for feeding animals and fish
Feed conversion ratio—Quantity of feed fed divided by fish weight gain over a specific time period (abbreviated FCR), with values typically ranging from 1.5 to 0.8 in intensive fish culture (0.8 is a better ratio)
Feed efficiency ratio—Fish weight gain divided by feed fed (reciprocal of feed conversion ratio; abbreviated FER)
Fines—Dust and small particles that result from crumbling or pellet disintegration
Fish hydrolysate—Enzymatically digested fish protein, supplied as wet or dry product
Flaked feed—Microdiet made into flakes, used to feed larvae and tropical aquarium fish
Food—Any material containing nutrients that can be consumed, absorbed, and used
Formulation—A formula listing the proportions (or percentages) of ingredients for a feed
Least-cost formulation—A diet formulation produced using computer software in which the formula represents the least-expensive combination of ingredients to achieve the established dietary nutritional specifications for a given diet
Mash—An unpelleted feed mixture
Microdiet—Feed made into very small particles for fish larvae
Moist diet—Same as wet diet
Nonspecific immunostimulant—Feed supplement added to enhance disease resistance
Open-formula diet—A diet formulation that is published and is fixed (does not change)
Pellet—A feed mixture mechanically forced into a formed shape, usually by compression or extrusion
Pelleted feed—Feed pelleted by compression, also known as steam-pelleted feed
Palatability—Quality of feeds associated with taste that affects feed intake
Practical diet—A diet composed of nonrefined ingredients (mainly commodities)
Premix—Microingredients, generally vitamins or minerals, mixed with a diluent that is added to feed mixtures, as a supplement
Probiotic—A feed supplement containing live microorganisms
Protein retention—Dietary protein fed divided by fish protein gain over a specified time period, with values typically ranging from 35 to 50%
Protein unit—A unit used to compare the costs of protein supplements having different prices and protein contents
Protein–energy ratio—The ratio of dietary protein to energy, expressed as grams of protein per kilocalorie or kilojoule

Proximate analysis—A system of analysis that fractionates feed components into six categories, based on their physical and chemical properties
Purified or semipurified diet—A research diet composed of highly refined ingredients
Ration—A share or allotment of feed over a given time period, typically 24 hr.
Semimoist diet—A diet containing 17–35% water (moisture)
Semipurified diet—A research diet composed of a combination of refined and nonrefined ingredients
Supplement—An ingredient rich in a particular nutrient or nutrients that is used to supply essential nutrients or other components to a feed
Therapeutant—A feed supplement added to prevent or combat disease
Top-dressing—The process of adding lipid or lipid suspensions to pellets
Wet diet—A diet containing more than 35% water

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10

Adventitious Toxins

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10.1 Introduction

Adventitious refers to any material, added extrinsically, not essentially inherent, acquired, accidental, casual, out of the usual, or arising sporadically. Obviously, these definitions are not totally appropriate to the subject at hand, since some of the materials that we will discuss, particularly those of plant origin, are inherent, genetically determined components of the plant. Other classes of compounds, such as those arising from human activities during growing, harvesting, processing, storing, or transporting of foodstuffs or from unwanted contamination, would more closely fit the definition of adventitious. True toxins are poisons formed as a specific secretion in the metabolism of a vegetable or animal organism that elicit deleterious effects in the exposed animals. In addition to these materials, it will also be necessary to discuss the effects of other toxic compounds such as synthetic organics and naturally occurring but frequently concentrated inorganics. Fish, for the purposes of this discussion, will be defined as cultured finfish species of the class Osteichthyes. Practically this will be further limited to primarily the salmonid and ictalurid families since the majority of nutritional research in fish has been studied in these intensively cultured groups. Finally, nutrition is a broad discipline encompassing the ingestion, absorption, assimilation, and excretion of food products. My primary concern is with the dietary constituents selected by fish husbandrymen, and the effects of toxicants within these constituents on the fish.

The formulation and preparation of pelleted fish diets are dynamic, everchanging processes, influenced strongly by the availability, price, and quality of constituents. Theoretically a broad spectrum of plant and animal foodstuffs is available to the fish diet formulator, but in actual practice the number of ingredients used is rather small. As a result, fish have never been exposed to a number of potential toxins, and their effects are unknown. A previous treatise (Friedman and Shibko 1972) described the effects of many compounds on birds and mammals and assumed that similar effects would occur in fish. In this paper I cover those food ingredients actually used in fish culture and concentrate on those toxins to which fish have been exposed and for which there are known effects.

10.2 Naturally Occurring Toxins in Formulated Fish Rations

10.2.1. Toxins of Plant Origin

10.2.1.1. Introduction

In the early days of fish diet formulation, animal products comprised the bulk of the ingredients used (Law *et al.* 1961; Sinnhuber *et al.* 1961). Today, with increased knowledge of the nutrient requirements of fish, the high prices of animal products, and the unavailability of high-quality animal products at certain times, the use of plant products has greatly increased. These plant materials are usually more reasonable in price than animal products, but they can present problems through the presence of naturally occurring antimetabolites. It is assumed that the function of these antimetabolites and toxins is as natural insecticides. However, these toxins can also have a variety of effects on fish and other animals, ranging from a mere reduced growth rate to severe toxicity, resulting in death. The plant commodities routinely used in salmonid and ictalurid diets in the United States include soybean meal; cottonseed meal; whole corn or corn screenings; wheat middlings, shorts, flour, and germ; distillers corn solubles; hominy meal; sorghum; rice bran; peanut meal; and oat meal or groats. In Canada rapeseed meal (canola meal) is used in at least some of the salmon diet formulations due to its ready availability and low cost.

10.2.1.2. Soybean Trypsin Inhibitor

Soybeans provide one of the most promising plant proteins for use as a substitute for fish meal in fish rations. Soybean meal is regularly used in trout, catfish, and other pelleted fish diets, but its use is restricted by the presence of several antimetabolites, the most common of which is a trypsin inhibitor. Soybean trypsin inhibitor is a crystalline globular protein with a molecular weight of 21,500 (Liener and Kakade 1980). It has been shown to depress the growth rate of chicks, rats, rainbow trout, channel catfish, carp, and other species of fish and to produce pancreatic hypertrophy in chicks and rats as an apparent compensatory response to the increased need for tryptic enzymes (Sandholm *et al.* 1976). It did not, however, cause pancreatic hypertrophy in calves, pigs, dogs, or adult guinea pigs (Liener and Kakade 1980). Robinson *et al.* (1981) examined pancreatic tissue from channel catfish fed soybean meal, with varying levels of trypsin inhibitor, and found no effects. There is evidence that the mechanism by which soybean meal decreases the growth rate may be more complicated than a simple interference

with protein digestion. The usual means of inactivating the trypsin inhibitor of soybeans is by heating the meal to a sufficient temperature. The optimum conditions of heat and moisture seem to vary from animal to animal. It is critical to determine this optimum temperature and time since overheating also will lower the nutritional value of soybean meal. Thus insufficient heating, for the inactivation of trypsin inhibitor, will interfere with protein digestion (Dabrowska and Kozak 1979), but overheating leads to a low availability (Sandholm *et al.* 1976) of certain amino acids, particularly lysine (Riesen *et al.* 1947; Evans and Butts 1951). A number of studies in several species of fish have been conducted on the general subject of substituting soybean meal for fish meal in fish diets. Some of these emphasize the importance of heat inactivation of trypsin inhibitor, and others concentrate on protein availability and fail to acknowledge the importance of trypsin inhibitor. As a result a variety of sometimes contradictory results is present in the literature. These are summarized in the following section, with the purpose of determining the role that trypsin inhibitor plays in practical fish nutrition.

Viola *et al.* (1982), working with carp, concluded that soybean meal contains 10–15% less available energy and lysine than required. They recommended only partial replacement of fish meal with soybean meal. Complete replacement would require expensive supplementation of individual amino acids and higher levels of lipid. They also concluded that fish meal contains some unknown growth factor(s) that is (are) missing in soybean meal. Viola *et al.* (1983) concluded that inadequate lysine, and not residual trypsin inhibitor, was the limiting factor in properly processed commercial soybean meals for the growth of carp. Dabrowski and Kozak (1979), working with grass carp fry, found that an increased soybean meal content of the diet resulted in growth depression, which they attributed to incompletely destroyed trypsin inhibitor.

Andrews and Page (1974) discovered that replacement of fish meal with soybean meal on an isonitrogenous basis resulted in significantly reduced growth and feed efficiency in channel catfish. Supplementation of soybean meal with a lipid extract, a nonlipid residue, or an ashed fraction of menhaden meal or the purified amino acid methionine, cystine, or lysine did not improve the growth significantly. Nothing was said about trypsin inhibitors. Robinson *et al.* (1981) tested the effects of different heat treatments on trypsin inhibitor levels in catfish diets. They reported depressed growth and poor feed efficiency in fish fed diets containing more than 1.9 TIU (trypsin inhibitor units)/mg sample. Robinson *et al.* (1985), on the other hand, observed no effect of trypsin inhibitor levels as high as 3.6 TIU in another experiment with catfish. Wilson and Poe (1985) treated uncooked, hexane-extracted soybean meal with heat for various lengths of time to produce meals with varying trypsin inhibitor activities. They fed fingerling channel

catfish test diets containing 25 or 35% crude protein and graded levels of trypsin inhibitor activity for 10 weeks. Growth rates and protein efficiency ratio values were reduced in fish fed raw or inadequately heated soybean meal at both protein levels, but more severely at the lower level. Fish tolerated more trypsin inhibitor activity on the 35% protein diets than on the 25% protein diets. They reported that 83% of the trypsin inhibitor activity in the soybean meal must be destroyed before the fish would grow at optimum rates. They also commented that channel can apparently tolerate higher trypsin inhibitor activity than carp. It was also apparent to these authors that other antinutritional factors in soybean meal, besides trypsin inhibitor, may be involved in less than optimal use of the available nutrients.

Dabrowska and Wojno (1977) found that rainbow trout can utilize soybean meal, supplemented with cystine (1%) and tryptophan (0.5%), almost as well as protein from fish; nothing was stated about inhibitors. Rumsey and Ketola (1975) supplemented soybean meal diets (80%) for rainbow trout with various individual or combinations of amino acids. Growth on the soybean meal diet alone was slow, but adding methionine, leucine, lysine, valine, and threonine or methionine, leucine, lysine, valine, threonine, histidine, tryptophan, and tyrosine, to provide amino acid levels in the diet that were comparable to those in fish protein, improved the growth. Nothing was mentioned about trypsin inhibitors. Ketola (1975) showed that supplementation of soybean meal diets with either dicalcium phosphate or ash, prepared from fish protein concentrate, increased growth significantly over that of the soybean-only control. It is assumed that this meal had been heat treated to destroy trypsin inhibitor. He emphasized that soybean meal was a poor source of calcium and phosphorous. Sandholm *et al.* (1976) developed a sensitive assay for antitrypsin activity in soybean meals based on the solubilization of calcium caseinate in agar plates. They showed the rainbow trout to be extremely sensitive to the soybean antitrypsin factor. Also, they observed a direct relationship between the effectiveness of heat destruction of soybean trypsin inhibitor and the availability of the energy and protein in soybean meal for rainbow trout. Smith (1977) demonstrated that properly cooked full-fat soybean meal was superior to solvent-extracted meal for use in rainbow trout diets. The critical factor was the temperature reached in the interior of the bean to assure destruction of the antitrypsin component. He found that full-fat soybean meal, when properly heated, was a satisfactory source of protein and energy, giving 80–100% of the growth observed with fish meal-containing diets. He emphasized that trout are very sensitive to antitrypsin activity and its content in soybean meals should be determined before use in trout diets. Reinitz *et al.* (1978) confirmed Smith's (1977) findings that properly heated full-fat soybean diets outperformed commercial

fishmeal-containing diets for rainbow trout. Fowler (1980) showed that full-fat soybean meal, even when properly heated, was not a satisfactory replacement for fish meal for chinook and coho salmon.

These studies reveal several important features about the effects of soybean trypsin inhibitor in fish husbandry. These include the following. (1) There seem to be varying degrees of sensitivity to the inhibitor among the various species tested. Rainbow trout appear to be highly sensitive, channel catfish are more resistant, while carp may be more intermediate in their response. Each species' sensitivity should be determined and the appropriate precautions taken. (2) Heat treatment of commercial soybean meals is not a standardized procedure, so the level of trypsin inhibitor varies from source to source. Several possible procedures could be recommended to avoid growth depression by trypsin inhibitors due to this variability. Results indicate both economic and biological advantages to the use of full-fat beans, at least for rainbow trout diets (Smith 1977). Perhaps diet formulators could purchase whole raw beans and properly heat-process them for the needs of the species for which they supply feed. Alternatively, soybean meals should be tested for antitrypsin activity before incorporation into fish diets. (3) Supplementation with amino acids and minerals provide variable results, probably due to both species differences and the quality of the soybean meal being used. Better standardization of meals and knowledge of species requirements would undoubtedly bring more understanding to this area. Supplementation of soybean diets with calcium phosphate appears to be important because of the low levels of these minerals in soybeans and the action of phytin (discussed later) in soybeans, which binds these nutrients and makes them unavailable. (4) Very little work has been done in fish on the effects of trypsin inhibitor on the pancreas. The pancreas was examined histologically in only one study (Robinson *et al.* 1981). Pancreatic hypertrophy and hyperplasia are predictable responses in some but not all mammals and chickens (Liener and Kakade 1980), and this needs to be studied in more species of fish. Soybeans are regularly used in many fish diets, often with less than optimal results due to these variables. Minimal efforts to prevent the antigrowth effects of residual trypsin inhibitor would seem to be prudent in light of the available evidence.

10.2.1.3. Hemagglutinins (Lectins)

Hemagglutinins or lectins are proteins which can interact in specific ways with certain carbohydrates. They can bind to free sugars or to sugar residues existing in polysaccharides, glycoproteins, or glycolipids, in either free or bound form, such as in cell membranes. It is this ability to bind to sugars on cell membranes that gives these proteins their agglutination properties. This is a very specific reaction, which can be inhibited if the specific sugar

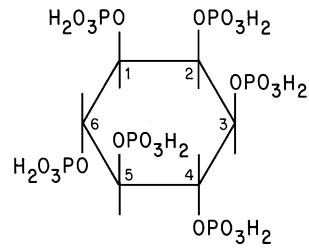


FIG. 10.1

Phytic acid.

is present in solution with the suspended cells. Hemagglutinins have been found in a number of edible plants, most notably in the legumes including soybeans (Jaffe 1980). Essentially nothing is known about the effects of hemagglutinins in fish. It is unlikely that these proteins affect the growth of fish since heat treatment inactivates the proteins and it is also readily inactivated by pepsin in the stomach (Liener 1958). We are not aware of any specific studies with hemagglutinins in fish.

10.2.1.4. Phytic Acid

Phytic acid, the hexaphosphate of *myo*-inositol (Fig. 10.1) is a constituent of all cereal products and oilseed meals such as soybean, rapeseed, and cottonseed, which are commonly used in salmonid and other fish diets (Jones 1979; Richardson *et al.* 1985). In rats, phytates consistently cause adverse effects on growth, especially in the presence of high calcium levels (Morris and Ellis 1980). Phytates have the capacity to bind tightly divalent cations such as calcium, magnesium, and zinc, and much of the phosphorous and part of the calcium, magnesium, and zinc, in plant ingredients containing phytates, are not available to the animal (Smith 1977). This may explain at least in part the beneficial effects of mineral supplementation of soybean-containing diets (Ketola 1975). Richardson *et al.* (1985) investigated the combined effects of varying levels of calcium, zinc, and phytic acid in chinook salmon. This study originated in response to an outbreak of cataracts in chinook and coho salmon in both British Columbia and Washington State hatcheries. Previous studies (Ketola 1979; Watanabe *et al.* 1980; Satoh *et al.* 1983) had shown an important role of calcium–zinc ratios in salmonid cataract formation. Thus Richardson *et al.* (1985) studied systematically the effects of different levels of calcium and zinc in combination with the strong divalent ion chelator, phytic acid, which is present in many commercial diets. They discovered that high dietary phytic acid depressed growth, food and protein conversion, and thyroid function, increased mortality, promoted cataract formation at low

zinc levels, and induced an abnormal pyloric cecal structure. They showed that high calcium accentuated these adverse effects and led to nephrocalcinosis in low- and high-phytic acid groups. They concluded that zinc was essential to normal eye development and that zinc deficiency could not be induced by high calcium alone, but required the presence of a stronger mineral chelator. Spinelli *et al.* (1983) conducted similar experiments on the combined effects of minerals (calcium and magnesium) and phytic acid (phytates) on growth in rainbow trout. They also found that phytic acid reduced the growth rate but that increasing levels of calcium and magnesium did not affect growth. High calcium alone also reduced the growth rate. They demonstrated that phytic acid binds with protein in the gut to produce partially digestible complexes. They concluded that reduced growth in fish fed phytic acid was related to a reduction in protein availability rather than a reduced bioavailability of zinc, iron, or copper. These experiments illustrate the potential adverse effects of phytic acid on growth as well as other essential body functions in salmonid fish fed diets high in this material. These studies utilized phytic acid added to purified diets, thus additional studies are needed to assess the effects of phytic acid, present in soybean or cottonseed meals in practical fish diets, on growth and other variables in salmonids as well as other fish.

10.2.1.5. Phytoestrogens

Phytoestrogens are compounds naturally occurring in many plants used for human and animal food. The compounds actually responsible for estrogenic activity include the isoflavones, the coumestans, and the resorcylic acid lactones. Foods used in fish diet formulation that contain one or more of these compounds include several grains (barley, oats, rice, and wheat), soybeans, and rape (Stob 1983). To our knowledge no one has investigated the effects of these rather weak estrogenic compounds on any species of fish. This would require the isolation of sufficient quantities of the compounds to conduct some short-term studies and compare the effects with those of estrogens such as 17β-estradiol. In rodents, vaginal cornification and increased uterine weights are the responses observed to detect estrogenic activity. In fish other end points would need to be assessed, but we do not believe that these materials present any serious threat to fish growth or health.

10.2.1.6. Glucosinolates

Glucosinolates (thioglucosides) are found naturally in all cruciform plants, including horticultural varieties such as cabbage, broccoli, and cauliflower, oilseed crops such as rapeseed, condiments such as mustard seed, and greens such as kale. Glucosinolates are the source of 5-vinyloxazolidinethione (goitrin) (Fig. 10.2), organic nitrites, isothiocyanates (Fig. 10.3), and

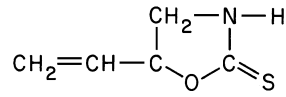


FIG. 10.2

Goitrin.

thiocyanate ions (Fig. 10.4). All of the hydrolysis products of glucosinolates (goitrin, nitrites, isothiocyanates, and thiocyanate ion) are antithyroid substances (Van Etten 1969; Tookey *et al.* 1980). Goitrin, the most potent, exerts its effects through the inhibition of the organic binding of iodine. The addition of iodine to the diet does not reverse the thyroid malfunction. In contrast, thiocyanate ion acts by lowering iodine uptake by the thyroid, and increased dietary iodine will reverse the condition. Less is known about the mode of action of organic nitrites and organic isothiocyanates but they are thought to function as goitrogens through the production of thiocyanate ion and subsequent lowering of iodine uptake by the thyroid (Van Etten 1969; Van Etten and Wolff 1973; Tookey *et al.* 1980). The glucosinolates are not particularly harmful themselves, but on hydrolysis by the constituent enzyme myrosinase, these other compounds are released that impair thyroid function and cause other deleterious effects (Tookey *et al.* 1980; Van Etten and Tookey 1983).

The glucosinolates have become important in fish nutrition with the increasing interest in rapeseed meal as a low-cost protein source for salmonid diets. In addition to glucosinolates, rapeseed meals also contain other toxic components including a high fiber content, erucic acid, tannins and other phenolics, trypsin inhibitor, and phytic acid. Yurkowski *et al.* (1978) showed that glucosinolates from three rapeseed ingredients caused thyroid hyperplasia and reduced plasma thyroxine (T_4) levels in rainbow trout. The three forms of rapeseed were rapeseed meal prepared by heat treatment (105°C) and solvent extraction, rapeseed protein concentrate prepared by dehulling and both solvent and water (100°C) extraction, and rapeseed flour prepared by dehulling and solvent extraction (no heat). In these feeding trials, the rapeseed flour-containing diets (no heat treatment) caused the greatest degree of thyroid hyperplasia, lowest T_4 levels, and poorest growth. Thyroids from these fish were extremely hyperplastic with cell hypertrophy. Thyroid

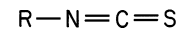


FIG. 10.3

Isothiocyanates.

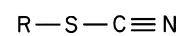


FIG. 10.4

Thiocyanate ion.

follicles invaded surrounding tissues including bone, muscle, and connective tissues. Follicle size and shape were variable but usually smaller and more irregular than in controls. Colloid was granular, eroded, scanty, and pale pink in color, and follicle cell nuclei were enlarged, with prominent nucleoli and frequent mitoses (Yurkowski *et al.* 1978). The water extraction step in the preparation of rapeseed protein concentrate removes up to 90% of the glucosinolates, producing a much less toxic and useful protein (Yurkowski *et al.* 1978; Jones 1979). As a total protein source for trout, however, it gave poor growth and still contained antithyroid activity. Heat treatment of rapeseed meal inactivates the enzyme myrosinase and reduces the antithyroid effect, but the meal still carries the glucosinolate content (Yurkowski *et al.* 1978).

The glucosinolate fraction of standard rapeseed meals varies from 3 to 8% (Fenwick and Hoogan 1976; Van Etten and Tookey 1983; Langer 1983). Plant geneticists have recently developed new varieties of rapeseed with a greatly reduced glucosinolate content (<0.2 mg/g) and have a goal of zero glucosinolate meals in the near-future (Slinger 1977). These low-glucosinolate meals are now referred to as canola meals (Higgs *et al.* 1982; Hardy and Sullivan 1983). Higgs *et al.* (1982) tested two canola meals, candle (*Brassica campestris*) and tower (*Brassica napus*), and a rapeseed protein concentrate as potential protein supplements for chinook salmon. Replacement of 10 and 13% of the protein in an Abernathy dry diet with one of the rapeseed meals or concentrate had no effect on the growth rate, food or protein utilization, body composition, thyroid or pituitary histology, osmoregulatory capacity, health, or mortality of chinook salmon. Twenty-five percent of the two meals did reduce the growth rate and food utilization and produced thyroid hyperplasia, but 25% of the rapeseed protein concentrate did not reduce growth or affect the thyroid. In a production diet, 13% of the tower meal in the diet supported growth comparable to that with the Oregon Moist Pellet, but there was a compensatory hyperplasia of the thyroid. The fish were apparently able to compensate for the antithyroid effect by thyroid hyperplasia because T_4 levels and growth were normal. These experiments show that properly treated low-glucosinolate rapeseed meals (at 16–20% of the dietary dry matter) are acceptable sources of protein for salmonid diets. They also show that rapeseed protein concentrate, without the interference of glucosinolates, high fiber, and phenolic compounds, is an even better source of protein.

Higgs *et al.* (1979) produced adequate growth of coho salmon in diets containing up to 22% tower rapeseed meal, but compensatory elevated thyroid activity was observed. They also stated that levels in excess of this could be used if 3,5,3-triiodo-L-thyronine was administered simultaneously to compensate for the antithyroid effects. Hardy and Sullivan (1983) produced similar results with canola meal in rainbow trout diets. They found that up to 20% canola meal in the diet did not affect the growth rate but did affect thyroid hyperplasia and thyroxin levels in the fish. Again, the fish were able to compensate for the antithyroid effect by increasing the activity and output of the thyroid gland. The authors pointed out that these trials had all been performed on juvenile trout for relatively short periods of time and that the effects of rapeseed protein should be tested on very young fish, in which thyroid activity is higher, and for longer periods of time such as in broodstock rations to determine the overall effects. At this point it appears that rapeseed protein is of a sufficiently high quality to support salmonid growth, if the antinutritional effects of the glucosinolates and other antimetabolites can be minimized. Continued plant breeding programs have goals of producing meals with a glucosinolate content of $\leq$0.2 mg/g, which, together with proper heat treatment, should greatly minimize or eliminate this restriction on rapeseed meal use in fish diets.

10.2.1.7. Erucic Acid

Erucic acid (*cis*-13-docosenoic acid) is a 22-carbon monounsaturated fatty acid (22:1 ω-9]) and is a normal constituent of rapeseed oil (Fig. 10.5). The level of this fatty acid in the oil from the standard varieties of rapeseed ranges from 20 to 55% (Mattson 1973; Slinger 1977). Some rapeseed varieties have been specifically bred for a high erucic acid content (>50%) in the oil, which is valuable for many industrial purposes. On the other hand, selective breeding in the opposite direction has yielded varieties that have an erucic acid content of <1%. The high erucic acid rapeseed oils were cardiotoxic to rats (Mattson 1973; Slinger 1977), causing early lipid accumulation followed by focal necrosis of the muscle fibers and infiltration by mononucleocytes. Unexpectedly, low-erucic acid rapeseed oils (1–2%) have also produced cardiac lesions in rats, and the causative agent has not been identified with certainty (Kramer *et al.* 1975). Beare-Rogers *et al.* (1974) showed that partial hydrogenation of high- or low-erucic acid rapeseed oils

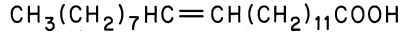


FIG. 10.5

Erucic acid.

reduced the cardiotoxicity. They also reported that linolenic acid was most affected by the hydrogenation, whereas erucic acid was only slightly affected. In fact, they postulated another toxic factor, other than erucic acid, which is removed by hydrogenation. McCutcheon *et al.* (1976) arrived at similar conclusions and postulated that linolenic acid played a role in the cardiotoxicity of rapeseed oils. Slinger (1977) reported that tower rapeseed oil with 0.9% 22:1 ω-9 gave only a slightly higher cardiotoxicity severity rating than corn oil, and on partial hydrogenation, there was no difference in the severity ratings of tower rapeseed oil and corn oil. He agreed with McCutcheon *et al.* (1976) that linolenic acid 18:3 ω-3 may be a factor in the etiology of cardiopathology.

With the development of rapeseed varieties with a low erucic acid content, and since rapeseed meals are solvent-extracted before use as protein supplements in fish diets, the amount of erucic acid in practical diets is probably negligible. In the reports of the use of rapeseed products in fish diets (Yurkowski *et al.* 1978; Higgs *et al.* 1979, 1982; Hardy and Sullivan 1983), no mention is made of the occurrence of erucic acid in the diets used or any pathology associated with it. Parker and Hendricks (unpublished) fed a high-erucic acid (50%) rapeseed oil to coho salmon. Fish were fed the oil at 6% for 6 months and 12% for 4 months and were killed after 10 months. We observed growth depression, mortalities, and histopathology in the skin, gills, kidney, and heart. Kidney lesions included fat accumulation in intertubular hematopoietic regions and glomerular atrophy and degeneration. Fat accumulation occurred in the epicardial connective tissue but there was no necrosis of the myocardium. Fatty acid analysis revealed a substantial increase in 22:1 ω-9, at the expense of 22:6 ω-3.

10.2.1.8. Alkaloids (Pyrrolizidine Alkaloids)

Alkaloids comprise a large and diverse group of plant toxins. Nearly 6000 of the compounds are presently known. Structurally, they exhibit great variability but all do contain nitrogen, usually as part of a heterocyclic ring (Cooper-Driver 1983). Hegnauer (1966) subdivided the alkaloids into three groups: the true alkaloids, which are derived biosynthetically from five amino acids (ornithine, lysine, phenylalanine, tyrosine, and tryptophan); the protoalkaloids, which do not contain heterocylic nitrogen other than the indole ring from tryptophan; and the pseudoalkaloids, which include compounds in which the nitrogen is introduced at a late stage in biosynthesis. The toxicity of alkaloids is usually directly related to their structural complexity. They are present in about 25% of all plant species (Cooper-Driver 1983). Most species of fish have never been exposed to the vast majority of the alkaloids, since alkaloids are found only in angiosperm plants and these do not comprise a significant portion of natural fish diets.

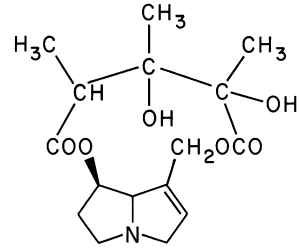


FIG. 10.6

Monocrotaline.

One group of true alkaloids, the pyrrolizidine alkaloids, which are formed from ornithine, has some potential for exposure to fish. Although it is unlikely that feedstuffs from plants containing pyrrolizidine alkaloids (PAs) would be intentionally placed in fish diets, it is possible that some of this plant material could enter fish diets as contaminants of soybean, cottonseed, or certain grain products. *Crotolaria spectabilis,* which contains the PA monocrotaline (Fig. 10.6), has been planted extensively as a cover crop in the southern United States. It is now spreading and growing wild in many areas and could easily contaminate crops such as soybeans or cottonseed which are mechanically harvested. Another plant, tansy ragwort (*Senecio jacobaea*), grows profusely throughout the Pacific Northwest and contains several PAs, the most abundant being jacobine (Fig. 10.7). It has been shown in rats that PAs are metabolized by the mixed-function oxidase system of the liver to the corresponding pyrroles, and present evidence indicates that the pyrroles are responsible for the toxic effects (McLean 1970). PAs are primarily hepatotoxins in mammals, but vascular lesions (venoocclusive disease) in the

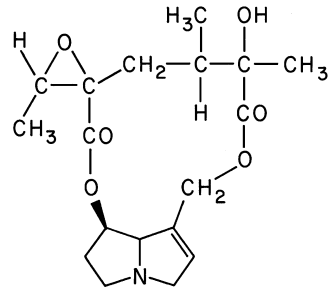


FIG. 10.7

Jacobine.

liver, lungs, heart, and kidney also occur (Allen *et al.* 1970; Allen and Carstens 1968). In addition, PAs are carcinogenic to the liver of chickens (Campbell 1956) and the liver and several other tissues of rats and mice (Rao and Reddy 1978; Johnson *et al.* 1978).

Hendricks *et al.* (1981a) tested the toxicity of a mixture of PAs extracted from tansy ragwort in rainbow trout. We discovered that rainbow trout metabolize PAs to the corresponding pyrroles and that they are primarily liver toxins. Growth rate was severely restricted by dietary PAs, especially at the high dietary level (100 ppm). Mortalities began during the fourth month on the high dose and the experiment was terminated after 6 months due to mortalities and the obvious sickness (malaise) of the fish. Trout fed 20 ppm PAs in the diet survived for 1 year with minimal mortalities, but high-dose fish, returned to a control diet after 6 months, continued to die and never recovered from the severe liver toxicity. Histologically, liver megalocytosis, necrosis, and extensive fibrous tissue scarring were prominent lesions. Venoocclusion of hepatic veins also occurred. Megalocytosis and scarring in the fish fed 100 ppm PAs persisted throughout the 6 months on control diet. No evidence of carcinogenicity was noted in the 12-month study. Rainbow trout embryo exposure to several concentrations of monocrotaline also failed to produce a carcinogenic response (Buhler and Hendricks, unpublished results).

As initially stated, the likelihood of PA poisoning of fish is remote, but the dramatic megalocytosis and liver scarring observed in rainbow fed PAs would warrant suspicion of PA exposure if similar lesions were observed in hatchery fish.

10.2.1.9. Gossypol

Cottonseed is another example of a high-quality plant protein, the use of which, for animal and human food, is restricted due to the presence of naturally occurring toxins. The best-known of these toxins is the yellow phenolic pigment gossypol (Fig. 10.8). It occurs naturally in the pigment glands of the cottonseed. Pigment glands comprise between 2.4 and 4.8% of the seed in varieties grown in the United States, and gossypol is between 39 and 50% of the weight of the glands (Berardi and Goldblatt 1980). Other gossypol-related pigments constitute another 2%. Commercial processing of cottonseed involves heating and moisture, which disrupt the glands and cause conversion of much of the free gossypol to bound forms. Bound gossypol is considered nontoxic to animals. Present processes for cottonseed meal production remove, destroy, or bind from 80 to 99% of the gossypol, making cottonseed meal acceptable as a dietary ingredient for most animals except the very sensitive, for instance, pigs (Singleton and Kratzer 1973). Genetic selection has also led to the development of glandless cottonseed that is

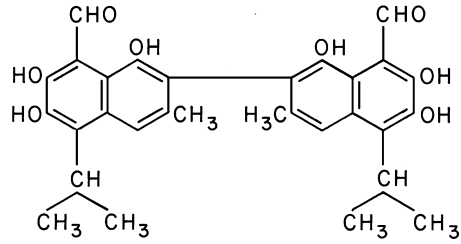


FIG. 10.8

Gossypol.

essentially free of all gossypol. Without the adverse effects of gossypol, this cottonseed meal may find great acceptance in animal as well as human feeds. Animals and humans vary in their sensitivity to gossypol, with rabbits and guinea pigs being very sensitive and ruminants very resistant to its toxicity. Humans also appear to be resistant to its toxicity (Berardi and Goldblatt 1980).

Herman (1970) tested the effects of gossypol from several sources (glandless cottonseed meal, normal glanded cottonseed meal, commercial cottonseed meal, raw cottonseed meal, low-heat cottonseed meal, and gossypol acetate) on rainbow trout. Growth suppression occurred on diets containing 290 ppm or more free gossypol. Fish receiving 531 ppm free gossypol had severe reductions in hematocrit, hemoglobin, and plasma proteins, in addition to growth suppression. Histological changes in the liver and kidney occurred in fish receiving 95 ppm or more gossypol. Thickening of the glomerular basement membrane, liver necrosis, and ceroid deposition in the liver, spleen, and kidney were the specific histological changes observed. It was concluded that 300 ppm gossypol was the maximal dietary level to minimize growth depression, but since histological changes occurred at 95 ppm, a maximum of 100 ppm or less would be desirable for rainbow trout. Consistent with these results were those of Roehm *et al.* (1967), who reported no adverse effect on the growth of rainbow trout fed gossypol acetate at 0.025% (250 ppm) for 18 months. They found relatively high concentrations of free and bound gossypol in the liver but low levels in the muscle. Fowler (1980) reported that chinook salmon grew well on diets containing 34% glanded cottonseed meal, while coho salmon tolerated diets containing up to 22% glanded cottonseed meal without growth depression.

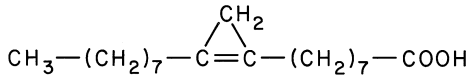
Dorsa *et al.* (1982) fed various levels of cottonseed meal or gossypol acetate to channel catfish and found growth depression at levels greater than 17% cottonseed meal. They concluded that cottonseed meal, contributing

not more than 0.09% gossypol, was acceptable for catfish diets as long as the lysine content of the diet was 1.6% of the diet. Lysine can be limiting in cottonseed meal since gossypol binds preferentially to lysine (Wilson *et al.* 1981). Robinson and Rowles (1983) tested the desirability of glandless cottonseed flour and meal for catfish diets and found that glandless cottonseed meal could replace soybean meal completely (50% of diet) with no reduction in growth. The glandless meal also did not require lysine supplementation. Glandless cottonseed meal thus appears to be a highly desirable plant protein, although production of this variety is not presently adequate to allow large-scale animal feeding. Robinson *et al.* (1984a) found similar results using glandless, defatted cottonseed meal for channel catfish. Glandless cottonseed meal could replace all the soybean and/or peanut meals and maintain adequate growth. With *Tilapia aurea,* however, Robinson *et al.* (1984b) found reduced growth with all cottonseed products, glanded as well as glandless, but the growth reduction was apparently not the result of gossypol. Lysine was supplemented to a presumed adequate level, but nutritive requirements for this species have not been determined. Thus, although *Tilapia aurea* tolerated gossypol levels up to 0.18% without growth reduction, cottonseed meals were not as high in nutritive value as either soybean or peanut meals.

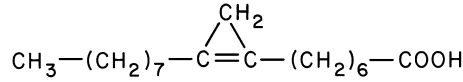
Effects of gossypol in other fish species are unknown. It is tolerated at variable levels in salmonids, channel catfish, and tilapia, but excessive levels can result in depressed growth, tissue accumulation, and histopathological changes.

10.2.1.10. Cyclopropene Fatty Acids

The cyclopropene fatty acids (CPFA), sterculic and malvalic acids (Fig. 10.9), are constituent fatty acids of plants of the order Malvales.



Sterculic Acid



Malvalic Acid

FIG. 10.9

Cyclopropene fatty acids.

Cottonseed meal provides the major source from which these acids may occur in fish diets. The level of these acids in crude cottonseed oil ranges from 0.6 to 1.2% (Bailey *et al.* 1966). Sterculic and malvalic acids are still found in the meal after processing cottonseeds to remove the oil. Their level depends on the amount of residual oil but is usually about 0.01% in meal obtained by modern processing methods (Levi *et al.* 1967). At this level a salmonid diet containing 20–30% cottonseed meal could contain 20–30 ppm CPFA. Cottonseed meal is routinely used in salmonid grower feeds but is not incorporated into either starter or brood rations (Charlie Smith, personal communication). Of the 0.01% CPFA in cottonseed meals, the approximate ratio of sterculic-to-malvalic acids is 2:1. Therefore only 7–10% of the more powerful sterculic acid would be present in salmonid grower diets. This level is probably not high enough to cause growth depression or other noticeable results, particularly in older fish. The use of full-fat glandless cottonseed meal, as reported by Robinson *et al.* (1984a,b), however, would result in much higher and potentially toxic levels of CPFA. Hendricks *et al.* (1980a), for example, fed glandless cottonseed meal, at 25% of the diet, to rainbow trout and observed growth depression as well as the production of liver neoplasms, discussed later. The glandless meal used in the experiment contained 39.8% lipid, resulting in 9.95% cottonseed oil in the diet. The CPFA content of this oil was 0.73%, yielding about 725 ppm CPFA in the diet. Thirty-five percent of that figure, or about 250 ppm, was sterculic acid.

One of the major effects of CPFA in mammals and birds is inhibition of the fatty acid desaturase system (Raju and Reiser 1967; Johnson *et al.* 1969). Roehm *et al.* (1970) found the same effect in rainbow trout. Other effects of CPFA that have been documented in rainbow trout include altered lipid metabolism (Struthers *et al.* 1975a; Malevski *et al.* 1974a), altered lipid composition and histology, including hepatocyte necrosis, unusual glycogen deposition, the appearance of "fibers" in the hepatocyte cytoplasm, proliferation of bile ducts, and fibrosis (Struthers *et al.* 1975b; Scarpelli *et al.* 1974; Malevski *et al.* 1974b); altered activities of liver mixed-function oxidases and other enzymes (Eisele *et al.* 1978, 1983; Taylor *et al.* 1973); increased liver-to -body weight ratios (Hendricks *et al.* 1980c); synergism with aflatoxin B1 (AFB1)-initiated hepatic neoplasia (Sinnhuber *et al.* 1968b, 1974; Lee *et al.* 1968, 1971; Hendricks *et al.* 1980c; Hendricks 1981; Schoenhard *et al.* 1981); and the apparent initiation of hepatic neoplasms alone (Sinnhuber *et al.* 1976; Hendricks *et al.* 1980b,c). The latter effect has been observed frequently using methyl sterculate at levels from 15 to 405 ppm (Sinnhuber *et al.* 1976; Hendricks *et al.* 1980b) and commercially available cottonseed products (glandless cottonseeds and cottonseed oil) (Hendricks *et al.* 1980b). Cottonseed oil, containing 0.35% CPFA, was used in the diet at 7.5%. Its use

resulted in about 30% of the fish having hepatic neoplasms at 12 months. This diet contained about 90 ppm sterculic acid. The glandless cottonseeds, fed at 25% of the diet, resulted in over 70% of the fish having neoplasms at 12 months.

Little is known about the effects of CPFA in species other than rainbow trout. Wales and Sinnhuber (1972) were able to produce liver neoplasms in sockeye salmon (*Oncorhynchus nerka*) using both AFB1 and CPFA, whereas AFB1 alone was ineffective in this resistant salmon species. Robinson *et al.* (1984a) found that glandless, full-fat cottonseed meal depressed growth in channel catfish and postulated that the growth depression could be due to the CPFA content in the full-fat meal. Robinson *et al.* (1984b) found a similar growth depression in channel catfish fed the full-fat glandless meal and acknowledged that it could be due to the CPFA content.

There is abundant evidence of the deleterious effects of CPFA in rainbow trout. Most research has been conducted using 50 ppm or more of sterculic acid or methyl sterculate. However, some experiments have shown distinct histological effects at levels as low as 10 ppm (J. Hendricks, unpublished data, 1979). It is known that young fish are more susceptible than older fish, but because of incorporation of CPFA into body lipids, CPFA effects can be transmitted into eggs and effect the offspring of CPFA-fed broodstock. Thus the current practice of not putting cottonseed meal into either starter or broodstock rations for rainbow trout is prudent. The levels of cottonseed meal currently used in production rations appear to be safe due to their low CPFA content.

10.2.1.11. α-Amylase Inhibitor

Wheat contains three molecular weight classes of albumins which can inhibit animal α-amylases (Silano *et al.* 1975). It is primarily the 60,000-Da (four-subunit) fraction that is responsible for inhibition of fish α-amylase. α-Amylase activity in the digestive tract of fish varies greatly depending upon their feeding habits. Carnivorous fish such as trout have low intestinal α-amylase activity, perhaps related to their poor utilization of dietary carbohydrate. Herbivorous fish, like carp, on the other hand, have α-amylase activities that are 10–30 times higher (Hofer and Sturmbauer 1985). A mixture of trout gut fluid with wheat flour inhibits 81% of the α-amylase activity. Proteolytic enzymes digest the albumin inhibitor, however, so the level of inhibition decreases with time, becoming 16% after 2 hr and 8% after 4 hr (Hofer and Sturmbauer 1985). Coarse-ground wheat is less efficient than flour in inhibiting α-amylase. Even though carp α-amylase is much more active than that of trout, the level of inhibition by wheat flour is similar (71–82%). The rate of recovery is slower in carp, possibly due to the lower levels of proteolytic enzymes in herbiverous fish (Hofer and Sturmbauer

1985). Thus the presence of wheat products in fish diets may have an important negative effect on the digestion and utilization of carbohydrates.

10.2.1.12. Other Toxins of Plant Origin

There are a number of other toxins which can have a significant impact on the rearing of other commercially important animals, such as favatoxin, cyanogenic glycosides, oxalates, toxic amino acids, phallotoxins, indoles, and potato glycoalkaloids, but the likelihood of the plants containing these toxins being used in fish diets is remote. Furthermore, there is little if any experimental evidence for the effects of these compounds on fish. As a result they are not considered in this chapter.

10.2.2. Toxins of Animal Origin

10.2.2.1. Introduction

The previous section lists the limited number of plant foodstuffs used routinely in pelleted fish rations. When we consider the number of animal-derived foodstuffs used in fish diets the number is even smaller: fish meal (herring, anchovy, menhaden and whitefish), whey, blood meal, poultry by-products meal, feather meal, meat meal, wet fish (hake, herring, bottom fish fillet scrap), shrimp or crab meal, krill, and fish oil. In general, there are no toxins, of a significant nature, associated with any of animals from which these products are derived. Two toxins found naturally in the liver, ovaries, and roe of certain fish could potentially enter fish diets if these fish species were inadvertently included with fish being prepared for fish meal or wet fish. These are tetrodotoxin and dinogunellin.

10.2.2.2. Tetrodotoxin

About 80 species of the order Tetraodontiformes (the puffer fish or globefish) produce and store tetrodotoxin, a complex cyclic compound (Fig. 10.10) which is highly toxic to all vertebrate animals tested except those species that produce it (Fuhrman 1974). I am not aware of tests that specifically determine its toxicity to other fish species. In humans and laboratory mammals it causes numbness of the lips, tongue, and fingers, muscular paralysis, and eventual respiratory paralysis and death. Most of the fish containing this toxin are from the western Pacific and Indian oceans, but some species of puffers also inhabit the coastal waters of the eastern United States (Fuhrman 1985). It is conceivable that these fish could enter the fishery and end up in fish meal or wet fish but the risk in fish diets is very low. The effects of the toxin on the various species of commercially fed fish are not known.

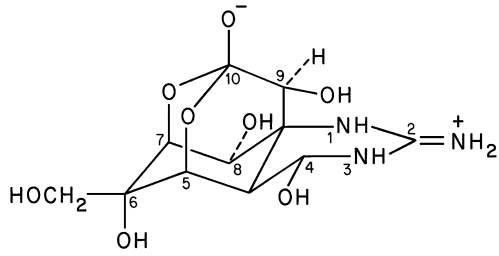


FIG. 10.10

Tetrodotoxin.

10.2.2.3. Dinogunellin

Dinogunellin is a toxic lipoprotein that is produced in the roe of two fish, the northern blenny (*Stichaeus grigorgjewi*), from northern Japan, and the cabezon or marbled sculpin (*Scorpaenichthys marmoratus*), a fish common to the Pacific coast of North America. This toxin is not as potent as tetrodotoxin, but it causes diarrhea, nausea, vomiting, epigastric distress, and liver and spleen necrosis (Fuhrman 1974, 1985). I am not aware of any exposures of fish such as trout to dinogunellin. Since the cabezon is a common inhabitant of the Pacific coastline, it is conceivable that some of these fish could end up in the fillet scrap of groundfish used as wet fish in Oregon Moist Pellets. In practice, however, these fish rarely enter the troll fishery since they prefer rocky bottoms near the coastline. Thus the potential for fish toxicity due to dinogunellin in pelleted fish diets is very low.

10.2.2.4. Dinoflagellate Toxins

Under undefined climatic and hydrographic conditions, certain blue–green algae known as dinoflagellates reproduce very rapidly to produce "blooms" which can actually turn the water of the estuary red or whatever color predominates in the organism. Several of these bloom-forming organisms are toxic and create dangerous conditions for fish as well as humans and terrestrial animals. Mollusks filter these organisms from the water, digest them, and accumulate the toxin in various tissues. Mollusks are not affected by the toxin but humans or other mammals that eat toxic shellfish are very sensitive and significant mortalities of humans, livestock, wild animals, and pets have resulted from eating these toxic animals. Fish also seem to be sensitive to algal blooms, since massive fish kills have occurred in bloom areas. Blooms of *Gymnodinium brevis* have resulted in large fish kills off the Florida coast, and the toxin appears to be released into the water because fish die soon after swimming into a bloom area. The dinoflagellate

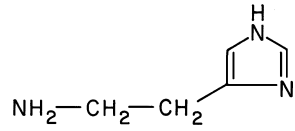


FIG. 10.11

Histamine.

Prymnesium parvum also releases toxins into the water that are extremely toxic to brackish-water fish.

The potential for these dinoflagellate toxins to appear in formulated fish diets is rare, though not impossible. Because mollusks are rarely used in fish diet formulations, and fish that would contain dangerous levels of these toxins would probably already be dead, the chances of these toxins entering fish diets is remote. Some crustacean products, such as shrimp and crab meal or oil, may be used in fish diets to impart carotenoid pigmentation to fish flesh, so these products could carry certain amounts of dinoflagellate toxins. Overall, however, it is not a significant problem.

10.2.2.5. Histamine

Under conditions of improper storage (warm temperatures and extended periods of time), scombroid fishes may develop high levels of histamine (Fig. 10.11). This is due to the action of the bacterium *Proteus morganii* on the high histidine content of these fish. Little is known about the effects of histamine on fish, but since the chances of scombroid fish (tuna, swordfish, and mackerel) being incorporated into fish diets is low, it is, again, only a potential problem that has little possibility of actually affecting cultured fish.

10.2.2.6. Oxidized Fats

Marine fish oils, high in polyunsaturated fatty acids, are very susceptible to autoxidation when exposed to atmospheric oxygen (Watanabe 1982). The use of oxidized or rancid fish oils in diet preparation, along with the additional oxidation that can occur during diet preparation and storage, results in the presence of undesirable lipid-derived toxins in the diet. These toxic components of the diets may affect fish by direct toxicity or indirectly through vitamin deficiencies caused by greater demands for vitamins, especially vitamin E (Hardy *et al.* 1983). The effects of oxidized lipids in fish diets have been investigated in at least three groups of cultured fish, the salmonids, catfish, and carp. Smith (1979) reported growth depression, increased mortality, microcytic anemia, and liver lipoid degeneration in

rainbow trout fed rancid diets deficient in both vitamin C and vitamin E. Supplementing the diets with adequate levels of vitamins C and E prevented the described symptoms. The symptoms are those of vitamin E deficiency (Woodall *et al.* 1964; Poston *et al.* 1976) in rainbow trout rather than vitamin C deficiency, indicating that adequate vitamin E was the most important factor in preventing the symptoms. Sinnhuber *et al.* (1968b) and Hung *et al.* (1980) also reported that adequate levels of vitamin E prevented adverse effects of feeding oxidized fish oils to rainbow trout. Murai and Andrews (1974) tested the effects of α-tocopherol and ethoxyquin on oxidized menhaden oil in channel catfish. Oxidized menhaden oil in diets, without supplemental α-tocopherol or ethoxyquin, resulted in poor growth, food conversion, and survival rates; high incidences of exudative diathesis, muscular dystrophy, and depigmentation; and fatty livers, anemia, and pronounced histological changes in muscle fibers, kidney, and pancreas. Adequate levels of α-tocopherol in the rancid diets reversed all the above symptoms, but ethoxyquin was only partially effective, not reversing the anemic and muscular dystrophic conditions. Hashimoto *et al.* (1966) described a muscular dystrophy condition in carp resulting from oxidized fat in the diet and reported that vitamin E would prevent the condition. Watanabe *et al.* (1966, 1967) reported that vitamin E was effective in preventing carp muscular dystrophy but that synthetic antioxidants such as ethoxyquin, BHA, methylene blue, ethyl gallate, and DPPD were not.

These results clearly indicate the deleterious effects of oxidized lipids on fish health. They also clearly demonstrate the essential requirement for adequate vitamin E in fish diets since at least some degree of lipid oxidation is almost inevitable, particularly in dry diets. The use of high-quality, unoxidized oils in diet preparation is recommended, but supplementation with adequate levels of vitamin E, which will vary depending on the fish species and the quality of the lipid, is absolutely essential.

10.2.3. Toxins of Microbiological Origin

10.2.3.1. Introduction

The biosynthetic products of molds that grow on foodstuffs used in the preparation of fish diets constitute an extremely important group of adventitious toxins. The epizootic of liver cancer in rainbow trout resulting from *Aspergillus flavus* contaminated cottonseed meal is legend. It not only alerted society to a new and powerful liver carcinogen with important consequences to man, but also revealed the necessity of carefully monitoring plant-derived foodstuffs for aflatoxin B_1 (AFB_1) prior to use in fish diets. Commodities that are particularly susceptible to *Aspergillus* infestation include cottonseed meal, peanut meal, corn products, and, to a lesser degree,

grains such as wheat, rice, barley, and oats. Because of the limited number of feed ingredients used in fish food, many of the potential mold-produced toxins, other than AFB_1, rarely find their way into fish diets. Extensive information is available on the effects of the aflatoxins to rainbow trout but less is known about their effects on other fish. Some other potentially important mycotoxins have been studied very little or not at all in any fish species.

Aflatoxins require metabolic activation by hepatic mixed-function oxidase enzymes prior to at least carcinogenic and possibly acutely toxic effects being observed. A significant research effort, particularly at our laboratory, has involved determining the metabolic products of AFB_1 in rainbow trout and the carcinogenicity of those metabolites (aflatoxicol, aflatoxin M_1, aflatoxicol M_1), as well as metabolites from other animals (aflatoxin Q_1, aflatoxin P_1), in the rainbow trout (Sinnhuber *et al.* 1974; Canton *et al.* 1975; Hendricks *et al.* 1980c; Schoenhard *et al.* 1976, 1981; Loveland *et al.* 1977, 1979, 1983, 1984, 1988; Bailey *et al.* 1994b, 1998). Although important for other reasons, these studies have little significance from a nutritional toxicity or carcinogenicity standpoint, since these metabolites do not appear in fish diets. For this reason I focus on the effects of naturally occurring aflatoxins that are produced when the molds *Aspergillus flavus* and *A. parasiticus* grow on foodstuffs. The major products of these molds are aflatoxins B_1, B_2, G_1, and G_2 (Ayres *et al.* 1971). Other intermediates in the biosynthetic pathway of these molds, namely, versicolorin A and sterigmatocystin, can also appear in contaminated feeds (Wong *et al.* 1977). Other species of *Aspergillus,* namely, *A. versicolor,* and even other genera of molds such as *Penicillium* and *Bipolaris* produce sterigmatocystin as a major metabolite (Schroeder and Kelton 1975; Rabie *et al.* 1977). Because sterigmatocystin and versicolorin A have both exhibited carcinogenicity, they also should be recognized as potential hazards to fish (Hendricks *et al.* 1980d).

10.2.3.2. Naturally Occurring Aflatoxins

Aflatoxins B_1 and G_1 (Fig. 10.12) are powerful acute toxins in rainbow trout (Halver 1965, 1967; Bauer *et al.* 1969), where LD_{50} values of 0.81 mg/kg for AFB_1 and 1.90 mg/kg for AFG_1 have been established (Bauer *et al.* 1969). Zebra fish are even more sensitive, with LD_{50} values of 0.44–0.58 mg/kg for AFB_1, 0.75–0.83 mg/kg for AFG_1, 0.24 mg/kg for sterigmatocystin, 1.0 mg/kg for AFB_2, and 4.2 mg/kg for AFG_2 (Abedi and McKinley 1968; Abedi and Scott 1969). In comparison, the acute LD_{50} for AFB_1 is 5.5–7.2 mg/kg in rats (Butler 1964; Wogan 1966) but only 0.36 mg/kg in the day-old duckling (Carnaghan *et al.* 1963). The LD_{50} for the more resistant coho salmon was estimated as between 5 and 10 mg/kg by Halver *et al.* (1969). Channel catfish have also been tested for AFB_1 acute toxicity and found to be relatively resistant, although we do not have LD_{50} values. These

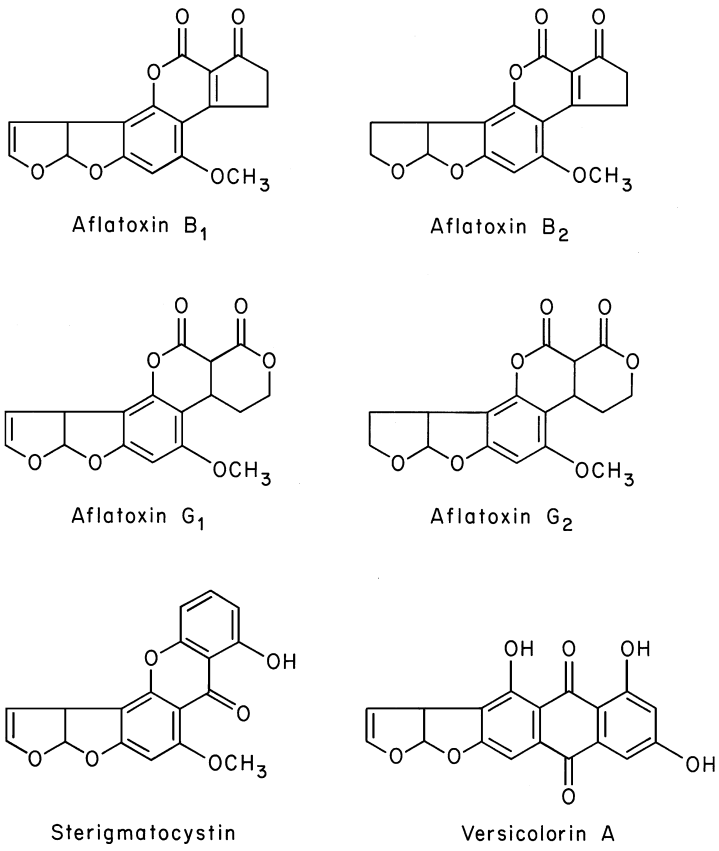


FIG. 10.12

Naturally occurring aflatoxins.

results indicate the variability in the sensitivity of fish to the acute toxicity of aflatoxin. For some fish, i.e., coho salmon and channel catfish, low levels of aflatoxin are of little concern, but for rainbow trout, even low-dose exposures could cause mortalities as well as delayed toxicity, as discussed next.

The carcinogenicity of dietary AFB_1 in rainbow trout has been observed numerous times, beginning with the original epizootic reports (Rucker *et al.* 1961; Heuper and Payne 1961; Wood and Larsen 1961; Snieszko 1961; Nigrelli and Jakowska 1961; Scarpelli *et al.* 1963; Wolf and Jackson 1967; Ashley and Halver 1963) and continuing with many experimental results (Halver 1965; Halver and Mitchell 1967; Lee *et al.* 1968, 1971, 1977;

Sinnhuber *et al.* 1968a,b, 1977a; Wales 1970; Ghittino 1976; Brekke *et al.* 1977; Hendricks *et al.* 1977, 1979; Shelton *et al.* 1983, 1984a; Nixon *et al.* 1984; Bailey *et al.* 1987; 1994a, 1998; Dashwood *et al.* 1988, 1989, 1991; Goeger *et al.* 1988; Breinholt *et al.* 1995; Orner *et al.* 1998; Oganesian *et al.* 1999). Rainbow trout appear to be the most sensitive vertebrate animal to the carcinogenicity of AFB_1 since oral doses of 0.4 to 1.0 ppb in the diet fed continuously for 1 year will produce a significant incidence of hepatic tumors (Lee *et al.* 1968; Shelton *et al.* 1984b). Conceivably, doses even lower than 0.4 ppb could produce tumors in trout if cyclopropenoid fatty acids in cottonseed meal were present to enhance the aflatoxin carcinogenesis (Hendricks *et al.* 1980a). This synergism between AFB_1 and CPFA necessitates extreme caution when using cottonseed meal in diets of young trout or brood trout, which may ingest the diet for long periods of time.

Aflatoxin G_1 also is carcinogenic to rainbow trout but it is less potent than AFB_1 (Ayres *et al.* 1971; Halver 1967). Aflatoxins B_2 and G_2, which do not have the double bond on the terminal furan ring, were not carcinogenic to trout in the experiments of Ayres *et al.* (1971), but Halver (1967) reported some tumors in trout fed AFG_2. Sterigmatocystin and versicolorin A have produced tumors in rainbow trout, but only by embryo exposure to aqueous solutions of the compounds (Hendricks *et al.* 1980d). Their carcinogenicity to trout via the dietary route is still unproven.

The Shasta strain of rainbow trout (*Oncorhynchus mykiss*), maintained as an outbred population at our laboratory, appears to be the most sensitive of the various strains of rainbow trout tested with AFB_1. Salmonid species of the Atlantic-based genus *Salmo, S. trutta* (the brown trout) and *S. salar* (the Atlantic salmon), are less sensitive to AFB_1 than the Pacific-derived rainbow (Wales 1970). Brook trout (*Salvelinus fontinalis*) are also less susceptible than rainbows but possibly more sensitive than brown trout (Wolf and Jackson 1967). Cutthroat trout (*O. clarki*) are also less sensitive than rainbows, while the extensively reared coho and chinook salmon (*O. kisutch* and *O. tschawytscha*) are very resistant to both the toxic (Halver 1969) and the carcinogenic effects of the aflatoxins (Halver 1969; Wolf and Jackson 1967; Bailey *et al.* 1988). Wales and Sinnhuber (1972) were able to produce hepatic neoplasms in sockeye salmon (*O. nerka*), but both aflatoxin B_1 and CPFA were required. Either embryonic exposure or very high dietary levels of AFB_1 (5 ppm for 3 weeks), alone, were required to produce neoplasms in coho salmon (Bailey *et al.* 1988). Another commercially important fish, the channel catfish, is relatively resistant to the carcinogenicity of aflatoxin, although liver cell cancer has been reported from this species (Ashley 1969).

Dietary exposure of other fish species to aflatoxin is limited. Sato *et al.* (1973) and Matsushima *et al.* (1975) fed AFB_1 to guppies (*Lebistes reticulatus*) at 0.6 ppm for 11 months and produced hepatic tumors in 7 of 11 fish. They

found no tumors in guppies fed 3 ppm AFB_1 for 2 months. Sterigmatocystin fed at 2 ppm for 2 months produced cholangiomas in guppies. Hatanaka *et al.* (1982) fed AFB_1 at 2.5 and 5 ppm to Japanese medakas (*Oryzias latipes*) for 24 and 6 weeks, respectively, and produced a low incidence of hepatic tumors. Sterigmatocystin actually was more carcinogenic to medakas than AFB_1, since 2.5 ppm dietary sterigmatocystin produced more tumors than 2.5 ppm AFB_1 for 24 weeks (Hatanaka *et al.* 1982). These two species of aquarium fish are definitely more resistant to AFB_1 than rainbow trout since 1000 times the dose (ppm vs ppb) produces incidences in guppies and medakas similar to those in rainbow trout. I am unaware of other fish exposures to dietary aflatoxin, either experimentally or accidentally.

10.2.3.4. Other Mycotoxins

Ochratoxin A (Fig. 10.13), the most toxic of the metabolites, produced by *Aspergillus ochraceus,* is a potential fish toxin since it occurs as a natural contaminant of corn and wheat (Shotwell *et al.* 1969; Scott *et al.* 1970). Doster *et al.* (1972) determined the acute intraperitoneal toxicity of ochratoxins A and B and their dihydroisocoumarin derivatives ochratoxins a and b in rainbow trout. Ochratoxin A was found to be lethal, with an LD_{50} of 4.67 mg/kg. It caused degenerative changes in the liver and necrosis of the proximal tubules, hematopoietic tissue, and glomeruli of the kidney. Ochratoxin B, the dechlorinated form of ochratoxin A, was nonlethal at doses up to 66.7 mg/kg, but the high dose caused some histological changes in the kidneys and liver similar to those caused by low doses of ochratoxin A. Ochratoxins a and b were nontoxic at levels up to 28.0 and 26.7 mg/kg, respectively. Both feeding trials and embryo exposure of rainbow trout to ochratoxins A and B have been negative with regard to carcinogenesis (unpublished results).

Patulin (Fig. 10.14), produced by several species of the fungal genera *Penicillium* and *Aspergillus,* grows readily on grain products and dry malt feed (Scott 1977). It is acutely toxic to microorganisms and all animals tested

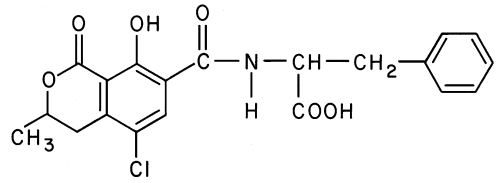


FIG. 10.13

Ochratoxin A.

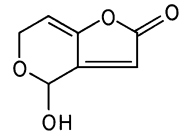


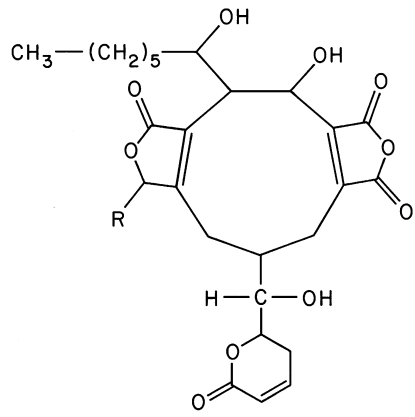
Fig. 10.14

Patulin.

and produces sarcomas at the injection site in rats (Carlton and Szczech 1977). To my knowledge, the acute toxicity of patulin has not been tested in fish, however, rainbow trout embryo exposure to several concentrations of patulin proved noncarcinogenic (unpublished results).

Rubratoxins A and B (Fig. 10.15), produced by *Penicillium rubrum,* are highly toxic to laboratory animals by i.p. injection but are much less toxic orally (Scott 1977). They are noncarcinogenic in rats (Wogan *et al.* 1971). There do not appear to be any acute toxicity tests of the rubratoxins in fish, although they could appear in fish diets through moldy corn. Embryo exposures of rainbow trout to rubratoxins A and B proved noncarcinogenic at our laboratory (unpublished results, 1976).

Citrinin (Fig. 10.16) is produced by species of *Penicillium* and *Aspergillus* which grow on a variety of grain products such as wheat, oats, rye, and barley (Scott 1977). It is primarily a kidney toxin in animals causing acute



Rubratoxin A – R = HOH
Rubratoxin B – R = O

Fig. 10.15

Rubratoxins.

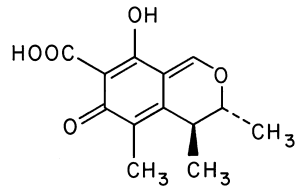


Fig. 10.16

Citrinin.

kidney necrosis (Scott 1977) but has also produced kidney tumors in rats (Shinohara *et al.* 1976). Acute oral toxicity in fish has not been determined but exposures of rainbow trout embryos to solutions of citrinin were not carcinogenic (unpublished results).

Vomitoxin is one of the naturally occurring trichothecene mycotoxins produced by genus *Fusarium* molds that grow on various cereal grains such as corn, barley, and wheat. Because wheat and corn products are used frequently in salmonid diets, vomitoxin is a potential problem for trout and salmon culture. Woodward *et al.* (1983) fed diets containing vomitoxin to rainbow trout to determine their response and sensitivity to this toxin. The primary response to vomitoxin was feed refusal within 5–7 days when vomitoxin levels were 20 μg/g or higher. When returned to a control diet after 4 weeks, the fish began feeding within 2 days and fed vigorously thereafter. Fish receiving low levels (1–12.9 μg/g) of vomitoxin ate the diets but demonstrated reduced growth and feed efficiency. Neither emesis nor mortalities were observed during the 8-week study. Thus vomitoxin depresses feed acceptance but does not have a residual effect on feed intake, weight gain, or feed conversion efficiencies when trout are returned to nontoxic diets.

T-2 toxin is another trichothecene mycotoxin produced by *Fusarium* molds growing on cereal grains. The effects of this mycotoxin on rainbow trout have been studied by Marasas *et al.* (1967, 1969) and Poston *et al.* (1982). A single acute oral dose (6.5 mg/kg body wt) given to rainbow trout fingerlings caused extensive shedding of the intestinal mucosa, severe edema in body cavities, and eventual death (Marasas *et al.* 1967). Long-term (12-month) ingestion of low doses (200–400 μg/g food) in older fish had no apparent adverse effect and actually promoted better growth than the control (Marasas *et al.* 1969). Poston *et al.* (1982) fed levels of T-2 toxin from 1 to 15 mg/kg and found that doses higher than 2.5 mg/kg depressed growth, efficiency of feed use, hematocrit, blood hemoglobin concentration, and feed acceptance. The 15 mg/kg dose caused hemorrhaging in the intestines and regurgitation. As with vomitoxin, the major effect was feed refusal, resulting in a low assimilation of T-2-contaminated diet.

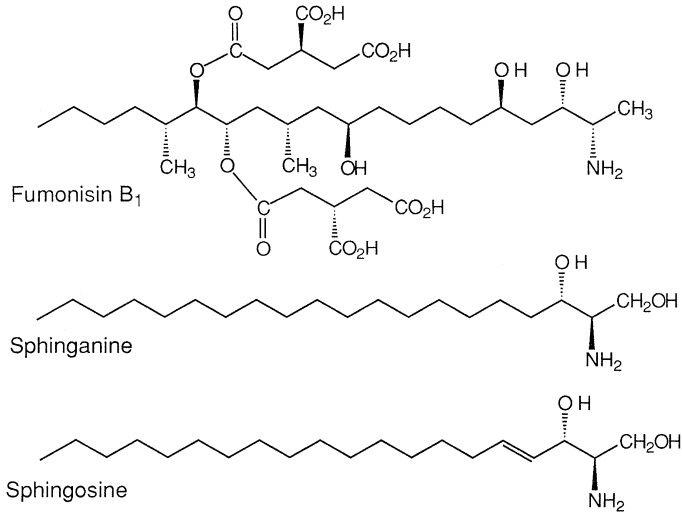


FIG. 10.17

Fumonisins.

Yet another group of mycotoxins produced by *Fusarium moniliforme* and other *Fusarium* species is the fumonisins. The best characterized of these toxins is fumonisin B_1 (FB_1) (Fig. 10.17). *Fusarium moniliforme* grows as a symptomless endophyte on the kernels of corn and other grain products (Bacon and Hinton 1996) and could easily contaminate fish diets made from such ingredients. FB_1 has been shown to inhibit the biosynthesis and metabolism of sphingolipids (Merrill *et al.* 1993). Specifically, FB_1 inhibits ceramide synthase, a key enzyme in sphingolipid biosynthesis, resulting in the accumulation of the sphingoid base, sphinganine, to toxic levels (Norred *et al.* 1997). Elevation of sphinganine in liver and blood is in fact diagnostic for exposure to FB_1 (Riley *et al.* 1993). Leukoencephalomalacia in horses (Wilson *et al.* 1992) and rabbits (Bucci and LaBorde 1996) and hepato- and nephrotoxicity in rats, mice, and poultry have been traced to FB_1 exposure (Voss *et al.* 1995). Although it appears to be a weak initiator (Gelderblom *et al.* 1991), FB_1 has been implicated in human esophageal cancer (Rheeder *et al.* 1992; Marasas 1993; Norred and Voss 1994) and hepatocarcinogenesis in rats (Wilson *et al.* 1985; Gelderblom *et al.* 1991a,b). It has also been shown to be a significant promoter of liver cancer in rats (Gelderblom *et al.* 1988) and rainbow trout (Carlson *et al.* 2001). The primary concern is for the effect of FB_1 as a promoter of AFB_1 carcinogenesis, since the two toxins could easily cocontaminate certain grain products, especially corn. FB_1 was

not carcinogenic to rainbow trout but significantly promoted AFB_1 hepatocarcinogenesis at doses of 25 and 100 ppm fed for 24 weeks post-AFB_1 exposure. Sphinganine levels in trout (Carlson *et al.* 2001) and channel catfish (Goel *et al.* 1994) tissues and were similar to those in other animals, showing that it has a similar action in all vertebrates tested. As with many of these toxins, the chance that a significant dosage of FB_1would occur in fish diets is remote but not impossible.

Numerous other mold-produced toxins occur that have produced toxicity in terrestrial animals. Many of these contaminate foods that are not used in fish diet formulation (e.g., ergot toxins in ryegrass and psoralens on celery) so their effects on fish are unknown. I have discussed most of the mycotoxins commonly associated with fish culture. However, this does not imply that others may not be important. As new food ingredients are identified and incorporated into fish diets, their mold contaminants will need to be identified and tested for possible deleterious effects. Whenever general pathological symptoms occur in hatchery fish, the role of a mycotoxin should not be overlooked. It is likely that the toxicities of several mold metabolites new to fish remain to be discovered and researchers are encouraged to test the toxicities of potentially important mycotoxins on various species of fish.

10.3 Nonnatural Components and Additives in Formulated Rations

10.3.1. Organic Chemicals

Contamination of food ingredients by organic chemicals occurs in two primary ways: long-term, low-level contamination of the environment from gradual diffusion of persistent chemicals and their subsequent uptake and bioaccumulation in the food chain and shorter-term, higher-level contamination stemming from industrial accidents and waste disposal. Both of these sources of contamination contribute prominently to the body burden of contaminants in feral fishes, and there are numerous examples of both elevated body burdens and resulting biological effects from fish inhabiting polluted waters (Nadeau and Davis 1976; McCain *et al.* 1977; Pierce *et al.* 1978, 1980; Smith *et al.* 1979; Klauda *et al.* 1981; Malins *et al.* 1984, 1985a,b; Stein *et al.* 1984; Murchelano and Wolke 1985). In feral fish, uptake from the water and sediments contribute to bioaccumulation, but Reinert *et al.* (1974) observed that the most important route of uptake is through the ingestion of contaminated food organisms. Jarvinen *et al.* (1977) disagreed with this conclusion and reported that fathead minnows accumulate more DDT from the water than the diet. However, they found that oral DDT is

more toxic than that absorbed from the water. In most culture situations, the water and sediments, if any, should be of a reasonably high quality, leaving the diet as the major route of exposure to organic chemicals. In most foodstuffs, levels of these contaminants should be low, so any observed effects would be of a low-grade, chronic nature and not distinguishable as organic chemical poisoning. Acute and chronic exposures of fish to these chemicals under laboratory conditions provide our best information on their effects on fish, but we realize that these studies are probably not relevant to exposures under present-day fish culture conditions. Most of the experiments conducted on pesticides in fish have utilized water rather than dietary exposure. Since these studies constitute much of the broad discipline of aquatic toxicology and are not directly associated with fish nutrition, I do not review them here, realizing that this eliminates most of such studies in fish. The organic chemicals for which there are fish dietary exposure data available are very limited, namely, DDT, toxaphene, dieldrin, mirex, the PCBs, and benzo[*a*]pyrene. These compounds are considered in this section.

10.3.1.1. DDT

DDT (Fig. 10.18), the most widely used of the synthetic organochlorine pesticides, was introduced in 1943. Until 1969, when its use in the United States was banned, millions of tons of DDT were sprayed in this country and around the world for the control of general vectorborne diseases and agricultural pests (Deichmann 1972). Because of its fat solubility and resistance to degradation, DDT has persisted in the environment and bioaccumulated in the food chain. By 1969, DDT and its metabolites were found in 99% of all fish samples taken from across the United States (Henderson *et al.* 1969). DDT exerts a number of effects on fish, including acute toxicity (Buhler *et al.* 1969; Macek and Sanders 1970), sublethal effects on the nervous system (Anderson and Peterson 1969; Bahr and Ball 1971), inhibition of gill and kidney Na^+,K^+-ATPase activity (Campbell *et al.* 1974), induction of liver cancer (Halver 1967), and enhancement of the carcinogenicity of other chemicals (unpublished data from our laboratory, 1982). DDT fails to

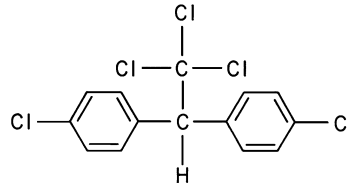


Fig. 10.18

DDT [1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane].

induce the mixed-function oxidase (MFO) system of trout (Addison *et al.* 1977), although it does induce the MFO system of rats (Street 1969).

Buhler *et al.* (1969) fed both technical and *p,p′*-DDT to juvenile chinook and coho salmon for up to 95 days. They discovered that *p,p′*-DDT was more toxic than technical DDT, that chinook salmon were two to three times more sensitive to DDT than cohos, that younger fish were more susceptible to DDT than older fish, and that the LD_{50} values for chinook and coho were 0.0275 and 0.064 mg/kg/day, respectively. DDT was extremely toxic to small chinook salmon at dietary levels above 10 ppm. Median survival times (MST) for chinook fed diets containing 37.5, 150, and 600 ppm were 26.6, 4.8, and 1.6 days, respectively. The MST for cohos receiving 400 ppm DDT in the diet was 7.5 days. Survivors of these trials developed necrosis of distal convoluted tubules along with necrosis and sloughing of the epithelial tissues of the nose or snout. Buhler and Shanks (1970) attributed the higher sensitivity of smaller salmon to at least two factors: (1) younger fish consume more diet per unit body weight than older fish and would consume more DDT per body weight, and (2) young fish have a lower percentage of lipid in which to store and dilute the toxic DDT. DDT in the diet of fathead minnows (*Pimephales promelas*) significantly reduced survival. Maximum acceptable toxicant concentrations for DDT were 0.9 μg/liter for fish exposed to DDT in the water alone and only 0.4 μg/liter for fish exposed to DDT in both the water and the diet (Jarvinen *et al.* 1977).

Since DDT is no longer used in the United States and environmental concentrations are decreasing, it is unlikely that any foodstuff constituents for fish diets would be sufficiently contaminated to cause acute or chronic toxicity in hatchery fish. In the unlikely event of DDT poisoning, the lesions produced by DDT in fish are nonspecific and variable but generally confined to the liver, kidneys, and intestine (Walsh and Ribelin 1975).

10.3.1.2. Toxaphene

Toxaphene is a mixture of chlorinated camphenes containing 67–69% chlorine. It has come into prominence since the ban on DDT and is presently used in much the same ways as DDT was used. In 1976, over 100 million lb was used, primarily on cotton but also on soybeans, corn, wheat, and peanuts. Since each of these commodities can be used in fish diet formulations, the possibility of toxaphene contamination in fish diets is evident. Even though toxaphene is sprayed on agricultural crops, the highest levels of toxaphene are found in fish due to bioaccumulation in the aquatic food chain [National Cancer Institute (NCI) 1979]. Toxaphene is more acutely toxic than DDT to some species of fish, i.e., ictalurids and cyprinids, but less toxic than DDT to others, i.e., centrarchids and salmonids (Macek and McAllister 1970). It appears to be less resistant to degradation than DDT, but it still accumulates in the environment. The Food and Drug Administration, which monitors

pesticide levels in food and feed commodities, has found rising toxaphene residues over the past several years. In 1976, it found toxaphene in over 6% of the samples tested, which were distributed over 16 food and feed categories (NCI 1979). For a period of time, toxaphene was used as a fish toxicant for the removal of unwanted fish, and levels between 0.05 and 0.2 ppm in the water gave complete kills. Its persistence and toxicity to other aquatic organisms were major reasons for its discontinuance as a piscicide in 1963 (NCI 1979). Most of the information on the toxicity of toxaphene comes from water exposures of fish, and little is known about its oral toxicity.

In a test for toxaphene carcinogenicity, we fed rainbow trout fingerlings a diet containing 100 ppm toxaphene and began to observe mortalities after 2 months. The fish exhibited extreme excitability and erratic swimming behavior prior to death. The toxaphene level was reduced to 50 ppm and fed at that level for an additional 16 months. The mortality rate decreased but some mortalities continued to occur throughout the experiment. The total cumulative mortality from the toxaphene exposure was 49% for the 18-month period. Histopathology samples have not been studied at this time, but there was no evidence of carcinogenicity in these fish (Hendricks, unpublished data).

The broken back syndrome, observed in fathead minnows and brook trout, as a result of water exposure to toxaphene (Mehrle and Moyer 1975a,b), was not observed with our dietary exposure. It would seem that channel catfish or species of bait fish that are reared in the southern United States have the greatest risk of being exposed to toxaphene, since most of the toxaphene used in agriculture is used in the South. Foodstuffs, such as soybeans, cottonseed, corn, and peanuts, grown in that region would have the greatest potential for contamination by toxaphene. In 1975, an analysis of wild fish in Louisiana revealed toxaphene residues of over 10 ppm (NCI 1979).

10.3.1.3. Dieldrin

The cyclodiene pesticide, dieldrin, (Fig. 10.19) is another chlorinated hydrocarbon that is no longer in use in the United States, but because of

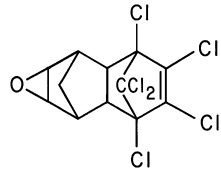


Fig. 10.19

Dieldrin.

its persistent qualities it still can be detected in the environment. As with other banned chemicals, it is unlikely that dieldrin would ever result in acute toxicities in cultured fish, but low-level chronic exposures may result in tissue accumulation or nonspecific chronic effects. Dieldrin is eliminated from fish much more rapidly than DDT. Reinert *et al.* (1974) reported that 89% of the dieldrin accumulated in lake trout was eliminated after 125 days, while DDT residues remained unchanged during the same time period. Several effects of dietary dieldrin on fish have been reported in the literature. Macek *et al.* (1970), Mehrle *et al.* (1972), and Mehrle and Bloomfield (1974) reported that dieldrin, at dietary levels of 1–10 ppm, did not cause reduced growth in rainbow trout. Argyle *et al.* (1975) did observe growth depression in channel catfish fed 4 ppm dieldrin. Hendricks *et al.* (1979) reported slight growth depression with 5 ppm dieldrin in rainbow trout. Other effects of dietary dieldrin in rainbow trout include (1) a reduction of ammonia detoxifying mechanisms (Mehrle and Bloomfield 1974), (2) a disruption of liver mitochondrial membranes (Mehrle and Bloomfield 1974), (3) increased serum amino acids (Mehrle *et al.* 1971), (4) a reduction of liver phenylalanine hydroxylase activity (Mehrle *et al.* 1972), (5) a reduced mortality from DDT intoxication (Moyer *et al.* 1972), and (6) a slight enhancement of aflatoxin B_1 carcinogenesis when fed simultaneously (Hendricks *et al.* 1979). Dieldrin has also been shown to inhibit brain ATPase activity in channel catfish (Desaiah and Koch 1975) and bluegill (Yap *et al.* 1975).

Hilton *et al.* (1983) prepared practical trout diets with Pacific Ocean and Great Lakes (Michigan and Ontario) salmon as the primary protein source. The diets were analyzed for DDT, chlordane, dieldrin, mirex, and polychlorinated biphenyls (PCBs) and then fed to rainbow trout for 24 weeks. Feed consumption and growth were observed during the trial and histology, thyroid hormone levels, and tissue contaminant residues were determined at the end. The authors found no significant differences in the final body weights, feed-to-gain ratios, or mortality rates. There were no histopathological abnormalities, thyroid hyperplasia, or any declines in serum T_3 or T_4 levels even though contaminant residues were elevated in Great Lakes salmon-derived diets. Therefore rainbow trout do not appear to be adversely affected by the uptake and accumulation of these contaminants, although Leatherland and Sonstegard (1982) observed a lowered thyroid function and reduced ability to mobilize liver glycogen in coho salmon fed similar diets. Levels of contaminants in the most contaminated diet (whole Lake Ontario salmon) and fish carcasses, respectively, after 24 weeks on the diet, were as follows: total DDT, 2.10 and 5.36 ppm; dieldrin, 0.11 and 0.23 ppm; chlordane, 0.15 and 0.29 ppm; and PCB, 3.60 and 7.62 ppm. These results reveal that three banned organochlorine compounds still accumulate significantly in the aquatic food chain of some environments, resulting in

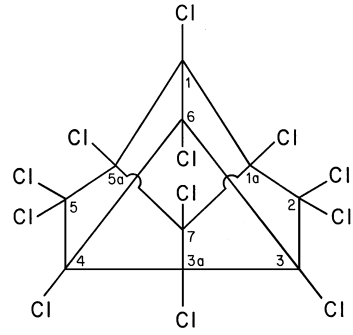


Fig. 10.20

Mirex.

unacceptable residues of these materials in cultured fish flesh. These materials may have only a minimal effect on the fish themselves but the contaminants may be unacceptably high for human consumption.

10.3.1.4. Mirex

Rainbow trout fed mirex (Fig. 10.20) at 50 mg/kg had serum T_3 levels which were significantly lower and a T_4:T_3 ratio which was significantly higher than those in control fish (Leatherland and Sonstegard 1980). Tenfold increases in dietary mirex (5, 50, and 500 ppm) resulted in only 4.56- and 1.35-fold increases in the carcass mirex content. Coho salmon responded to 50 mg/kg dietary mirex with similar effects, i.e., reduced serum T_3 levels, increased serum T_4:T_3 ratios, and reduced body weights (Leatherland and Sonstegard 1978). These studies comprise all that is known about dietary exposure to mirex. Since it is not highly toxic and expected levels of mirex in feed ingredients would be low, it is unlikely that any observable effects would result from mirex contamination.

10.3.1.5. Polychlorinated Biphenyls

PCBs are ubiquitous, persistent, industrial chemicals that biomagnify in the food chain and cause a variety of effects in humans and animals, including fish. Although industrial production of PCBs has been discontinued in the United States, their persistent nature ensures that PCBs will continue to contaminate soils, bodies of water, sediments, and living organisms in contact with these substrates, for years to come.

Acute oral toxicities of PCBs to cultured fish are not relevant except in extremely rare cases of unknown contamination of feed. These acute toxicities have been determined in several fish species, mostly by the water exposure

route (Stalling and Mayer 1972). One of the primary sources of PCBs in fish diets is the fish oil used as the lipid source. It is virtually impossible to find marine or freshwater source feed components without some PCB residue (Hansen *et al.* 1976). PCB levels in marine fish oil are usually low, but continuous feeding can result in detectable levels of PCB in cultured fish. Rainbow trout broodstock at our facility are fed an Oregon Moist Pellet-like diet containing as major ingredients herring meal, ground whole shrimp or krill, ground wet fish, and supplemental herring or salmon oil with a total lipid content of 15–20% on a dry weight basis. Eggs taken from females fed this diet for 2 years had PCB residues of approximately 0.5 ppm on a whole-egg basis or 11.4 ppm in egg lipids at the time of spawning (Hendricks *et al.* 1981). This PCB contamination most likely originated in the herring oil or lipids from the fish or shrimp carcasses. Two-year-old rainbow trout gravid females that were fed a 200 ppm Aroclor 1254-containing diet for 2 months prior to spawning produced eggs that contained 1.64 or 45.3 ppm PCBs on a whole-egg or total lipid basis, respectively (Hendricks *et al.* 1981b). Leatherland and Sonstegard (1980) and Cho *et al.* (1974) have observed similar PCB residues in trout fed commercial rations. We did not observe any reduction in egg viability, hatchability, or fry survival compared with controls, but Jensen *et al.* (1970) reported that PCB levels of 7.7 or 34 ppm in the lipid of Atlantic salmon eggs caused 34 and 100% mortality of the fry, respectively. Aroclor 1242 at about 41 ppm in the lipid of rainbow trout eggs caused 75% mortality of the fry by 30 days posthatch and 60–70% of the fry were deformed (Hogan and Brauhn 1975). Our experimental fish are fed a semipurified (Sinnhuber *et al.* 1977a,b) diet consisting of casein, dextrin, gelatin, and salmon oil (10%) as the major ingredients. Rainbow trout fed this diet have low but detectable (<1-ppm) levels of PCBs on a whole-body basis (Hendricks *et al.* 1977, 1980a; Shelton *et al.* 1983, 1984b). This contamination almost certainly originates with the salmon oil component of the diet. In these experiments, trout fed diets containing 100 ppm PCB as Aroclor 1254 for 1 year accumulated PCB body burdens of 75–100 ppm on a whole-body basis (Hendricks *et al.* 1977, 1980a). This level of PCBs (100 ppm) had no effect on growth rate, but this level, as well as a lower level (50 ppm), of PCBs does result in a pronounced induction of the trout hepatic MFO system (Voss *et al.* 1982; Shelton *et al.* 1983, 1984b). Other investigators have also observed hepatic MFO induction in various salmonid species by orally administered PCBs (Addison *et al.* 1977, 1978, 1979; Lidman *et al.* 1976; Gruger *et al.* 1977).

In another unpublished study, we fed high doses (500 ppm) of three PCB mixtures, Aroclor 1242, Aroclor 1254, and Aroclor 1260, for 18 months to test for the carcinogenicity of these compounds to rainbow trout. No carcinogenicity was observed with any of the mixtures, and only Aroclor 1242

reduced the growth rate. We have observed minimal histopathology in trout fed 50 and 100 ppm Aroclor 1254. The high (500-ppm) doses caused hyperplasia of the thyroid, but no grossly evident goiters. This observation supports the findings of Leatherland and Sonstegard (1978, 1980), who reported depressed serum thyroxine (T_4) and triiodothyronine (T_3) levels in rainbow trout fed a diet containing 500 ppm PCB for 1 to 3 months. Epizootiological studies of Great Lakes coho salmon suggest that goiters in these feral fish may be the result of goitrogens, such as the PCBs, acting in concert with low environmental iodine levels in Great Lakes water (Black and Simpson 1974; Sonstegard and Leatherland 1976; Moccia *et al.* 1977, 1981). In our experiments with rainbow trout, PCB doses of 100 (Hendricks *et al.* 1977) and 500 ppm (unpublished results) reduced hepatic glycogen storage, giving the livers the appearance of a higher cellularity per unit area. This may be due to the smaller cell size resulting from the depletion of glycogen. Nestel and Budd (1975) described similar effects of Aroclor 1254 on rainbow trout liver. Nestel and Budd (1975) also described a kidney nephrosis condition in rainbow trout fed 100 ppm PCB but we have not experienced this effect. We have observed hyperemic spleens with reduced amounts of white pulp tissue in trout fed 100 ppm or 500 ppm PCB. Ultrastructurally, Hacking *et al.* (1977) described irregular and bizarre hepatic nuclei in rainbow trout fed 10 or 100 ppm Aroclor 1254 for up to 11 months. They also observed increases in the smooth endoplasmic reticulum, an altered rough endoplasmic reticulum, increased lysosomes, reduced glycogen, increased lipid, hypoxic vacuoles, and concentric membrane arrays and myelin figures in intracellular spaces and Golgi cisternae. Hawkes *et al.* (1980) and Hawkes (1980) described intestinal pathology in chinook salmon fed 5 ppm PCBs, along with proliferated smooth and rough hepatic endoplasmic reticulum, but no reduction in growth. Freeman *et al.* (1982) described pathological changes in the testes, gills, and liver of Atlantic cod fed 5–50 ppm Aroclor 1254 for 5.5 months.

Klaunig *et al.* (1979) administered Aroclor 1254 to channel catfish by a single intubation (1000 mg/kg body weight) followed by sacrifice 21 days later. They reported MFO enzyme induction and extensive proliferation of the smooth endoplasmic reticulum in hepatocytes examined ultrastructurally. Hansen *et al.* (1976) fed channel catfish a diet containing 20 ppm Aroclor 1242 for 20 weeks and observed reduced weight gain and liver hypertrophy. The same fish were then given a control diet for 8 weeks followed by 20 ppm Aroclor 1254 for another 8 weeks. During this time, liver weights approached control values and the fish gained faster than the controls. PCB accumulation was stopped but levels remained constant during the 8 weeks of control diet. After resumption of PCB feeding, accumulation proceeded as during the first exposure period. Zitko and Hutzinger (1976) fed PCB or

polybrominated biphenyl (PBB)-contaminated food to Atlantic salmon and found the accumulation to increase with increasing substitution by halogens. They also found the concentration of chloro- and bromo-substituted compounds to be similar. Zitko (1977) found that fish accumulate less PBBs from the water than PCBs, but equal or greater amounts of PBBs from the food than PCBs.

Another biological effect of PCBs that we have observed in rainbow trout is the inhibition of AFB_1 carcinogenesis. When PCBs and AFB_1 were fed simultaneously (Hendricks *et al.* 1977; Shelton *et al.* 1984b) or when PCBs were fed prior to AFB_1 (Shelton *et al.* 1983), there was a significant reduction in the number of fish with hepatic tumors and the number of tumors per fish. Simultaneous feeding of PCBs with diethylnitrosamine, however, enhanced the hepatic tumor response in rainbow trout, indicating that the nature of the carcinogen is important in determining the effect (Shelton *et al.* 1984a).

These examples illustrate the opening statement that PCBs have a variety of effects in fish. Probably the dietary exposure to PCBs in most culture situations is minimal and would not cause overt effects, but fish culturists need to be aware of the potential problems and insist on high-quality aquatic source dietary ingredients.

10.3.1.6. Polycyclic Aromatic Hydrocarbons

Very little is known about the toxicity of polycyclic aromatic hydrocarbons (PAHs) within the fish culture environment. As mentioned earlier in this section, the accumulation of PAHs from contaminated sediments, water, and food organisms is substantial in polluted environments and results in harmful effects on the resident fish. Expected levels in formulated fish diets would be very low, however, and recognizable effects would not be expected. Dietary exposures of fish to naturally occurring PAHs are limited. A continuous 18-month dietary exposure of rainbow trout to 1000 ppm benzo[*a*]pyrene (Fig. 10.21) (Hendricks *et al.* 1985) reduced the growth rate, caused the induction of liver microsomal mixed-function oxidase enzyme activities, and caused a 25% incidence of hepatocellular carcinomas at 18 months. More recently, the more potent dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P)

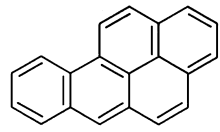


Fig. 10.21

Benzo[*a*]pyrene.

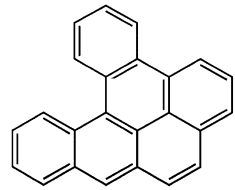


Fig. 10.22

Dibenzo[*a*,*l*]pyrene.

(Fig. 10.22) has been shown to produce tumors of the liver, stomach, and swim bladder of rainbow trout at much lower doses (200–500 ppm) and exposure times (2–4 weeks) (Reddy *et al.* 1999). Thus fish are susceptible to these compounds but no response would be expected at the low levels to which cultured fish might be exposed.

10.3.1.7. Nitrosamines

Nitrosamines, compounds which are carcinogenic to a wide range of experimental animals, can form whenever nitrite and secondary or tertiary amines occur together. This occurrence happens routinely in the preparation or storage of fish by-products, such as fish meal, fish protein concentrates, and fish hydrolysates. Thus the presence of at least low levels of nitrosamines in these fish diet constituents is not uncommon. In most cases these levels are low, but if nitrite was used as a preservative for fish meal, nitrosamine levels could potentially reach toxic levels (Ender *et al.* 1964; Sakshaug *et al.* 1965). Little is known about the oral toxicity of nitrosamines in fish. Grieco *et al.* (1978) determined the median lethal dose of dimethylnitrosamine (DMN) to rainbow trout to be 1770 mg/kg body weight by i.p. injection. This is a high dose, indicating a low acute toxicity, but it may not reflect oral toxicity. The nitrosamines (DMN) and diethylnitrosamine (DEN) have been shown to be carcinogenic by dietary exposure to rainbow trout (Ashley and Halver 1968; Grieco *et al.* 1978; Shelton *et al.* 1984; Hendricks *et al.* 1994) at doses which could have relevance under fish culture conditions. However, I am unaware of any outbreaks of cancer in hatchery trout that have been attributed to nitrosamines.

10.3.2. Heavy Metals

In the field of aquatic toxicology, the effects of waterborne heavy metals on fish have been well documented. Dietary exposure to these metals, however, is rare, a fact that is reflected in the paucity of information available

in the literature. Fish have been exposed to a few dietary heavy metals (i.e., mercury, cadmium, and lead). The observed effects of these exposures are presented as examples of heavy metal oral toxicity in fish. The potential for contamination of fish diet ingredients by these toxicants is low, with the exception of some fish by-products which may have significant levels of mercury.

10.3.2.1. Mercury

Rainbow trout were fed diets containing 4, 8, 16, and 24 ppm mercury as methyl mercury chloride over a 105-day period (Wobeser 1975). The two highest doses produced higher hematocrits and gill epithelial hyperplasia but no mortalities. Muscle tissue accumulated up to 30 ppm mercury. Phillips and Buhler (1978) studied the relative importance of uptake of methylmercury from water and food in rainbow trout. They reported a 68% assimilation of dietary methylmercury and a 10% assimilation of methylmercury from the water. They also reported no mortalities in the course of their experiments. Spinelli and Mahnken (1976) reported the uptake of mercury by coho salmon from diets containing dogfish meal. A diet containing 60% dogfish meal for 210 days resulted in muscle mercury levels of 0.45 ppm, near the U.S. Food and Drug Administration tolerance level of 0.5 ppm. The mercury content of the livers of these fish reached and surpassed 0.5 ppm after 150 days.

These limited studies indicate a low toxicity of methylmercury to fish when accumulated slowly over time. However, fish that ingest mercury can accumulate relatively high levels of mercury which could be toxic to humans. The long half-life of mercury, reported to be over 200 days in rainbow trout (Giblin and Massaro 1972), 700 days in northern pike, and over 1000 days in flounder (Jarvenpaa *et al.* 1970), creates a long-term hazard from mercury-contaminated fish and should caution us not to expose food fish to sources of mercury.

10.3.2.2. Cadmium

English sole (*Parophrys vetulus*) were given cadmium chloride at 5 μg/g body weight by stomach intubation at weekly intervals for 4 weeks. Another group of fish was given the same cadmium dose plus 25 μg Aroclor 1254/g body weight for the 4 weeks. Cadmium alone produced liver necrosis and karyomegaly, while PCB reduced the incidence of these lesions in the fish exposed to cadmium plus PCB. Mortalities occurred in both groups, with a maximum of 50% in the cadmium plus PCB group (Rhodes *et al.* 1985). This is the only experiment of which I am aware that looked at the effects of orally administered cadmium.

10.3.2.3. Lead

Hodson *et al.* (1978) fed lead to rainbow trout and concluded that dietary lead was not absorbed by fish. The lead content of these fish was not elevated above control levels, and the majority of lead consumed was present in the feces.

10.3.3. Nonnutritive Additives

A number of materials are added to diets for purposes other than nutrition. These include antioxidants, hormones (growth promoters), antibiotics, binders, preservatives, and carotenoid supplements. All of these categories are described in Chapter 9 with regard to their purposes in practical fish diets. Since these ingredients are added intentionally, at predetermined levels, it is unlikely that they would result in toxicities under production conditions. A great deal of research has been conducted, especially on steroid hormones, to determine both desirable and undesirable effects, and then dietary levels have been selected accordingly.

10.4 Summary

I have discussed the effects of a wide range of chemicals or other materials that occur either intentionally or unintentionally in fish diets. Several of these have caused severe losses to fish culturists in the past (i.e., the aflatoxins), but losses related to diet toxicity are becoming more and more unusual. This improvement is the result of several factors: (1) improved knowledge of fish dietary requirements and the foodstuffs that best supply those needs, (2) increased knowledge of the constituents of commonly used foodstuffs, (3) improved awareness of the toxicology of both the naturally and the unnaturally occurring antimetabolites in fish diets, (4) elimination of problem-causing ingredients from fish rations, (5) improved diet preparation equipment and storage facilities, and (6) improved analytical and fish cultural facilities. The possibility of new toxins entering the fish cultural arena should keep culturists and nutritionists on the alert, but dietary toxicity should become less and less troublesome as fish culture continues to improve.

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11

Special Feeds

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11.1 Introduction

As new data on the nutrient requirements of both finfish and shellfish accumulate, the day is approaching when feeds will be individualized for different farmed species as well as for each life-history stage of each species. Then all feeds will be "special feeds." Until that day, although a number of feeds at present are formulated for a specific maturation stage of a specific species (e.g., certain prawn diets), the application of scientific and engineering principles to the design and manufacture of fish[1] feed is a limiting factor to a worldwide economically viable aquaculture industry. The overall profitability of raising and marketing animals depends to a large degree on preventing mortality during the early stages of life and maximizing the efficiency of feed utilization during the entire life cycle. This requires feed that is specially formulated and manufactured for each specific situation. Hence, the economic success of the commercial terrestrial animal industry has been dependent largely on "special feeds." The physical and chemical properties of feeds are controlled to ensure that the nutritional value of all components in the feed is utilized by the animal to the fullest extent. The catfish industry in the United States is probably the leader in pioneering this type of economics as applied to aquaculture. Furthermore, aquaculture must be based on sustainable ecological balances, with integrated use of resources, especially finite resources, including fresh water and fish meal. These topics are covered in more detail in chapters 7, 9, 12, and 13. The purpose of the present chapter is to present an integrated overview of these topics, showing how they are interdependent and linked to sustainable, successful fish production.

11.2 Formulation of Special Feeds

11.2.1. Factors Affecting Formulation and Manufacture of Feeds

11.2.1.1. Species

Different species of fish have various preferences or habits in consuming food. The position in the water (e.g., bottom, midwater, or surface), color, odor, texture, shape, and size, and other factors control their desire and/or ability to consume feed and flourish.

Fish exhibit diversity in food gathering but may be classified into a limited number of feeding types (Hyatt 1979). Salmonids prefer floating or slowing

[1] Throughout this chapter the term "fish" refers to both finfish and shellfish.

falling particles, whereas eels and crustaceans are bottom feeders. Channel catfish are normally bottom feeders but can be trained to consume salmonid-type feeds (Mgbenka and Lovell 1984). Although bottom feeding is natural and beneficial for some species, others, such as salmon, waste feed and grow poorly when forced to feed off the bottom of tanks and pens.

Of particular importance to the future of aquaculture, especially shrimp production, is the feeding of larvae to eliminate reliance on the capture of berried females and postlarval forms. Although many larval species have been successfully grown on live food organisms, this source of feed is expensive and sometimes inadequate. Research to develop water-stable diets for larvae continues (Pigott *et al.* 1982; Pigott 1999c).

11.2.1.2. Nutritional Components

A properly compounded diet must contain adequate energy as well as all of the essential amino acids, vitamins, minerals, and other required growth factors. Quite often the quantitative requirements for these compounds are not known, and therefore, a trial-and-error approach to formulation of diets has been employed. A diet is formulated to give maximum growth and yet be economically available to commercial operators. A major problem with selecting components for feed formulation is balancing the nutritional requirements of the fish with the availability and cost of the ingredients. This is the essence of feed formulation, and the balance between optimum nutrition and economy will change with the life-history stage and the purpose of fish rearing, e.g., food production, broodstock, and fish raised for stocking.

11.2.1.3. Aquaculture Environment

Many environmental factors must be considered in preparing feeds. This is particularly true in formulating and manufacturing "special feeds," where these factors have a major impact on the cost and efficiency of feeding fish, regardless of the diet. The water temperature and temperature gradient, water flow rates, salinity, turbidity, depth, bottom and side conditions, materials of construction, fish density, fish size and size distribution, and state of the growing cycle all have a bearing on the requirements for specific physical and chemical properties of a feed.

Ecological preservation has become a worldwide concern. Aquaculture is the focus for many groups opposing pollution of the environment. Metabolic wastes and feces, as well as uneaten feed, contribute to and are blamed for environmental pollution. Additionally, wasted feed increases production costs and pollution affects animal health. Several possibilities exist and are being researched to reduce the pollution associated with aquaculture. As more accurate data are assembled regarding actual nutrient requirements for each species, feeds can be formulated to increase the retention of specific

polluting ingredients (e.g., nitrogen and phosphorus), thereby lowering the amount not retained and discharged into the aquatic environment. Increasing palatability will decrease feed waste, as will improvement of management techniques. Use of feeding trays, for instance, is one recommended method. More careful attention to feeding times and appropriate feeds for specific stages of maturation is also ecologically friendly (Lee and Lawrence 1997). The best possible feed will yield poor results if it is not handled, stored, and used properly.

Much of the research on reducing the use of polluting ingredients has been with salmonid feeds. Partial replacement of fish meal with corn or wheat gluten yielded a low-phosphorus feed which created no flavor changes in the flesh of rainbow trout (Skonberg *et al.* 1998). Maximum retention of both phosphorus and nitrogen from commercial feeds will reduce both in effluent. Sugiura (1998) found that citric acid increased the availability of many minerals in fish meal-based diets and also increased the effect of supplemental phytase in soybean meal-based diets, thereby reducing the phosphate pollution to the environment. In Tahiti and New Caledonia, intensive shrimp culture has increased the need for a "low-pollution" diet. Research to decrease nitrogenous waste by reducing crude protein levels in practical diets is being conducted (Baillet *et al.* 1997).

The high cost and dwindling supply of fish meal necessitate the use of alternative protein feed ingredients. Nutritionally, the best feed ingredients have a composition similar to that of the animal being fed. This is especially important with essential amino acids. Numerous studies report partial substitution of fish meal in diets of finfish and crustacean species. Salmonid diets with varying percentages of plant products such as wheat gluten meal, corn gluten meal, soybean meal, and soy protein concentrate have been reported (Hardy 1996; Haard *et al.* 1996; Krogdahl *et al.* 1994; Kaushik *et al.* 1995). Growth responses of shrimp at various maturation stages for feeds containing different protein sources have been measured (Sadhana and Neelakantan 1997; Che 1992; Stickney *et al.* 1996; Akiyama 1991; Lim *et al.* 1997).

11.2.1.4. Physical and Chemical Properties of Feeds

Many of the feed components being used in conventional fish diets are marginal contributors to the nutritional value because of the chemical or physical state in which they are fed. Most of these limiting conditions can be altered by judicious selection of processing techniques used to prepare feed ingredients. Considering the relatively few basic processes and operations available for preparing feed components or formulations, it is important to combine the various methods of manufacturing to maximize the feed quality. Not only is it important that fish can efficiently metabolize the ingested

feed, but the nutrient components must be stable under the conditions that are encountered during shipping, storing, and feeding. Some of the factors that limit the efficacy of a fish feed are as follows.

(1) A particle size larger than the mouth or esophagus of a fish or larva that cannot tear or chew the particles to reduce the size.
(2) Leaching of nutrients by the water before the fish can eat the feed.
(3) Selective leaching of specific important nutrients by the water.
(4) Severely reduced nutritional properties of a diet or its components due to uncontrolled processing.
(5) A short shelf life of feeds.
(6) Poor binding characteristics of feeds.
(7) Improper density, odor, taste, appearance, etc.

As is true of all animals, sensory responses control the acceptability of a particle of fish feed. These include visual perception, electrodetection, mechanoreception (sound and turbulence), chemoreception, odor, and taste (Hyatt 1979). Affected stimuli are size, movement, shape, color and contrast, and odor. These factors in a feed are related to the feed composition and manufacturing techniques.

Search behavior is adaptive and related to the particular sensory capacities of each species. For example, coprophagy is an important element in feeding many caridian and decopod crustaceans. Panaeid shrimp feed most readily on small-diameter, ribbon-like feeds resembling fish and invertebrate fecal materials (Costa-Pierce and Laws 1985).

11.2.1.5. Water Stability

One of the most important requirements for a feed is that it remains intact, without losing any nutrient components, for a specified period after it enters the water. Since the length of time for which the diet should remain intact in the water depends on the fish species and growing facility, requirements for stability vary for different feeds. An extreme case can occur with crustaceans, which are nibblers and intermittent feeders and often allow food to remain on the bottom for several hours before consumption (Goldblatt *et al.* 1979).

11.2.1.6. Particle Density

The density of a diet particle controls its position in the water, an important consideration depending on whether the species being fed is a surface or bottom feeder or prefers to consume feeds in the water column. The conventional compacted pellet is dense and quickly settles to the bottom. Hence, special density control is an important factor in the preparation of diets. One type of floating ration is produced by extrusion during which

starch binders gelatinize and trap air. Utilization of this bulky extruded feed is more efficient (Mgbenka and Lovell 1984).

11.2.1.7. Color and Contrast

Adding color pigmentation to simulate natural foods can control the visual perception of a feed particle by the fish. Colors can also be used to cause light refraction that simulates motion of live prey (Meyers 1979). Color can be added as an inert additive or can be from a material that also includes volatile compounds to give additional attraction. Krill acts in the latter manner, supplying both the reddish attractant color and the natural krill odor.

11.2.1.8. Odor

In a symposium review, Meyers (1979) summarized the effects of feed odor on the attraction of various species. Catfish are attracted by amino acids, especially alanine. Turbot show a preference for inosine and inosine monophosphate. Fish oil attracts some but not all fish. Carp are less responsive to odor and more responsive to color and shape.

Some attractants for crustacea are powdered *Artemia,* shrimp by-products, glucosamine, and glycine. Feeding stimulants are low molecular weight compounds such as certain amino acids, peptides, and betaine (Teshima *et al.* 1993; Tidwell *et al.* 1998). Certain amino acids and nucleotides added to feed improve shrimp flavor and increase market desirability (Kanazawa 1997). Adult shrimp seem to respond to chemicals released by prey, while larvae, which use new food sources with each stage, respond to a broader range of feeding cues (Lee and Meyers 1997). Intensive culture of shrimp carries with it an increased risk of disease. Since antibiotic use is limited, alternatives such as antioxidant nutrients and omega-3 highly unsaturated fatty acids (ω-3 HUFA) can increase the immune response and disease resistance. Carotenoid pigments, especially astaxanthin, must be included in shrimp diets not only to provide coloration, but also to provide a metabolically active antioxidant. The stress of intensive and semiintensive shrimp culture requires antioxidants for disease resistance, optimal growth, and coloration (Meyers and Latscha 1997).

11.2.1.9. Digestibility

Obviously, no matter how well formulated nutritionally, feeds are of no use unless they are digestible and appealing. Additionally, due to environmental needs for low-pollution feeds, they must be highly assimilated by the animal being fed. Poor digestion is the most important factor limiting nutrient availability. A number of factors influence digestibility such as species and age, environment (salinity, temperature, hypoxic conditions),

palatability, fiber content, and feed processing. How the feed is processed is significant in relation to nutrient availability. Some binders contribute compounds which act as negative feeding stimulants.

11.2.1.10. Particle Texture, Size, and Shape

A major problem with formulated or synthesized diets is the optimization of certain physical properties of the individual feed particle to ensure that it is not only attractive but also ingestible by the fish. Texture is important to the control of density, water absorption, and leaching, as well as other physical factors that alter the acceptability of a feed particle. Larvae can consume larger particles of live zooplankton than those in a dry diet, which are neither elastic nor compactable after swallowing (VanLimborgh 1979). This property of deformation or collapsibility is most difficult to incorporate into a manufactured feed particle, particularly a dry particle that has a "toughened" texture due to drying. Many fish larvae have esophagus diameters of 50 μm or less. A larger particle may be attractive to the fish as a food but not available to the individual that cannot chew or tear the particle apart. Conventional machine reduction of particle sizes to less than 50 μm is difficult, especially in dry feeds. However, homogenization of a slurry (e.g., hydrolyzed fish protein) followed by drying and sizing of the resulting flakes can enable the manufacture of complete feeds for larvae (Pigott *et al.* 1982; Tucker 1999).

The geometry of the particle, as well as the texture and composition, is important as related to the amount of leaching that takes place in the water. Extremely small particles have a much larger surface area per unit weight of the feed. Also, those of an extremely irregular geometry have more surface area than round particles. Physical parameters that increase surface area also increase leaching of unstable ingredients, unbalancing the nutritional value of the portion of the diet that is consumed.

11.2.1.11. Storage Stability

The best diet for fish is, like that for humans, one that is prepared fresh every day. However, this is not practical in most situations involving the rearing of large quantities of fish. Therefore, feeds that must be purchased and stored for periods of time must be stable under the storage conditions. These, of course, vary in time, temperature, and humidity with both the time of the year and the geographical location.

The economic viability of commercial aquaculture is dependent on the use of inexpensive rations. This is best accomplished in the mass manufacture of feed by dry diets that are easy to transport and store. Also, the nutritional components must be stable over the shelf life of the products. These requirements can be met by a combination of obtaining the best

ingredients for a feed, using good manufacturing procedures, and packaging the final product properly. A dry diet stored in a cool, dry environment in a package that allows minimal exposure to the air will normally be stable for long periods of time.

Improper handling and storing of feed by on-site hatchery personnel can negate good processing methods. Maintaining the nutrient quality of the feed during on-site storage is equally as important as all other factors involving the quality and effectiveness of a given formula.

11.2.2. Special Ingredients

11.2.2.1. ω-3 Highly Unsaturated Fatty Acids (HUFA)

A major selling point for fish is the health value of the highly unsaturated ω-3 fatty acids in the fish oil. These oil fractions are unique in that primarily plankton and plants grown in the aqueous environment produce these highly desirable fatty acids. Fish consume the plankton or other smaller fish or shellfish that feed on plankton and, therefore, have a high ω-3 (or *n*-3) content in their oil. At the present time, many aquaculture fish have low values of *n*-3 fatty acids since their source of feed is a prepared diet in which the oil is of plant origin. However, including fish oil in the diets can substantially increase preferred types of fatty acids.

Although only plants and algae make *n*-3 fatty acids, elongation and desaturation of HUFA occur along the food chain as fish metabolize and store these compounds. Furthermore, terrestrial plants, such as soybeans and rapeseed, containing small amounts of *n*-3 fatty acids, provide only linolenic acid, C18:3, *n*-3, not HUFA. This fatty acid is not elongated and desaturated efficiently by mammalians (Tucker 1995). Likewise, crustaceans appear to have a limited capacity for bioconversion of *n*-3 PUFA to *n*-3 HUFA (Jones *et al.* 1997; Boonyaratpalin 1998).

ω-3 fatty acids (*n*-3), particularly those found in high-quality fish oil, EPA (C20:5 *n*-3) and DHA (C22:6 *n*-3), are essential for the health, growth, and survival of larval as well as older fish and shellfish. DHA is essential for growth and optimal development of the brain and nervous system in humans as well as other animal species. Meyers (1979) speculated long ago that these long-chain HUFA might provide the intrinsic value of *Artemia* for larval diets. Certainly, a purely artificial diet for larvae must include *n*-3 fatty acids, preferably HUFA. Immune system functions are also enhanced by *n*-3 fatty acids so these lipids should improve survival as well. HUFA also seem to be required for ovarian maturation and spawning of crustacea (D'Abramo 1998). Wild larvae and broodstock of *Penaeus vannemei* had lipid profiles showing 27% of the total lipids as HUFA (Pedrozzoli *et al.* 1998), and therefore, artificial diets, at least for marine shrimp species, should attempt to

maintain these levels. To ensure that aquaculture products are nutritionally equivalent to, or better than, wild-caught, supplemental marine oils are essential in feed—especially when formulations contain a significant percentage of agricultural products (Pigott *et al.* 1987; Pigott 1989; Tucker 1999). Freshwater species typically contain a higher *n*-6:*n*-3 ratio of fatty acids. Additionally, the environmental temperature influences the levels of various essential fatty acids, with colder-climate fish and shellfish incorporating higher levels of *n*-3 HUFA than warmwater species. Accordingly, feed should be adjusted. The HUFA requirement for freshwater crustaceans appears to be about one-tenth that for marine species, although water temperature also plays a role (D'Abramo 1997). HUFA incorporation into tissues of all animals reflects the diet.

One good source of essential fatty acids is fish meal made from fish; however, the essential fatty acid content alone can be misleading since the meal from a low-fat fish (e.g., white fish) is actually much lower in total fat than that from a high-fat fish, such as herring, anchovy, and menhaden. Table 11.1 shows a comparison of HUFA between wild fish and farmed fish that have no significant HUFA in their diets. Although the wild fish have a much higher HUFA content than the farmed fish, note that farmed crayfish have about the same HUFA content as wild crayfish. This is due to the fact that crayfish are in contact with the bottom of the ponds and tend to eat algae and other naturally growing vegetation. Wet diets, containing a high percentage of fresh fish portions, having traditionally been fed to hatchery-raised Pacific salmon fry and fingerlings. The flesh of these fish contains a much higher HUFA content than does that of commercially raised trout fed a dry diet.

Table 11.1

Relationship between *n*-3 and *n*-6 Contents of Oil from Edible Portions of Wild versus Pond-Reared Shrimp, Crayfish, and Catfish[a]

Source	Total PUFA (%)	Fatty acids (%)		Ratio	
		n-6	*n*-3	*n*-3/*n*-6	HUFA/*n*-3
Marine shrimp	45.15	16.88	28.28	1.67	1.33
Pond-reared prawns	41.64	23.04	18.60	0.81	0.66
Wild crayfish	50.12	16.38	33.74	2.06	1.55
Pond-reared Crayfish	47.50	16.64	30.84	1.86	1.49
Wild catfish	39.77	12.13	27.64	2.54	2.00
Pond-reared catfish	26.07	15.85	10.22	0.62	0.48

[a] Adapted from Channugam *et al.* (1986).

There is also a large variation between the *n*-3 and the *n*-6 fatty acid content in wild fish. In addition to the variations between species, there are major differences within a given species or group at different periods in growth and development, especially during the spawning cycle. This most likely accounts for many of the differences in results of research involving the effects of HUFA in human and animal nutrition, especially during the early work when the product was simply cited as "fish oil." It is extremely important that a complete analysis of any given fish oil be made prior to feeding in nutritional or clinical tests. Furthermore, over long, extended tests, the oil should be periodically analyzed to check on chemical and oxidative changes taking place over time.

The interest that has developed over the past few decades in the *n*-3 HUFA contained in fish oil has stimulated worldwide research on methods of refining oil for human consumption. This has resulted in numerous projects, ranging from the recovery and stabilization of high-quality oils to clinical investigations studying the effects of *n*-3 fatty acids on reducing heart and other diseases. It is interesting to note that the major efforts in producing high-quality "heart-healthy" products from fish oils are paralleled by an increase in the use of fish oil for fuel in the high-sea fleets processing fish. This is especially true for the surimi operations, where the oil from fish is removed during washing of the flesh. Although the fish, such as pollock, used for surimi are low in oil content, the large volume of fish processed results in considerable oil recovery from the wash water. It has proven more economical at the present time to burn the oil with the ship fuel than to transport it to the distant, currently low-priced, markets where it is used for industrial purposes or for production of margarine.

Although there is a considerable amount of fish oil in the fish meals fed to animals, the specific feeding of fish oil has not been practiced to a large extent. Fish meal containing fish oil is fed in the diets of poultry, pigs, fish, crustaceans, ruminants, fur-bearing animals, and pets. Special efforts are being made to improve the quality of the protein and oil in fish meal so that many commercial animals, currently not fed significant amounts in their diet or needing better-quality meals, can utilize this high-protein supplement (Pigott 1997; Bimbo and Crowther 1992). These products, produced from menhaden, have proven to be beneficial for early-weaned pigs, high-yielding dairy cows, and aquaculture-raised fish.

Poultry could be a major source of *n*-3 fatty acids for humans if poultry feeds include high-quality fish oil high in HUFA content. Recent studies have indicated that chickens can be a source of *n*-3 fatty acids that is equal to, albeit different from, that of cod fish (Opstvedt 1985; Hulan *et al.* 1988). These studies have shown that significant amounts of *n*-3 HUFA can be incorporated in poultry diets without affecting the meat flavor. However, it

should be emphasized that only fish meal specially processed to minimize heat degradation and oil oxidation is utilizable. Many conventional meals are limited as to the amount that can be fed in poultry diets without the fish flavor, caused primarily by rancid oil and other degradation products, being transferred to the meat.

Since a large part of the world's population consumes poultry, especially chickens, as a significant portion of the meat protein in their diets, considerable effort is being expended to produce low-cholesterol, high-*n*-3 fatty acid eggs from layer hens (Hulan *et al.* 1989; Stadelman 1989). Up to 6% refined menhaden oil can be added to the total diet without affecting the flavor (Yu and Sim 1987). A layer hen diet containing 3% menhaden oil was shown to increase the EPA and decrease the ratio of *n*-6 to *n*-3 from 18 to 3 (Oh *et al.,* 1988). Other experiments have shown that regular menhaden oil, stabilized with antioxidants, could be fed to layer hens at a level of 3% of the diet without causing a "fishy" flavor (Hargis *et al.* 1991).

11.2.2.2. *n*-6/*n*-3 Ratios

The importance of the ratio of *n*-6- to -*n*-3 fatty acids has only recently been seriously addressed in human nutrition. The dramatic increase in dietary vegetable oils (*n*-6) during the past half-century has resulted in increased immunosuppression and inflammation. Human diets are now 20–40:1 *n*-6:*n*-3, whereas for hundreds of thousands of years the ratios had been 1–4:1. Lim *et al.* (1997) showed that *n*-6 and *n*-3 fatty acids are essential for juvenile *P. vannemei,* but *n*-3 fatty acids promoted faster growth than *n*-6 fatty acids. The *n*-3 HUFA had better growth-promoting effects than linolenic acid. Preferential incorporation and conservation of HUFA in polar lipids of crustacean tissue have been demonstrated (D'Abramo 1998). Perhaps one reason that alternate sources of protein from plants, with their differing *n*-6:*n*-3 content, have limited usefulness, especially for marine species of crustacea, is due to an alteration of the *n*-6:*n*-3 ratio.

11.3 Feed Manufacturing

11.3.1. The Challenge

The world's fish meal production reached a maximum in the late 1980s and has been more or less fixed at 6–7 million metric tons per year for the last decade. Meanwhile, the use of fish meal in fish feeds has increased dramatically, mostly at the expense of its use in poultry and swine feeds. Obviously, higher levels of alternate protein sources, from both animal and plant sources, must be used in fish feeds as aquaculture production

increases. A trial-and-error approach to formulating diets has given way to a more scientific method for development of manufactured diets. However, most of the normal ingredients are whole industrial fish, discarded by-catches, processing waste, and inexpensive by-products from other food industries of commodity-type agricultural products. Feeds have been formulated based on protein quantity rather than quality. Balancing nutritional requirements with the availability and cost of ingredients is often less than satisfactory.

The manufacture of fish meal has demonstrable effects on the nutritional value of several ingredients, especially protein. High manufacturing temperatures degrade or damage protein molecules, resulting in a loss of availability of certain essential amino acids. Consequently, a higher percentage of crude protein must be incorporated into diets to provide adequate levels of essential amino acids. Additionally, much heat destruction and oxidation of valuable ω-3 (n-3) fatty acids occur. Hydrolyzed fish protein (HFP), in contrast, is prepared at ambient temperatures and is not "cooked," thereby maintaining the protein and lipid quality. HFP is functional and may be used as a high-quality protein binder in fish (and larval) feeds (Pigott *et al.* 1982).

11.3.2. Conventional Fish Meal

For the past 50, or more, years the conventional meal manufacturing process has not changed. The quality of the commercial meal produced throughout the world varies tremendously depending on the age and maintenance of plants, the type of processing equipment, and the quality of raw materials. High drying temperatures result in protein degradation and oil rancidity. In addition, the quality of the raw material is often quite low, the result of spoilage before the raw material is processed. Hence, although current fish feed formulations are dependent on fish meal as the animal protein ingredient, a different form of fish protein would be an advantage for many formulations.

The severe processing conditions of conventional fish meal production reduce the nutritional value of both protein and lipids and, also, result in a completely nonfunctional protein. The search for improved processes to produce fish protein for diets has been stimulated by the drive to utilize the harvested aquatic raw materials better. The large portion of the world fisheries going to the human consumption market often results a recovery of 30% or less. Since a fish has approximately 60% edible flesh, 20% or more visceral portion, and the remainder trimmings and bone, the economics of processing fish dictates that a larger portion of the raw material be utilized for profitable products. Furthermore, the disposal of the high-protein

Table 11.2

Percentage Yields of Fish for Various Market Forms

Market form	Yield (%)
Fillet	18–35
Steaks	60–70
Smoked	40–70
Canned	60–75
Gutted (head on)	70–85
Headed–gutted	60–80

content waste from processing plants represents a major nonrecoverable cost (Pigott and Tucker 1990).

11.3.2.1. Total Utilization

There is no such thing as true waste from processing raw materials harvested or grown in the oceans and freshwater bodies of the world. If the world aquatic food industry would discard the mind set of "waste," it would be realized that the dreaded materials are valuable "secondary raw materials." Furthermore, total utilization of foods from the marine and freshwater bodies of the world not only can be of major economic value to the industry, but also can minimize environmental effects and current costs of disposing the so-called waste. As shown in Table 11.2, a significant portion of the whole fish is currently wasted or reduced to fish meal.

11.3.2.2. Fish Protein Concentrate (FPC)

The early development of processes for preparing highly concentrated proteins from fish was directed toward high-quality products for human food prepared from the edible portion of the fish. During the 1970s, following efforts by the National Marine Fisheries Service (NOAA) to produce FPC economically by alcohol extraction of edible fish flesh, there was much interest in producing a functional FPC by proteolytic digestion. Countries particularly interested in this area were Denmark, Japan, Norway, the United States, and the United Kingdom. The 1960–1970 efforts to utilize total waste for human food resulted in frustration for many when the U.S. Food and Drug Administration (FDA) would not allow anything but edible flesh to be used in human food. This left a major portion of the processing waste for production of relatively cheap fish meal or discharge to the environment. However, environmental concerns and economic problems within the fishing industry have encouraged a recent renewal of interest in efficiently utilizing secondary raw materials.

11.3.3. Hydrolyzed Fish Protein

11.3.3.1. Enzyme Digestion

Over the years, further development of the process for FPC for human consumption has paralleled the development of other processes to ensure that all portions of a fish are totally utilized for profitable products. For many years programs have been carried out to utilize better these secondary raw materials currently being discarded or made into products that do not maximize the nutritional value. Tryptic digestive enzymes, pepsin hydrolysis, papain, and many other enzymatic processes were tried in an effort to produce a highly functional protein concentrate.

The use of enzymes to hydrolyze fish flesh has been studied extensively for preparation of a highly functional protein powder that can be used in products for human consumption (Tarky and Pigott 1973: Pigott *et al.* 1976). However, this entails adding an enzyme, which significantly increases the cost of production, since there are no concentrations of natural enzymes in the flesh. The basic process consists of adding an enzyme to homogenized flesh, controlling the pH, temperature, and other processing conditions, and then allowing the digestion of proteins in the flesh to take place.

In general, acid pepsin digestion with continuous pH control has proven to be one of the best procedures for producing a high-quality, low-cost hydrolyzate that can be used as an animal or fish feed supplement (Tarky and Pigott 1973). Fish waste, containing the visceral portion, has endogenous enzymes that can be controlled to produce a hydrolyzed product. The primary enzyme in the viscera of a fish is pepsin, the same enzyme that prevails in the human stomach for digesting meats and other protein products. Pepsin requires an acid environment to be active so that, by controlling the pH of a homogenized fish waste, natural enzyme digestion will take place. This eliminates the requirement for adding expensive purified enzymes. Research has shown that the resulting products from natural enzyme digestion can be valuable for animal feed supplements and foliage spray fertilizers (Pigott 1999a,b).

In many fish processing plants the volume of fish waste does not warrant the investment of a fish meal facility for converting the waste to animal feed. Furthermore, meal made from fish waste does not have the nutritional value of conventional meal, which is normally made from whole industrial fish. Because fish meal is nonfunctional due to its having been heated to a high temperature during cooking and drying, it cannot be solubilized in a water solution. The knowledge gained from previous studies on fish protein hydrolyzate for human foods was used to develop a process for converting fish waste into a most effective hydrolyzed fish protein (HFP) for animal feed and fish fertilizer. Used as a foliage spray, this product has

proven to be a superior fertilizer for a wide variety of crops. Research has only recently begun to concentrate on this high-quality protein for fish diets (Pigott 1999c).

11.3.3.2. Animal Feed and Fertilizer

Numerous projects are being directed toward developing highly nutritional feed supplements for animals and well-balanced fertilizers for a wide variety of agriculture crops. Successfully reaching these goals would eliminate the environmental concerns of waste discharge and improve the overall economics of commercial fish processing as well as reducing the damaging runoff from petrochemical fertilizers.

A wide variety of products can be prepared from the normally wasted portion of processed fish. These include high-quality proteins for animal and fish feed. Raw material for production of high-quality products include

(1) discarded by-catch from present fisheries,
(2) industrial fish of high nutritional value but currently being reduced to fish meal and oil,
(3) waste from primary and secondary fish processing operations, and
(4) species not being commercially exploited at the present time.

Acid enzyme hydrolysis not only provides highly functional protein but also includes important macro- and micronutrients that are often lost in other processes carried out at high temperatures or in a neutral or basic environment that does not remove these compounds from bones and cartilage.

There are particular advantages to reclaiming normally wasted portions from aquaculture fish (Fig. 11.1). The fish can be processed shortly after harvest and this prevents the mechanical breakdown and spoilage products found in commercially caught fish that have been held for varying periods of time. This is also true of the minced flesh removed from aquaculture fish for human food products. In fact, the requirement for rapid processing is the reason much of the surimi manufactured from pollock, hake, and other species is processed on high-seas processing vessels. However, the economics of processing aquaculture fish on-site (Fig. 11.2) or in nearby plants is a major advantage over high-seas processing. Also, the utilization of unwashed minced flesh in finished products greatly improves the yield and reduces the cost compared to those prepared from washed flesh (Nielsen and Pigott 1996). A wide variety of products can be prepared from the normally wasted portions of processed fish. The high quality of reclaimed meat from aquaculture fish used in value-added products can significantly increase the profitability of the industry.

For the past several decades there have been active research programs designed to utilize totally the raw materials from the marine and freshwater

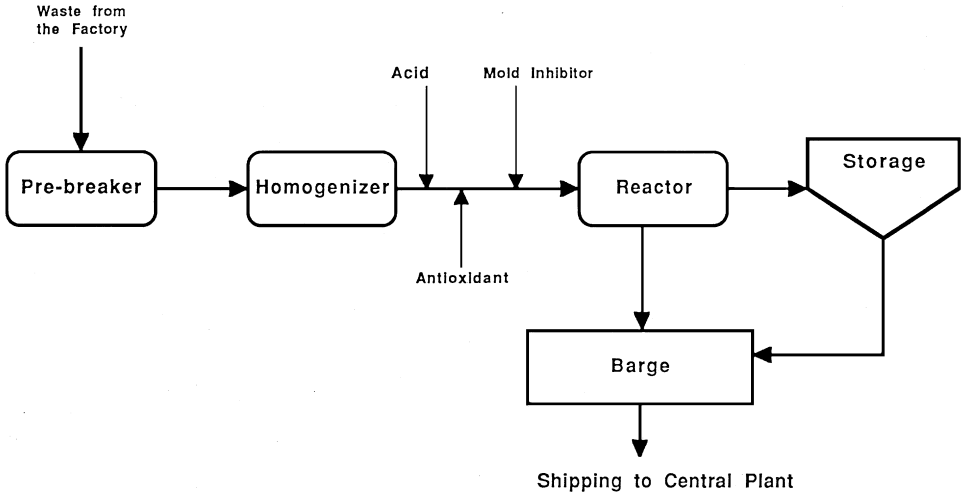

FIG. 11.1

Flow diagram for on-site processing.

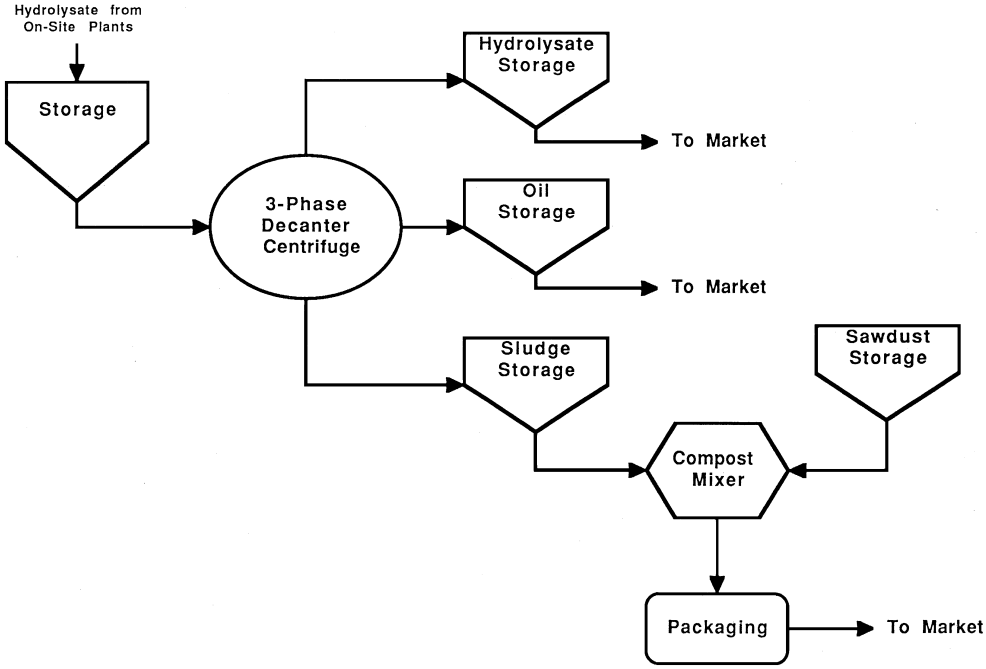

FIG. 11.2

Flow diagram for processing at the central plant.

bodies of the world. This work has covered a wide spectrum of processing technology applicable to aquaculture fish as well as those harvested from wild stocks. The waste from processing operations includes edible flesh, visceral portions, and bones and cartilage. A wide spectrum of products has been developed and studied, including human and animal foods, superior-quality fertilizer, and nonfood commercial products. Of particular interest is processing aquaculture waste and then recycling the superior high-quality proteins in on-site feed manufacturing. Hence, not only is total utilization of the fish accomplished but also there is a tremendous economic advantage in such a concept.

Recent research has demonstrated that HFP can be substituted for fish meal in high-quality fish diets (Pigott 1999c). Initial results indicate that the functional proteins in HFP are more efficient and reduce the cost of feed.

11.4 Summary

Fish nutrition and feed production have improved greatly over the past 15 years, mainly in connection with the growth and demands of the aquaculture industry. Different sectors of the aquaculture industry are more advanced than others, and in the case of the catfish industry, feed formulation, production, and use are approaching the degree of sophistication seen in the poultry feed industry. On the other hand, farming of new species, such as tuna or temperate marine fish species, is relatively new, and virtually nothing is known of the nutritional requirements of these species. In may cases, these species are fed diets containing high levels of frozen or fresh raw fish, rather than diets prepared from common feed ingredients, similar to the situation in salmon, trout, and catfish production in the early stages of their development as farmed species. If aquaculture is to realize its potential to produce high-quality seafood for the expanding world population, it must do so by following the lead provided by other animal production sectors of agriculture. In other words, aquaculture must produce safe, healthy, competitively priced food, and do so in a sustainable manner. Sustainability means different things to different people, but in agriculture and aquaculture, sustainability generally refers to minimizing the environmental cost of production and using raw material inputs, including feeds, that can sustain production increases. Since fish meal is a finite resource, finding alternative protein sources for fish meal is clearly a high priority for aquaculture. Similarly, developing production diets that result in a high retention of polluting nutrients, mainly nitrogen and phosphorus, is a high priority. The use of basic engineering approaches to find solutions to these

problems is an approach that should be encouraged, especially with species for which the basic nutritional requirements are known. Chapter 9, "Diet Formulation and Manufacture," and Chaper 13, "Diet and Fish Husbandry," provide detailed information on the state of the art of topics covered in general in this chapter and serve as a guide for the expansion of aquaculture production in an environmentally benign and sustainable manner.

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12

Nutrition and Fish Health

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12.1 Introduction

Aquacultural production of fish and crustaceans has continued to expand throughout the world over the past decade, and additional growth is expected in the years to come due to the increasing demand for fisheries products and limited supplies from capture fisheries (Anonymous 1999). In conjunction with the general increase in aquacultural production has been a trend toward more intensification of culture practices due to scientific and technological advancements, as well as economic incentives to increase the amount of salable product per culture unit. As a result, there has been a tendency for marginal environmental conditions and high fish densities, which are commonly encountered in intensive aquaculture, to increase the likelihood of disease and other adverse health effects on cultured organisms. It also has become increasingly apparent that under the conditions of intensive aquaculture, proper nutrition plays a critical role in maintaining normal growth and health of aquatic organisms. All of the essential nutrients discussed in the preceding chapters of this book should be provided in the diet in adequate quantities to sustain the health of fish. A number of these nutrients and other dietary components, as well as various feeding practices, have been shown to influence the susceptibility of fish to various infectious and noninfectious diseases. In addition, prepared diets also may serve as a primary method of administering chemotherapeutics and immunostimulants to fish that are infected with certain pathogenic organisms. Thus, this chapter has been structured to describe general aspects of fish health and elaborate on the various roles that nutrition and feeding play in sustaining the health of fish produced in aquaculture. Throughout this chapter, the term fish is intended to include shrimp, unless otherwise noted.

12.1.1. Conditions of Health and Disease

A primary goal but significant challenge in aquaculture is to maintain fish in a condition of optimum health, which, in the broadest sense, means freedom from illness of any kind. A healthy condition is most conducive for rapid growth and high survival of fish to marketable size, which are generally two of the most important goals in aquaculture. In contrast to health, the condition of disease represents various states of illness or sickness. Disease can be induced by a variety of factors and manifested in many different forms. The duration and severity of disease also may vary considerably depending on the underlying cause.

12.2 Factors Affecting Fish Health

The various diseases which are encountered by fish may be caused by living or nonliving agents. Living agents of disease include various infectious organisms which may be communicable and pathogenic. Organisms known to cause disease in fish include bacteria, fungi, viruses, and various kinds of parasites. Nonliving agents that adversely affect fish health may originate from inside or outside the fish. External factors such as environmental conditions may alter normal physiological processes and cause disease. Likewise, endogenous factors such as the genetic constitution of a fish may influence its susceptibility to less than optimal environmental conditions or infectious agents. It is well established that various fish species, and certain strains within a given species, may exhibit widely different tolerances to various environmental conditions and infectious agents (Plumb 1994; Wiegertjes *et al.* 1996).

12.2.1. Environmental Conditions

There is a variety of environmental conditions which, if allowed to exceed certain limits, can impair normal physiological processes of fish and thus compromise their health. An inverse relationship between environmental quality and fish disease is well established in aquaculture (Plumb 1994). The water in which a fish lives is the principal environmental component that influences its health. Some of the most critical water quality conditions that are readily influenced by biological activity, and thus of primary concern in intensive aquaculture, include dissolved oxygen, un-ionized ammonia, nitrite, carbon dioxide, and pH. Maximum and minimum tolerable and lethal concentrations for many of these metabolites and constituents have been established for numerous fish species (Stickney 1994). Other water quality characteristics which are not influenced by biological activity such as alkalinity, hardness, salinity, temperature, and turbidity also may affect the health of fish, especially if they are not within normal tolerable limits. Different fish species may vary considerably in terms of specific tolerance limits for various water quality characteristics (Evans 1993). In addition to these natural components which comprise water quality, there is a variety of natural and synthetic chemicals which may contaminate water and adversely affect the health of fish.

A primary task in aquaculture is to maintain water quality conditions within tolerable levels for the species being cultured, but this is not always achieved. Even if lethal concentrations of certain metabolites or other water quality variables are avoided, fish health can be compromised by stress that

may be imposed if fish are required to tolerate less than optimum water quality conditions (Roberts 2000). Thus, the adverse water quality could be viewed as a stressor and the resulting stress would be a physiological state or condition in which normal functioning is impaired. Stress also may be imposed by other conditions in aquaculture such as crowding, antagonistic interactions among fish, and handling such as harvesting, sorting, or grading. Inadequate nutrition in the form of nutrient deficiencies, improper feeding regimes, or the presence of toxins in the diet also may impose stress on fish. All forms of stress caused by various environmental offenses tend to reduce the health of fish and make them more susceptible to various kinds of pathogenic organisms. This is generally believed to result from physiological changes that suppress the fish's natural resistance and immunity.

12.2.2. Infectious Agents

Infectious diseases are a major cause of economic loss in commercial aquaculture. There are various disease-causing organisms that may infect fish including bacterial, fungal, parasitic, and viral pathogens (Noga 1996). The biology of these various pathogens, including their environmental requirements, host specificity, virulence, and methods of control, has been reviewed extensively in several publications (Stoskopf 1992; Plumb 1994; Stickney 1994; Noga 1996).

Some disease-causing organisms are obligate pathogens in that they must have a host or intermediate host to survive. In contrast, facultative pathogens are able to live and multiply in a host or in the environment without a host being present. Infection by a pathogenic organism can occur without a diseased condition being produced. But when conditions are favorable, infection by a pathogenic organism will result in disease. Infectious diseases are most commonly manifested in aquaculture when fish become stressed due to less than optimal environmental conditions. Thus, it is of utmost importance to avoid conditions that induce stress in fish to maximize their resistance to various disease-causing organisms.

12.2.3. Immunity and Disease Resistance

Fish have a reasonably well-developed immune system consisting of both nonspecific and specific components (Iwama and Nakanishi 1996). The nonspecific component is considered natural or innate immunity and consists principally of phagocytic mechanisms associated with macrophages and granular leukocytes such as neutrophils which are designed to attack microorganisms that invade the fish's skin or mucous. In addition to phagocytes, there are other soluble factors such as lysozyme and complement which may assist in destroying invading pathogens.

The specific component of the immune system consists of humoral and cell-mediated responses which may provide specific immunological memory, although this immune memory of fish is generally considered to be less developed than that of mammalian species. The rate of induction and the level of response are readily influenced by temperature. In the specific immune response, macrophages serve as antigen-presenting cells, while B lymphocytes are involved in antibody production. In addition, T lymphocytes are involved in cell-mediated immunity by stimulating the differentiation and proliferation of B lymphocytes. Once antibodies are produced to a specific pathogen, they bind to the pathogen's membrane so that it can be destroyed via activation of the complement system through its classical pathway.

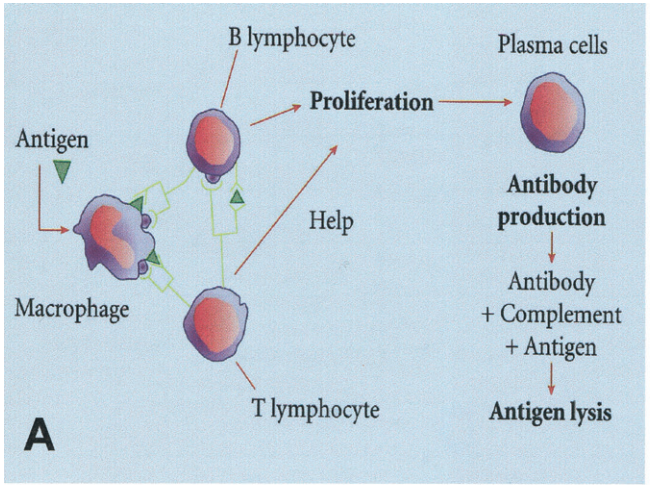
There has been a growing body of evidence in recent years which indicates that various immune responses (Fig. 12.1) and disease resistance of fish can be influenced by both nutrients and nonnutritive components of the diet. Enhancement of the immune responses of fish and their associated resistance to disease through dietary means has been viewed as an attractive alternative to treating fish, given the very limited number of efficacious and approved chemicals which are available for use after they become diseased.

12.3 Dietary Components Influencing Fish Health

Proper nutrition has long been recognized as a critical factor in promoting normal growth and sustaining health of fish. Artificial diets manufactured from various feedstuffs are the primary source of nutrition in intensive aquaculture. Prepared diets not only provide the essential nutrients that are required for normal physiological functioning but also may serve as the medium by which fish are exposed to other components which may affect their health either positively or negatively.

12.3.1. Nutrients

Previous chapters in this book have described in detail the various nutrients and their specific functions, dietary requirements, and signs of deficiency in fish. It is well established that all essential nutrients are required in adequate quantity to sustain normal health. A deficiency of any nutrient, if severe enough, can adversely affect fish health either directly, by impairing metabolic functions, or indirectly, by making fish more susceptible to opportunistic disease-causing agents. Thus, artificial diets used in aquaculture are typically formulated to provide adequate quantities of all nutrients to prevent deficiency. However, dietary supplementation of certain nutrients at levels above those which satisfy minimum requirements has allowed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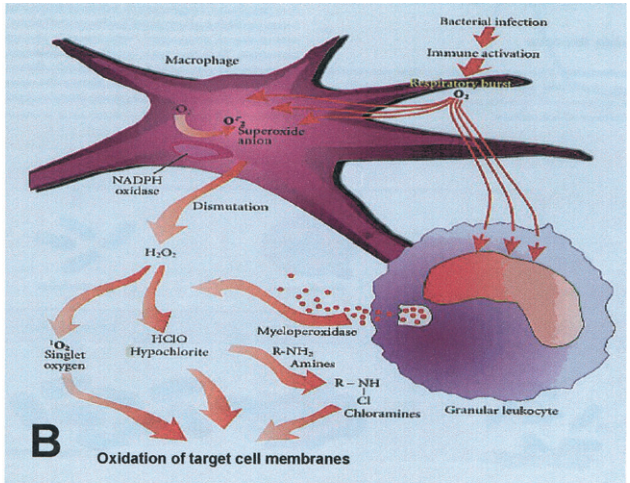
Bacterial infection
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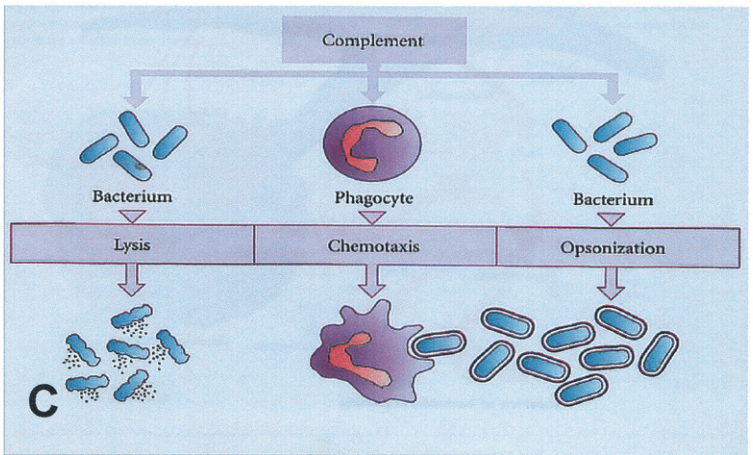
Complement
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Lysis
Chemotaxis
Opsonization
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enhancement of some immune responses and disease resistance of numerous terrestrial animals (Reddy and Frey 1990) as well as several fish species (Landolt 1989; Lall and Olivier 1991). This section considers the various nutrients groups with regard to their ability to enhance the immunity, disease resistance, and health of fish. Many of the published studies concerning immune responses of fish fed certain nutrients above minimum requirement levels are summarized in Table 12.1.

12.3.1.1. Energy-Yielding Nutrients

12.3.1.1.1. Protein and Amino Acids. The minimum dietary requirement for protein or a balanced mixture of amino acids is of primary concern in aquaculture because satisfying this requirement is necessary to ensure adequate growth and health of fish, while providing excessive levels is generally uneconomical, as protein is the most expensive dietary component. As such, most studies have been concerned with determining minimum dietary protein requirements for maximum weight gain and accretion of body protein. Dietary requirements of fish for the 10 indispensable amino acids also have received considerable attention. However, there have been limited investigations concerning manipulation of dietary protein and amino acids to enhance fish health even though immunoglobulins and other components of the immune system are proteinaceous. In one study (Kiron *et al.* 1995a), rainbow trout (*Oncorhynchus mykiss*) fed a diet containing only 10% protein had reduced lysozyme activity and C-reactive protein in serum compared to fish fed diets with 35 and 50% protein, but antibody production was not affected by dietary protein level. Mortality associated with a controlled exposure to *Aeromonas salmonicida* also was elevated in fish fed the diets with 10 and 50% protein compared to those fed the diet with 35% protein. Reduced parasitism was observed in rainbow trout fed a protein-deficient diet (19% crude protein) compared to a control diet with 38% protein (Thomas and Woo 1990). More research concerning the effects of dietary protein on immune responses of fish is certainly warranted.

Arginine is one amino acid whose influence on health has received considerable attention with experimental animals (e.g., Saito *et al.* 1987; Madden *et al.* 1988) and recently, to a more limited extent, with fish (Neumann *et al.* 1995; Buentello and Gatlin 1999). This amino acid is

FIG. 12.1

Several nutrients and nonnutritive diet additives have been documented as influencing various components of the fish immune system including (A) specific immunity via antibody production, (B) macrophage killing ability via oxidative reactions, and (C) functioning of the complement system. (Adapted from V. Verlhac and J. Gabaudan, "The Effects of Vitamin C on Fish Health," Centre for Research in Animal Nutrition, Societe Chimique, Paris, France, 1997, with permission.)

Table 12.1

Immune Responses and Disease Resistance of Fish Fed Selected Nutrients at Dietary Concentrations Above Those Required for Normal Growth

Nutrient	Species	Dietary concentration (mg/kg)	Duration of feeding	Immune response[a]	Reference
Vitamin C[b]	Channel catfish	150 AA	14 weeks	+ *Edwardsiella tarda*	Durve and Lovell (1982)
		3,000 AA	20 weeks	+ Complement hemolytic activity + Antibody	Li and Lovell (1985)
			13 weeks	+ *E. ictaluri*	
		4,000 AA	9 weeks	− Complement hemolytic activity − Antibody + *E. ictaluri*	Liu *et al.* (1989)
		1,000 APP	7 weeks	− Neutrophil bactericidal activity − Phagocytosis	Johnson and Ainsworth (1991)
		>2,000 APP	8 weeks	− *E. ictaluri*	Li *et al.* (1993)
		250 APP	10 weeks	− Antibody − *E. ictaluri*	Li *et al.* (1998)
	Rainbow trout	2,000 AA	12 weeks	+ Antibody + *Vibrio anguillarum*	Navarre and Halver (1989)
		2,000 AMP and SCAA	8 weeks	+ *Ichthyophthirius multifilis* − Specific immunity	Wahli *et al.* (1995)
		4,000 APP	2 weeks	+ Complement (alternative) + Chemiluminescence + Macrophage − Lysozyme − Antibody − Complement (classical)	Verlhac *et al.* (1996)

		1,000 APP	2 weeks	+ Chemiluminescence + Pinacytosis + Lysozyme + Complement (classical) + Antibody − Complement (alternative) − Lymphocyte proliferation	Verlhac *et al.* (1998)
	Atlantic salmon	2,750 AA	26 weeks	+ Complement + *A. salmonicida* + Lymphokine − Antibody − Phagocytosis − Respiratory burst	Hardie *et al.* (1991)
		5,000 AA and AS		− Antibody	Sandnes *et al.* (1990)
		2,980 AA and 4,770 AS	72 days	+ Antibody − *Yersinia ruckeri* − *Vibrio salmonicida*	Erdal *et al.* (1991)
		4,000 AMP	24 weeks	+ Complement + Lysozyme + *A. salmonicida* + Antibody	Waagbo *et al.* (1993)
		1,000 APP		+ Hydrogen peroxide + Lymphocyte proliferation	Verlhac and Gabaudan (1994)
		3,170 AA	23 weeks	− Respiratory burst − Bactericidal activity + *A. salmonicida* killing − Leukocyte migration − − Antibody	Thompson *et al.* (1993)

(*continues*)

Table 12.1 *(Continued)*

Nutrient	Species	Dietary concentration (mg/kg)	Duration of feeding	Immune response[a]	Reference
	Red sea bream	1,000 AA	6 weeks	− Complement + Hemagglutinin titer	Yano *et al.* (1988)
	Turbot	2,000 CA	127 days	+ Phagocytic index + Lysozyme	Roberts *et al.* (1995)
	Sockeye salmon	10,000 AS	231 days	− *R. salmoninarum* − Antibody	Bell *et al.* (1984)
Vitamin E	Channel catfish	240	120 days	+ Macrophage intracellular superoxide anion	Wise *et al.* (1993a)
		2,500	180 days	+ Phagocytic index − Antibody	Wise *et al.* (1993b)
	Rainbow trout	806	22 weeks	+ *Y. ruckeri* − Antibody	Furones *et al.* (1992)
	Atlantic salmon	800	20 weeks	− Leukocyte count − Serum protein − Lysozyme + Phagocytosis − Respiratory burst − Lymphokine production	Hardie *et al.* (1990)
	Turbot	500	12 weeks	+ Phagocytosis	Pulsford *et al.* (1995)
Vitamin A	Atlantic salmon			+ Antiprotease + Migration + *A. salmonicida*	Thompson *et al.* (1994)
	Rainbow trout	18 9,000 IU/kg	16 weeks	− IgM − Lysozyme − Respiratory burst	Thompson *et al.* (1995)

Phosphorus	Channel catfish	8,500	10 weeks	+ *E. ictaluri* + Antibody	Eya and Lovell (1998)
Magnesium	Atlantic salmon	5,000	28 weeks	− Antibody − Lysozyme − Complement hemolytic activity + Spontaneous hemolytic activity	El-Mowafi *et al.* (1997)
Iron	Channel catfish	180	8 weeks	− Antibody − Chemiluminescence − Macrophage migration − *E. ictaluri*	Sealey *et al.* (1997)
	Atlantic salmon	400	20 weeks	− Total antibody − Specific hemolytic complement − Spontaneous hemolytic complement − Lysozyme	Andersen *et al.* (1998)
Selenium	Channel catfish	0.4 (Selenomethionine and selenoyeast) (Had a higher potency than Na_2SeO_3)	9 weeks	+ *E. ictaluri* + Antibody + Macrophage chemotaxis	Wang *et al.* (1997)
Zinc	Channel catfish	200	10 weeks	− *Aeromonas hydrophila*	Scarpa and Gatlin (1992)
		30 (Zinc methionine had a higher potency than zinc sulfate)	10 weeks	+ *E. ictaluri* + Antibody	Paripatananont and Lovell (1995)
		60	16 weeks	+ Neutrophils + Chemotaxis − Phagocytosis of *E. ictaluri* − *E. ictaluri*	Lim *et al.* (1996)

[a] +, increase; −, no change; and − −, decrease.

[b] Provided as ascorbic acid equivalents by either crystalline L-ascorbic acid (AA), ascorbate-2-monophosphate (AMP), ascorbate-2-polyphosphate (APP), silicone-coated AA (SCAA), calcium ascorbate (CA), or ascorbate sulfate (AS).

involved in the synthesis of nitric oxide via nitric oxide synthase, which is inducible in fish (Schoor and Plumb 1994; Neumann *et al.* 1995). Nitric oxide has many physiological functions including enhancement of macrophage cytotoxicity. Arginine has been shown to have numerous beneficial effects on T cell-mediated immunity in various animal models and in humans (Madden *et al.* 1988). Thus its involvement in fish health is certainly worthy of further investigation.

12.3.1.1.2. Carbohydrate. Due to the fact that fish do not have a specific dietary requirement for carbohydrate, its inclusion in diets is to provide a relatively inexpensive source of energy. However, the ability of fish to utilize dietary carbohydrate for energy varies considerably (NRC 1993; Wilson 1994), and excessive soluble carbohydrate (principally starch) in the diet of some carnivorous fish has been associated with impaired health, due principally to glycogen accumulation in the liver (e.g., Hilton and Hodson 1983). In one study with Atlantic salmon, digestible carbohydrate at from 0 to 30% of the diet had minor effects on humoral immune responses after vaccination with *Vibrio salmonicida,* although cumulative mortality after exposure to this organism was lowest in fish fed 10% carbohydrate (Waagbo *et al.* 1994). Oligosaccharides in feedstuffs such as soybean meal have been shown to affect adversely the health of Atlantic salmon due to intestinal disturbance (e.g., van der Ingh *et al.* 1996) but other species appear to be more tolerant of these compounds.

In contrast to the soluble and fibrous carbohydrates associated with common feed ingredients included in fish diets, some structural carbohydrates associated with the cell walls of yeast and fungi such as β-glucans and mannan oligosaccharides have been shown to enhance the immune response of some fish species. These compounds are considered further in the section concerning nonnutritive dietary components.

12.3.1.1.3. Lipids and Essential Fatty Acids. Lipids are important components of fish diets because they provide a concentrated source of energy that is typically well utilized. In addition, dietary lipids supply essential fatty acids which cannot be synthesized by the fish. Adequate quantities of these essential fatty acids must be provided in the diet to sustain normal growth and health of fish, but excessive levels have been shown to have growth-suppressing effects (NRC 1993). Another potentially negative health effect associated with dietary lipid can be attributed to rancidity of the lipid. Many fish diets contain relatively high levels of polyunsaturated fatty acids, which are particularly susceptible to oxidation. Various products of lipid oxidation may react with proteins, vitamins, and other dietary components to limit their nutritional value. Vitamin E is particularly susceptible to destruction by oxidized lipid due to its antioxidant properties. Thus, feeding oxidized lipid

may result in overt signs of vitamin E deficiency such as membrane fragility, ascites, and anemia, as well as liver degeneration and steatitis (Klontz 1992). The immune system also may be adversely affected by oxidized lipid. For example, turbot (*Scophthalmus maximus*) fed a diet containing oxidized fish oil with marginal levels of vitamins C and E had reduced chemiluminescent responses of phagocytes and elevated mortality after infection by *Vibrio anguillarum* compared to fish fed a control diet, although antibody production was not impaired (Obach and Laurencin 1992).

It is well established in humans and a number of animal species that dietary lipid and its constituent fatty acids may affect several aspects of the immune response including lymphocytes, macrophages, and other immunocompenent cells (Hummel 1993; Kelley and Daudu 1993; Miles and Calder 1998). The modulating effect of dietary fatty acids on immune responses has been attributed in part to their influence on prostaglandin and leukotriene production from macrophages, as well as their effect on oxidative stress, lipoprotein profiles, and membrane fluidity of cells, which in turn affects membrane receptors, receptor binding affinity, and enzyme activities. A deficiency of essential fatty acids has been reported to impair the antibody production and killing ability of macrophages in rainbow trout (Kiron *et al.* 1995b) as well as depleting serum complement activity (Tort *et al.* 1996; Montero *et al.* 1998) and agglutinating serum activity (Tort *et al.* 1996) in gilthead seabream. More surprising is that dietary supplementation of *n*-3 fatty acids for several fish species known to require these fatty acids has resulted in impaired immune responses. For example, dietary supplementation of *n*-3 fatty acids to Atlantic salmon was immunosuppressive in that it reduced survival after controlled infection with *Yersinia ruckeri* as well as reducing antibody production after vaccination (Erdal *et al.* 1991). Excessive *n*-3 highly unsaturated fatty acids also increased the mortality of rainbow trout infected with *Aeromonas salmonicida* (Kiron *et al.* 1995b). Channel catfish (*Ictalurus punctatus*) fed diets containing either 7% menhaden oil or 7% linseed oil (both providing *n*-3 fatty acids) had a reduced survival when exposed to *Edwardsiella ictaluri* compared to fish fed diets with 7% beef tallow, corn oil, or an equal mixture of beef tallow, corn oil, and menhaden oil in two separate experiments; however, fish fed 7% menhaden oil had higher antibody production 2 weeks after immunization (Fracalossi and Lovell 1994). In another study (Li *et al.* 1994), channel catfish fed diets supplemented with menhaden oil at 2% had 15 and 21% higher mortality after *E. ictaluri* exposure compared to fish fed diets supplemented with catfish offal oil at 2%, although antibody titers were not influenced by diet 3 weeks after immunization. The immunosuppressive effects of menhaden oil in these studies was attributed to its *n*-3 fatty acids, which competed with arachidonic acid and altered the kinds of leukotrienes produced in macrophages and neutrophils. In contrast to those studies, Blazer (1991),

however, reported enhanced killing ability of macrophages from channel catfish fed menhaden oil compared to those from fish fed soybean oil, beef tallow, or a combination of those lipids. Hemolytic complement activity and antibody production of cod (*Gadus morhua*) also have been reported to be affected by the type of dietary lipid (Waagbo *et al.* 1995). It is obvious from these studies that dietary lipid can affect a variety of immune functions in fish but a much better understanding of these relationships is needed.

12.3.1.2. Vitamins

Of the 15 vitamins and vitamin-like compounds established as being essential for fish, vitamin C or ascorbic acid is the water-soluble vitamin most noteworthy in its ability to influence immunity and disease resistance when overfortified (typically 10 to 100 times the fish's minimum requirement) in diets. Vitamin E is the fat-soluble vitamin whose effects on immune responses of fish have been most widely studied. These two vitamins have several distinct metabolic functions but they both have antioxidant properties. One hypothesis relating to the enhancement of immune responses suggests that the presence of these nutrients above maintenance levels provides a readily available reservoir for use by the host defense system upon activation. These vitamins have been shown to affect complement and antibody production, as well as various aspects of macrophage function including enzymes involved in respiratory burst and intracellular killing and protective mechanisms to prevent tissue damage from free radicals (Blazer 1991).

In channel catfish, megadose levels of vitamin C added to the diet has been shown to improve the antibody response, complement activity, and survival following infection with *E. ictaluri* in aquaria (Li and Lovell 1985) and enhanced resistance to infection by *Edwardsiella tarda* (Durve and Lovell 1982). However, other studies have failed to show positive responses of channel catfish fed diets overfortified with vitamin C when reared under laboratory conditions (Li *et al.* 1993, 1998). Liu *et al.* (1989) reported no effect of dietary vitamin C level on complement hemolytic activity or antibody production of channel catfish reared in ponds, but fish fed 1000 mg vitamin C/kg had increased resistance to *E. ictaluri.* In some studies with rainbow trout, relatively high concentrations of vitamin C in the diet enhanced wound repair (Halver 1972) and immune responses (Blazer 1982; Navarre and Halver 1989; Verhlac *et al.* 1998). Rainbow trout fed 2000 mg vitamin C/kg diet as either silicone-coated ascorbic acid or ascorbyl phosphate also had a significantly reduced mortality when infected with *Ichthyophthirus multifilis,* but specific immunity did not appear to be affected (Wahli *et al.* 1986). Rainbow trout fed 1000 and 4000 mg vitamin C/kg in combination with yeast glucan had enhanced complement activation by the alternative pathway and chemiluminescence response of macrophages, but lysozyme and complement levels activated by the classical pathway were not different

from those of fish fed 150 mg vitamin C/kg (Verhlac *et al.* 1996). Hardie *et al.* (1991) observed an increase in complement activity of Atlantic salmon with dietary supplementation of 2750 mg vitamin C/kg. In another study with Atlantic salmon, supplementation of 4770 mg ascorbate sulfate/kg enhanced antibody production after vaccination but did not confer increased survival against *Y. ruckeri* infection (Erdal *et al.* 1991). Verhlac and Gabaudan (1994) also observed in Atlantic salmon an increase in hydrogen peroxide production of macrophages at a dietary supplementation level of 1000 mg vitamin C/kg. Very impressive results were reported by Waagbo *et al.* (1993), who observed an increased resistance of Atlantic salmon to furunculosis following supplementation of 4000 mg vitamin C/kg. In addition, specific antibody production was elevated in fish fed this level of vitamin C along with lysozyme activity of head kidney, serum complement activity, and serum iron in fish surviving bacterial challenge. In contrast to those findings, Lall *et al.* (1989) did not observe an effect of elevated dietary vitamin C on antibody production, bactericidal activity of serum, or susceptibility of Atlantic salmon to *Vibrio* or *Aeromonas salmonicida*. Sandnes *et al.* (1990) reported a slight reduction in antibody production of Atlantic salmon deprived of vitamin C, but no differences in antibody responses of fish fed vitamin C at 500 or 5000 mg/kg supplied as either ascorbic acid or ascorbate-2-sulfate. Chinook salmon (*Oncorhynchus tshawytscha*) fed diets with graded concentrations of vitamin C up to 2500 mg/kg did not have increased resistance to *Aeromonas salmonicida* or *Renibacterium salmoninarum* (Leith and Kaattari 1989).

Dietary supplementation of vitamin C has been evaluated in conjunction with subjecting fish to stressful conditions due to the immunosuppressive nature of stress. In one study with Atlantic salmon, fish fed as much as 3170 mg vitamin C/kg showed no differences in leukocyte respiratory burst activity, bactericidal activity, or leukocyte migration compared to fish fed 82 mg/kg (Thompson *et al.* 1993). Gilthead seabream held at a high density and fed a diet supplemented with 250 mg ascorbyl phosphate/kg had reduced serum lysozyme compared to fish fed the unsupplemented diet, although alternative complement activity and agglutination responses were not affected (Montero *et al.* 1999). Some *in vitro* studies also have shown enhanced immune responses such as leukocyte proliferation and macrophage activation (Hardie *et al.* 1993) as well as leukocyte migration and phagocytosis (Mulero *et al.* 1998b) when vitamin C was added to culture media.

The rather variable results concerning the effects of vitamin C on immune responses and disease resistance of various fish species indicate the need for additional research using standardized experimental protocols, especially with regard to immune responses and disease challenge.

Vitamin E and its potential immunomodulatory effects have not been studied as extensively in fish as vitamin C, but some influences on immune responses have been established. A dietary deficiency of vitamin E has been

shown to reduce various specific and nonspecific immune responses in rainbow trout (Blazer and Wolke 1984a) and increased the susceptibility of Atlantic salmon to *Aeromonas salmonicida,* due principally to reduced complement activity (Hardie *et al.* 1990). The complement activity of gilthead seabream also was adversely affected by a vitamin E deficiency (Tort *et al.* 1996; Montero *et al.* 1998), as was agglutinating serum activity (Tort *et al.* 1996). Enhanced disease resistance to *Yersinia ruckeri* was observed in rainbow trout fed a diet with 806 mg vitamin E/kg but the serum antibody level was not influenced by the vitamin E level (Furones *et al.* 1992). Dietary supplementation of 250 mg vitamin E/kg maintained serum alternative complement activity in gilthead seabream subjected to crowding stress (Montero *et al.* 1999). However, supplementation of 299 mg vitamin E/kg diet did not influence the outcome of a natural *Renibacterium salmoninarum* infection of chinook salmon (*Oncorhynchus tshawytscha*) (Thorarinsson *et al.* 1994). In a separate study (Leith and Kaattari 1989), dietary supplementation of vitamin E did not influence immune responses of chinook salmon or resistance to *R. salmoninarum.*

The only other fat-soluble vitamin which has received limited consideration in terms of its influence on immunocompetence of fish is vitamin A. In one study with rainbow trout (Thompson *et al.* 1995), fish were fed diets supplemented with either vitamin A (18 mg/kg) or astaxanthin (100 mg/kg) or both. Fish fed the basal diet without supplements of either component had reduced total serum antiprotease activity and classical serum complement activity. The lack of vitamin A supplementation in the presence or absence of astaxanthin also tended to reduce leukocyte migration, while the total serum immunoglobulin level, specific immunolglobulin level following immunization with *Aeromonas salmonicida,* serum lysozyme activity, and respiratory burst of macrophages were not affected by vitamin A or astaxanthin intake. In contrast to these findings, Atlantic salmon fed a diet supplemented with 60 mg astaxanthin/kg had greater resistance to challenge from *Aeromonas salmonicida,* although serum hemolytic activity, specific antibody level, and lysozyme activity in the head kidney were unaffected (Christiansen *et al.* 1995). Higher levels of retinol and α-tocopherol were observed in liver and muscle of fish fed the astaxanthin-supplemented diet.

Other water-soluble vitamins that have been investigated with regard to their effects on immune function and disease resistance include folic acid, pyridoxine, riboflavin, and pantothenic acid. Dietary levels of these vitamins that provided optimum growth of chinook salmon also were adequate to provide the best immune functioning in these fish (Leith and Kaattari 1989). However, an earlier study indicated that elevated pyridoxine concentration in a high-protein diet increased the resistance of chinook salmon to *V. anquillarum* (Hardy *et al.* 1979). Channel catfish fed diets with folic acid at 0.4 or 4.0 mg/kg along with vitamin C at 200 mg/kg had maximal survival

and antibody production against *E. ictaluri,* but when the diet contained vitamin C at 20 mg/kg, folic acid at 4.0 mg/kg was required to improve survival (Duncan and Lovell 1994).

12.3.1.3. Minerals

It has been established that fish generally require the same minerals as terrestrial animals for tissue formation and a variety of metabolic functions including osmoregulation (NRC 1993). However, dissolved minerals in the aquatic environment may contribute to satisfying the metabolic requirements of fish and interact with dietary requirements. Over 14 mineral elements have been established as essential nutrients for fish, and dietary requirements for several of these minerals have been quantified. However, excessive levels of some dietary and waterborne minerals may cause toxicity as discussed in Chapter 8. Dietary supplementation of certain minerals at concentrations above those required to support normal growth but below those causing toxicity has been shown to enhance immunity of various animals including some fish species, although much less research has been conducted with minerals in this regard as compared to vitamins.

Phosphorus is one of the most important macrominerals to include in fish diets to support proper growth and tissue mineralization; however, very limited research has investigated the effects of this mineral on disease resistance of fish. In a recent study (Eya and Lovell 1998), graded levels of phosphorus were fed to channel catfish which were exposed to *E. ictaluri.* Resistance to *E. ictaluri* challenge and antibody production were both negatively affected by inadequate phosphorus intake. The dietary phosphorus requirement for maximum resistance against bacterial challenge was 0.4% of the diet and similar to that required for maximum weight gain, while 0.5% of the diet was needed to maximize antibody production. Another macromineral that has received limited consideration in terms of immune response is magnesium. Atlantic salmon fed graded levels of magnesium and vaccinated against *Vibrio anguillarum* had similar antibody titers and lysozyme and complement hemolytic activity (El-Mowafi *et al.* 1997).

Several of the microminerals have received at least some consideration as to their effects on immunity and disease resistance of fish. Selenium is of particular interest because of its physiological interaction with vitamin E and established role in improving disease resistance of terrestrial animals (Van Vleet and Watson 1984). Supplementation of selenium in conjunction with vitamin E/kg was not able to influence the outcome of a natural *Renibacterium salmoninarum* infection of chinook salmon (*Oncorhynchus tshawytscha*) (Thorarinsson *et al.* 1994). In a more recent study with channel catfish (Wang *et al.* 1997), dietary selenium concentration and supplemental form were found to influence different aspects of the immune response. Resistance to *E. ictaluri* was maximum in fish fed 0.20 mg Se/kg from

selenomethionine and 0.40 mg Se/kg from selenoeast or sodium selenite. Antibody production generally increased in response to graded levels of selenium in the diet but was greatest for fish fed selenoyeast. Macrophage chemotactic response also was enhanced in fish fed selenoyeast and selenomethionine.

Iron is a trace mineral of particular interest with regard to disease because its availability to microorganisms affects their ability to cause infection. One study conducted with different families of Atlantic salmon indicated that families with high levels of serum iron were more susceptible to *Vibrio* infection; however, no relationship was apparent with regard to furunculosis or bacterial kidney disease (Ravndal *et al.* 1994). In another study with Atlantic salmon, supplementation of 400 mg iron/kg to a basal diet containing 160 mg/kg had no effects on serum total protein, serum total antibody, hemolytic complement activity, or lysozyme activity in serum, head kidney, or spleen (Andersen *et al.* 1998). Iron supplementation did increase catalase activity in the head kidney. Channel catfish fed a diet deficient in iron had increased mortality due to *E. ictaluri* exposure and reduced chemotactic migration of peritoneal macrophages (Sealey *et al.* 1997; Lim and Klesius 1997), but antibody production was not affected by dietary iron level (Sealey *et al.* 1997). Diets containing 60 mg Fe/kg from either iron methionine or iron sulfate provided the highest chemotactic index (Sealey *et al.* 1997). More research is needed to delineate the effects of dietary iron supplementation and iron status of fish on their resistance to disease.

Other trace minerals such as copper, manganese, and zinc have received limited attention to date with regard to their effects on immunity and disease resistance of fish. It was noted in one study that hatchery-reared coho salmon had significantly lower concentrations of whole-body copper and zinc than their wild counterparts (Felton *et al.* 1994). Deficiencies of manganese and zinc reduced leukocyte natural killer activity of rainbow trout, and supplementation of these minerals restored that activity (Inoue *et al.* 1998). Supplementation of both manganese and zinc at over 100 mg/kg diet did not enhance resistance of sockeye salmon to bacterial kidney disease from *Renibacterium salmoninarum* (Bell *et al.* 1984). Zinc supplementation at 200 mg/kg diet did not enhance resistance of nonimmunized channel catfish to *Aeromonas hydrophila;* however, dietary zinc deficiency and calcium excess reduced mortality during disease challenge (Scarpa and Gatlin 1992). Those results are in contrast to those of Paripatananont and Lovell (1995), in which zinc deficiency caused 100% mortality in channel catfish challenged with *E. ictaluri.* In that study, maximum survival after challenge was achieved with 5 mg Zn/kg provided by zinc methionine or 30 mg Zn/kg provided by zinc sulfate. Maximum antibody production was achieved with 15 mg Zn/kg from zinc methionine or 30 mg Zn/kg or more from zinc sulfate. Thus, the organic chelate of zinc methionine increased protection from *E. ictaluri* and elevated antibody production relative to zinc sulfate. In a similar study

(Lim *et al.* 1996), channel catfish fed zinc methionine at 20 and 60 mg/kg diet and zinc sulfate at 60 mg/kg had higher chemotactic responses of macrophages; however, dietary zinc had no influence on phagocytic activity of macrophages for *E. ictaluri*. In addition, the source or level of dietary zinc did not provide protection against *E. ictaluri* infection.

Iodine and fluorine are two other trace minerals that were established as essential for protecting Atlantic salmon from *R. salmoninarum* infection (Lall *et al.* 1985). In that study, both minerals were provided at 4.5 mg/kg diet.

12.3.2. Nonnutritive Compounds

Two promising nonnutritive dietary supplements to potentially aid in disease control of fish are immunostimulants and probiotics. Immunostimulants increase disease resistance by enhancing host defense mechanisms against pathogenic microorganisms, while probiotics decrease the frequency and abundance of pathogenic or opportunistically pathogenic organisms in the environment.

12.3.2.1. Immunostimulants

As indicated previously, the immune system of fish is similar to that of mammals in that it is divided into both specific and nonspecific branches (Iwama and Nakanishi 1996). An immunostimulant may be defined as an agent which stimulates the specific immune system when given with an antigen or the nonspecific immune system when given alone (Anderson 1992). A variety of heterogeneous biochemicals including some nutrients has been established as immunostimulants (Sakai 1999). Recently there has been growing interest in the application of various immunostimulants to fish in aquaculture to enhance disease resistance and improve health.

12.3.2.1.1. Stimulation of Specific Immune Responses. A specific immune response requires the host to have prior exposure to an antigen such that recognition and subsequent activation occurs through a coordinated effort of antigen-presenting cells, B lymphocytes and T cells. Vaccination is the best-known method of specific immunostimulation in humans and animals including fish (Iwama and Nakanishi 1996). The most common vaccines used in aquaculture are preparations consisting of live attenuated bacteria, killed cells, or cellular extracts which are administered to fish primarily by injection or immersion. Intraperitoneal injection of antigen is generally most effective in stimulating the immune response but this type of administration is somewhat labor intensive and may impose excessive stress on the fish. Immersion vaccination consisting of either short-term exposure to a concentrated solution of antigen, longer exposure to dilute suspensions of vaccine (bath method), or hyperosmotic infiltration of the vaccine have

all been shown to be effective in immunizing fish. Administration of vaccines to fish via the diet also has been practiced, but primarily as a means to provide periodic booster doses of vaccine in conjunction with the initial vaccine exposure by injection or immersion. Destruction or alteration of the vaccine in the gastrointestinal tract may influence the effectiveness of oral vaccination; however, various means of protecting the vaccine from degradation in the gut have been devised and proven effective. For example, oral vaccination of fish with microparticles (Piganelli *et al.* 1994) or antigens encapsulated in alginate has been shown to provide an enhanced immune response (Joosten *et al.* 1997).

A variety of immunostimulatory products has been demonstrated to improve specific immune responses through their ability to act as adjuvants, which, when used in conjunction with an antigen, increases the intensity and duration of the specific response against that antigen (Anderson 1992). Oil-based immunostimulants such as Freund's complete adjuvant, which also contains killed mycobacteria, and various modifications without the bacterial cells were initially used with fish and yielded positive responses. Adjuvants are able to intensify antibody production by holding the antigen in tissues for slow release as well as by increasing the activity of cell types involved in the specific immune response.

In recent years there has been heightened interest in the ability of β-glucans, polysaccharide derivatives from yeast and fungi, to act as immunostimulants in fish based on some positive results. These compounds are insoluble polysaccharides consisting of repeating glucose units which can be joined through β1–3 and β1–6 linkages when derived from yeast and mycelia fungi (Raa *et al.* 1992), or β1–3 and β1–4 linkages when derived from barley (Wang and Wang 1997). Atlantic salmon exposed to β-glucan had increased production of antibodies to the immunodominant antigen of *A. salmonicida* but not against the lipopolysaccharide layer of *A. salmonicida* (Rorstad *et al.* 1993). Aaker *et al.* (1994) reported a similar increase in antibody production of glucan-injected and vaccinated fish and hypothesized that the increased antibody response may be due to a T cell-independent mechanism. An improvement in specific immune response was reported when glucan was fed to vaccinated rainbow trout (Verhlac *et al.* 1996); however, when β-glucan was fed to turbot, *Scopthalmus maximus,* in conjunction with a *Vibrio anguillarum* vaccine, no improvement in survival over that of the orally vaccinated controls was observed (Baulny *et al.* 1996).

12.3.2.1.2. Stimulation of Nonspecific Immune Responses. Most immunostimulatory compounds examined in fish have been shown to have immunoenhancing potential by improving nonspecific immune responses, which are considered to be the first line of defense against invading organisms (Anderson 1992). Responses of macrophages such as cell migration,

phagocytosis, and bactericidal activity as well as changes in the numbers of leukocytes and activation potential of cells on stimulation as measured by tissue levels of oxidative radicals and enzymes are often used to assess nonspecific immunity.

Immunostimulants which have been examined most frequently for their ability to increase the nonspecific immune responses of fish include glucans (Yano *et al.* 1989; Robertsen *et al.* 1990; Nikl *et al.* 1991; Jeney *et al.* 1997; Siwicki *et al.* 1994) and the synthetic drug levamisole (Mulero *et al.* 1998a). Thus far the β-glucans appear to show the most promise. The source and extraction process by which these glucans are produced have been shown to affect their immunostimulatory capacity. A large amount of variation also has been observed in the ability of glucans to enhance disease resistance in various fish species (Wang and Wang 1996, 1997). Engstad *et al.* (1992) suggested the mechanisms through which these agents enhanced immune responses of Atlantic salmon were increases in lysozyme and complement activation. Similar results have been observed in other fish species including common carp (Yano *et al.* 1989), turbot (Santarem *et al.* 1997), and yellowtail (*Seriola quinqueradiata*) (Matsuyama *et al.* 1992). Increased oxidative capacity of phagocytic cells also has been included as a mechanism through which β-glucans enhances nonspecific resistance as well as elevates production of superoxide anion in leukocytes (Anderson and Siwicki 1994, Dalmo *et al.* 1996. Duncan and Klesius (1996a) reported that channel catfish fed diets with either 0.2% β-glucan or 2.7% of the yeast *Saccharomyces cerevisiae* had enhanced macrophage and neutrophil migration and phagocytosis but not increased resistance to infection by *E. ictaluri*. In other studies, no effect on oxidative capacity or lysozyme activity of blood leukocytes has been observed in fish fed β1–3- and β1–6-glucans (Efthimiou 1996).

Various *in vitro* studies have attempted to examine further the potential of glucans to stimulate the phagocytic cells of fish and the mechanisms by which they exert their effects. The addition of laminaran, a β1–6-branched β1,3-D-glucan, to isolated head kidney macrophages of Atlantic salmon caused macrophages to undergo spreading and membrane ruffling indicative of activation (Dalmo and Seljelid 1995). These cells also exhibited increased pinocytosis as well as elevated superoxide anion and acid phosphatase levels. Similar results were observed when Atlantic salmon head kidney macrophages (Jorgensen and Robertsen 1995) and turbot head kidney cells (Figueras *et al.* 1997) were cultured with glucan *in vitro*.

There also has been considerable interest in the immunostimulatory potential of levamisole in the United States for fish because it is already registered by the U.S. Food and Drug Administration for treatment of helminth infections in ruminants (Anderson 1992). This compound has been shown to enhance nonspecific immune responses. For example, increased serum bactericidal activity, complement activation, phagocytosis, and natural killer

cell activity were observed in rainbow trout injected with levamisole (Kajita *et al.* 1990). Head kidney phagocytes of common carp immersed in levamisole had an increased chemotactic ability, phagocytosis, and oxidative capacity (Baba *et al.* 1993), and a similar response was observed when gilthead sea bream were fed levamisole (Mulero *et al.* 1998a).

There also are other animal products and extracts which have nonspecific immunostimulatory potential. Increased phagocytic response and natural killer cell activity were observed in rainbow trout injected with an abalone extract (Sakai *et al.* 1991) and with chitin (Sakai *et al.* 1992). Similar results on leukocyte function, as well as increased killing activity, total serum protein, and immunoglobulin levels were observed in rainbow trout fed diets containing chitin, and increased protection against *A. salmonicida* was manifested when brook trout (*Salvelinus fontinalis*) were injected with chitin (Anderson and Siwicki 1994). Dietary supplementation of the iron-binding glycoprotein bovine lactoferrin has also been reported to increase resistance of red sea bream to *Cryptocaryon irritans* infection by enhancing nonspecific defense mechanisms (Kakuta and Kurokura 1995).

Products derived from various algal species also have shown nonspecific immunostimulatory potential. For example, a processed form of the blue–green algae *Spirulina platensis* enhanced phagocytosis and chemotaxis of channel catfish phagocytes when included in the diet; however, no increase in disease resistance was observed following challenge with *Edwardsiella ictaluri* (Duncan and Klesius 1996b). Carrageenans, linear sulfated poly-D-galactans composed of repeating disaccharide units, have been reported to elevate migration of phagocytes from common carp head kidney but had no effect on complement activity or phagocytosis, although resistance to both *E. tarda* and *A. hydrophila* was increased (Fujiki *et al.* 1997). Alginates with a high mannuronic acid content enhanced the resistance of juvenile turbot to *V. anguillarum* infection when fed via *Artemia* (Skjermo *et al.* 1995).

Another group of biochemicals that participates in many biological processes, including immunological responses, is the nucleotides. The purine and pyrimidine nucleotides are involved in essentially all cellular processes and have important structural, metabolic, energetic, and regulatory functions. Dietary supplementation of nucleotides has been shown to positively affect various specific (Jyonouchi 1994) and nonspecific (Carver 1994) immune responses of mammals, especially during early development and recovery from injury. Most recently, Burrells *et al.* (2001a, 2001b) described the influences of nucleotides in fish. A series of experiments was conducted in which a commercial diet was supplemented with a nucleotide mixture consisting of cytidine-5′-monophosphate, disodium uridine-5′-monophosphate, adenosine-5′-monophosphate, disodium inosine-5′-monophosphate, disodium guanidine-5′-monophosphate and RNA at a combined inclusion level of 0.03% (Burrells *et al.* 2001a). Feeding this nucleotide-supplemented

diet for 3 weeks prior to disease challenge positively affected responses of rainbow trout against challenge infection with *Vibrio anguillarum,* Atlantic salmon against infectious salmon anaemia virus, and coho salmon against *Piscirickettsia salmonis.* In addition, sea lice (*Lepeophtheirus salmonis*) infestation was reduced in Atlantic salmon fed the diet supplemented with nucleotides. Additional experiments (Burrells *et al.* 2001b) reported positive effects on vaccination efficiency of Atlantic salmon and subsequent challenge infection when the same mixture of nucleotides was supplemented to a diet at 0.03%. Enhanced immune responses of tilapia to *Aeromonas hydrophila* vaccine also were observed in fish fed a diet supplemented with a mixture containing nucleotides (Ramadan *et al.* 1994). In addition, to improved immune responses, Burrells *et al.* (2001b) reported enhanced osmoregulation of Atlantic salmon fed a nucleotide-supplemented diet following transfer to seawater. Weight gain of Atlantic salmon following disease challenge and seawater transfer was 15–22% greater in fish fed the nucleotide-supplemented diet compared to that observed in fish fed the control diet after 8 weeks. Intestinal fold morphology also was observed to increase in fish fed the nucleotide-supplemented diet (Burrells *et al.* 2001b).

Based on research to date, there is potential for incorporation of certain types of immunostimulants into disease prevention and control regimes, in particular, the β-glucans and levamisole. However, there currently are a number of factors that need to be understood more thoroughly before these compounds can be routinely used in aquaculture. Many of these factors such as the type of immunostimulant, dosage level, method of delivery, fish species, and disease organism(s) against which protection is sought contribute to determining the success or failure of an immunostimulation regime. Proper dosage of the immunostimulant is critical because failure to administer a sufficient amount may result in no enhancement of response, while excessive quantities can lead to immunosuppression. Considerable variations in immunostimulant effectiveness have been noted with different routes of administration, with injection protocols producing the best results. Oral application of immunostimulants would be the most convenient means of administration to fish; however, oral applications generally have had the most varied success rates. Further research addressing the dosage and timing of prophylactic and therapeutic oral treatments as well as measuring the adjuvant activity of many of these compounds seems warranted.

12.3.2.2. Probiotics

Probiotics have been defined as live organisms, principally bacteria, administered as a diet supplement which beneficially affect intestinal microbial balance of the host organism (Fuller 1989). Some microbial products designed for administration in the aquatic environment rather than the diet are technically not probiotics unless the added microbes survive in the

gastrointestinal tract (Gatesoupe 1999). A wide variety of mechanisms has been proposed to explain the beneficial effects of probiotics on the host organism including their production of antimicrobial substances, competition for adhesion receptors and/or nutrients, and direct immunostimulatory effects.

One strategy often followed for administering probiotics includes isolating bacteria from mature fish and including these favorable bacteria in the feed of juvenile fish of the same species. Lactic acid bacteria such as *Carnobacterium sp.*, which produce bactericidins, are often used for such purposes in this manner. For example, the lactic acid bacteria isolated from the intestine of Atlantic salmon have been shown to inhibit growth of pathogenic bacteria such as *Vibrio anquillarum* (Gildberg and Mikkelsen 1998) and *Aeromonas salmonicida* (Gildberg *et al.* 1995). Other positive results such as improved initial resistance to mortality from *Vibrio* infection have been reported for larval turbot fed rotifers enriched with lactic acid bacteria (Gatesoupe 1994) and cod (*Gadus morhua*) fry fed a dry diet containing lactic acid bacteria or peptides from a fish protein hydrolyzate (Gildberg and Mikkelsen 1998). In contrast, no effect on protection of salmon fry against *A. salmonicida* was noted when lactic acid bacteria were added to a dry diet (Gildberg *et al.* 1995). The mechanism(s) through which protection may be conferred by probiotics appears to be rather complex and has not been completely resolved at this time.

One concern with implementing probiotics into the food chain or environment of larval fish is that their colonization and proliferation within the host must remain under control. Gatesoupe (1994) observed a negative correlation between high concentrations of lactic acid bacteria and survival in turbot larvae fed $>2 \times 10^7$ colony forming units, indicating the need for careful determination of inclusion levels of lactic acid bacteria to avoid detrimental effects. At this time the successful isolation and cultivation of probiotic bacteria is a labor-intensive effort which seems best suited to larval culture when the environment or diet provided to mass quantities of fish may be treated with less probiotics and less labor.

12.4 Feeding Practices Affecting Fish Health

Conventional wisdom has generally held that a fish's health is optimally maintained by feeding it a nutritionally complete diet on a daily basis. However, some recent research findings as well as empirical evidence from commercial aquaculture have indicated that certain deviations from feeding to satiation on a daily basis may significantly influence fish health and resistance to disease. For example, a common practice in commercial aquaculture of channel catfish is not to feed fish once an infection of a bacterium such

as *Edwardsiella ictaluri* is manifested (Robinson and Li 1996). Such action generally has been shown to limit the severity of infection.

Another feeding regimen experimentally shown to influence disease resistance is an extended period of feed deprivation. In channel catfish reared in ponds, it was found that year 2 fish (~0.5 kg) had increased resistance to enteric septicemia of catfish caused by *E. ictaluri,* following a 3-month period of food deprivation (November through January), compared to fish fed throughout this period (Kim and Lovell 1995). However, year 1 fish (initially 22 g) not fed for the same 3-month period had increased mortality when challenged with *E. ictaluri.* Similar findings were reported in a separate study by Okwoche and Lovell (1997) in which feed deprivation of year 2 fish for a period of time similar to that in the earlier study caused reduced mortality after challenge with *E. ictaluri* along with higher antibody production, while nonfed year 0 fish had increased mortality and reduced antibody production. Nonfed fish in each age group had a reduced phagocytic index. Differential responses to disease challenge of the two sizes of fish in these studies complicate recommendations for implementing periods of feed deprivation in commercial aquaculture. However, it should be noted that fish previously not fed for 3 months experienced a compensatory gain in the subsequent 3 months (February through April) of feeding such their final weight was similar to that of fish fed throughout the study. Application of feed restriction for enhanced production efficiency and improved health is certainly worthy of additional investigation.

12.4.1. Manipulation of Nutritional Condition

As discussed in the previous section, feeding practices may influence the nutritional status of fish, which may in turn affect their disease resistance by altering immune function or by retarding growth of pathogenic organisms (Lall and Olivier 1993). It was noted that age 2 channel catfish deprived of feed for a 3-month period had a reduced iron content in circulation (Okwoche and Lovell 1997). This may have contributed to their increased resistance to disease when exposed to *E. ictaluri* because iron is a critical nutrient sequestered by bacteria from their host to sustain infection.

A study with Atlantic salmon also has demonstrated a relationship between feeding a low-iron diet and increased resistance to disease caused by *V. anguillarum* (Lall *et al.* 1996). The low-iron diet did not affect humoral immunity and the complement system but did lower the level of iron in the organs of fish following experimental challenge. Taken together these studies seem to indicate that elevated tissue levels of certain micronutrients may predispose fish to infection and that feeding regimes to lower tissue levels of certain nutrients may improve the disease resistance of fish. However, there is a need for much more research in this area to define clearly

the relationships among feeding regimes, manipulation of tissue nutrient levels, and disease resistance of fish.

Another factor known to influence nutritional condition and potentially health of fish is the dietary composition of energy-yielding nutrients. Fish vary greatly in their ability to metabolize dietary protein, carbohydrate, and lipid, as well as in the distribution and storage of these components in the body. Storage and distribution of lipid between muscle and liver tissues of assorted fish species may vary considerably due to differences in natural history (Sheridan 1988). Variation in nutrient utilization also may influence the health of certain species when given artificial diets. For example, excessive levels of soluble carbohydrate in the diet have been shown to affect adversely the hepatic function of rainbow trout due to glycogen accumulation (NRC 1993). Other species such as the channel catfish maintain hepatic glycogen within more narrow limits even when fed very high levels of soluble carbohydrate such that liver function and health are not adversely affected. Elevated dietary lipid has been associated with excessive fat deposition in the liver of some species; however, the adverse effects of this condition appear to vary greatly among species. For example, red drum (*Sciaenops ocellatus*) fed a diet containing 15% lipid had over 25% lipid in the liver, which did not adversely affect liver function (Craig *et al.* 1999). In contrast, fatty infiltration of the liver has been noted in other fish species fed high levels of lipid (Mosconi-Bac 1990). Thus, the proper balance of energy-yielding nutrients in diet formulations is important not only for sustaining acceptable growth and feed utilization of fish but also for maintaining body composition within certain limits to allow normal functioning and health.

12.4.2. Seasonal Feeding Regimes

As indicated in the previous section, manipulation of diet formulations and/or feeding schedules as functions of fish size or production objective has not been as well developed in the culture of various fish species as it has with terrestrial animals. Thus considerable opportunity exists for further refinement in these areas to increase the production efficiency in aquaculture. Seasonal factors also may influence feeding regimes in certain types of aquaculture, particularly where fish are cultured in systems in which environmental conditions vary seasonally. It is well established that the prevalence of certain infectious diseases varies with the season, as most pathogenic bacteria have specific temperature ranges in which their virulence in greatest (Plumb 1994). It also is thought that a fish's immunity may be altered along with its metabolism as a result of seasonal temperature changes. Therefore, certain modifications of feeding schedules and diet composition in relation to season have been evaluated to a limited extent. One such modification seen in pond culture of channel catfish in the southeastern United States is overfortification of diets with vitamin C during the spring of the year. This

modification is intended to enhance the disease resistance of fish by providing higher than normal levels of this vitamin during the time of the year when some of the most serious bacterial epizootics are prevalent. The marginal efficacy of such practice has limited its continued application in the industry today. Experiences in Chile with jaw deformities in rapidly growing salmon smolts transferred to the sea during periods of high water temperature suggest that extra dietary supplementation with vitamin C before and after the smolt transfer period is beneficial. However, further research on the seasonal application of some of the immunostimulants discussed in previous sections appears warranted.

12.4.3. Use of Medicated Feeds

Effective control of disease-causing organisms is a primary concern in aquaculture. Currently there are only a limited number of government-approved chemotherapeutics in the United States which can be used to treat diseases of fish intended for human consumption (Noga 1996); however, many other countries have less stringent rules governing use of chemotherapeutics in aquaculture. Administration of antibiotics in the diet is one of the most effective means of treating numerous fish in large volumes of water. Two antibiotics which are approved in the United States for treatment of *Aeromonas salmonicida* infections in salmonids and *Edwardsiella ictaluri* in catfish are Romet and Terramycin. Romet, produced by Hoffmann-LaRoche, Inc., is a potentiated sulfonamid consisting of sulfadimethoxine and ormetoprim. Terramycin is the trade name for oxytetracycline hydrochloride produced by Pfizer.

Several factors can limit the effectiveness of antibiotic treatment. One such factor is that sick fish generally do not feed vigorously and therefore may not consume enough medicated feed to ensure proper dosage. In addition, some antibiotics may have limited palatability to certain species. The palatability of Romet was increased for channel catfish by increasing fish meal to approximately 16% of the diet (Robinson *et al.* 1990). In addition, reducing the concentration of antibiotic in the diet in conjunction with feeding at a higher rate to provide the recommended dosage has been shown to be effective (Robinson and Li 1996). Romet is heat stable and thus can be used in diets manufactured by extrusion processing. Terramycin is a broad-spectrum antibiotic that is effective against a variety of bacteria but is heat labile such that its use is restricted to diets manufactured by compression pelleting.

Another potential limitation to antibiotic treatment is the development of resistance by certain strains of bacteria. Such antibiotic resistance requires proper isolation of the disease-causing bacteria and determination of its sensitivity to various antibiotics. Such diagnosis must be completed in a timely

manner so that treatment with an appropriate antibiotic can be initiated before the infection progresses.

Antibiotics also have been used as growth promoters for various production animals including some fish species. It is generally believed that positive responses to antibiotic supplementation in diets are due to altering the microflora of the gastrointestinal tract. Enhanced digestibility of some unsaturated fatty acids was observed in rainbow trout fed diets containing chloramphenicol, oxolinic acid, and oxytetracycline (Cravedi *et al.* 1987).

In three separate studies conducted in aquaria (Ahmed and Matty 1989), cages (Viola and Arieli 1987), and ponds (Viola *et al.* 1990), weight gain of common carp was generally improved with inclusion of the antibiotic Virginiamycin in the diet. In contrast, neither Romet nor oxytetracycline inclusion in the diet improved the weight gain of channel catfish (Rawles *et al.* 1997). Due to the inconsistent improvements in performance of fish fed antibiotics, this practice generally has not been implemented in commercial aquaculture. The potential for antibiotic resistance and residues in the fish also has restricted the use of antibiotics in fish diets. Other complications also may result from prolonged administration of antibiotics in the diet. For example, spinal deformities were produced in Atlantic salmon but not in Arctic charr after prolonged feeding of Terramycin (Toften and Jobling 1996).

12.5 Concluding Remarks and Research Needs

Dietary immunomodulation has the potential to aid aquacultural production greatly through the prevention and/or treatment of diseases. Supplementation of diets with nutrients and nonnutritive immunostimulatory compounds to optimize immune function and disease resistance holds promise and may be cost-effective if reductions in mortality can be consistently demonstrated. However, additional study using standardized protocols is needed at this time to address the questions of fish and pathogen species variability, nutrient and nonnutrient immunostimulatory compound dosages necessary to elicit protection, and the duration of the various treatments.

Variations in experimental protocols currently limit the ability to determine precisely the effects of dietary modulation on immune responses and disease resistance. Diet composition, feeding rate, feeding duration prior to pathogen exposure, genetics, and environmental conditions are just some of the factors known to influence the responses of fish to immunomodulators. In addition, the immune responses that can be measured are numerous and may be differentially affected by various immunostimulants. Thus, a greater understanding of the fish's immune system is needed along with standardized experimental protocols to allow a systematic evaluation of various

immunostimulants. Further advancements in these areas may allow nutritional modulation of the immune response to be used as an effective and relatively inexpensive alternative to chemical therapy in combating diseases of fish in aquaculture.

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13

Diet and Fish Husbandry

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13.1 Introduction

Nutrient requirements for normal growth and function of the major cultured fish species are essentially known and the amounts required for optimum performance under standard conditions have been fairly well identified. However, optimum methods of formulating, processing, and feeding diets for a diverse group of commercial aquaculture species, in a variety of culture environments, and for different production functions present a myriad of challenges to the practicing fish nutritionist.

This chapter deals with feeding strategies for several important commercial aquaculture species with diverse culture environments and technologies. Feed costs account for over 50% of the variable costs in most aquaculture operations, therefore applying the best feeding strategy can have a significant impact on optimizing profit, which is the primary goal of commercial aquaculture. Since commercial aquaculture and livestock industries have similar goals, to provide a high-quality consumer product at a profit, it may be well to compare feeding fish with land animals. Subsequently, different levels of fish farming are examined and then applicable feeding practices are discussed.

13.1.1. Levels of Aquaculture

Several authors have recommended different systems of classification of the various stages of aquaculture. Some classify according to the level of intervention. i.e., how much the culture environment has been modified. This ranges from the impoundment of natural waters and harvest of any and all animals therein, without adding seed stock or nutrients, to the use of a closed system in which water is recirculated. Some classify on the basis of the quantity and quality of nutrients utilized by aquaculturists, such as extensive, where no nutrients are added; fertilization, to enhance production of aquatic organisms; supplemental feeding, using incomplete feeds; intensive feeding using nutritionally balanced feeds; and hyperintensive feeding, where high inputs of concentrated, nutritionally complete feeds are used. Some classify on the basis of the energy input (labor, fossil fuel, feed) or technology input (harvesting, stocking, feeding, pumping, aeration, biofiltration). Generally, the higher the level of intervention in the production of aquatic animals, the more important is the feed to the success of the operation.

13.1.1.1. Production of Fish Exclusively from Natural Aquatic Foods

Some fish obtain their food exclusively from plankton. These fish are usually continuous grazers and have mechanisms for filtering or concentrating

FIG. 13.1

Tilapia grown in combination with ducks. The pond receives only manure from the ducks. The fish yield is approximately 1200 kg/ha annually.

the suspended animal and plant organisms from the water. An example is the silver carp. Others, such as some of the tilapias, have the ability to feed on plankton but also feed on bottom materials. The common carp is an efficient bottom feeder. Some fish, such as grass carp, have herbivorous appetites and consume large quantities of higher aquatic plants. Such fish can be cultured without artificial feeds, as shown in Fig. 13.1, but usually with pond fertilization. This level of production is most applicable in environments where supplemental feeds are expensive or unavailable.

13.1.1.2. Supplementation of Natural Foods with Feed

This level of fish farming essentially involves taking full advantage of natural aquatic productivity and using various feedstuffs or prepared feeds as a supplement to increase the yield further. An example is semiintensive shrimp culture in Central and South America, where large ponds are fertilized to enhance natural productivity and also receive pelleted feeds. Usually with species that will accept supplemental feeds, the additional yield of fish

resulting from the additional feeding is profitable. For example, the yield of common carp in fertilized ponds was 390 kg ha^{-1}, the addition of grain or grain by-products increased the yield to 1530 kg ha^{-1}, and formulated feed further improved the yield to 3000 kg ha^{-1} (Lovell *et al.*, 1978).

Where natural aquatic food may make a relatively small contribution to the total protein and energy requirements of the cultured fish, it can provide essential micronutrients that will allow nutritionally incomplete supplemental feeds to be used. As the biomass of fish in the pond increases, however, the fish will become more dependent on the supplemental feed for all nutrients. Channel catfish grown in earthen ponds to maximum standing crops of 2000 kg ha^{-1} grew normally and showed no deficiency signs when vitamin C was deleted from their feed. However, when the fish density was increased to 4000 kg ha^{-1} and above, growth was normal but resistance to bacterial infection was reduced and subclinical deficiency signs occurred when no vitamin C was fed (Lovell and Lim, 1978).

13.1.1.3. Intensive Culture of Fish in Highly Modified Environments

With these systems, natural foods are an insignificant source of nutrients. Maximum yield per unit of space and effort and minimum accumulation of unretained nutrients in the culture system are primary concerns. Thus, highly concentrated, nutritionally complete feeds are justified. Examples of this type of production are rainbow trout cultured in spring-fed raceways and Atlantic salmon grown in net pens in the coastal areas of the sea. Also, channel catfish or marine shrimp in intensively stocked ponds may obtain negligible amounts of nutrients from natural foods.

13.1.2. Feeding Fish versus Feeding Land Animals

Feeding fish in their aqueous environment takes on dimensions beyond those considered in feeding warmblooded food animals. These include the nutrient contribution of natural aquatic organisms in pond cultures, the effects of feeding on water quality, and the interaction between feed allowance and optimum dietary nutrient concentration. Because fish cannot be fed *ad libitum*, the feeder, not the fish, decides how much feed is fed, and thus a higher level of management is required to feed fish. However, the concept of feeding is the same as that applied in feeding other food animals; to nourish the animal to the desired level or form of productivity as profitably as possible. Thus, application of knowledge of the nutritional requirements of fish and the husbandry of feeding various cultured species is essential to successful aquaculture.

Unlike intensively reared livestock and poultry, which are fed *ad libitum,* fish are given a restricted feed allowance that will minimize waste. Because

feedlot farm animals eat as much and as often as they want, their nutrient allowances are based upon satiation feeding. Fish are often (perhaps usually) not fed to satiation and the daily feed allowance has been shown to affect the fish's response to various dietary nutrient concentrations. Li and Lovell (1992) found that the optimum dietary protein allowance for channel catfish fed to satiety was 26%, while fish fed to less than satiation responded to higher protein concentrations. For example, a study at the Tunison Fish Nutrition Laboratory in Cortland, New York (Rumsey, 1993), showed that the arginine requirement for maximum growth for young rainbow trout was significantly lower when the fish were fed to satiation than when the fish were given a restricted feed allowance. This interaction between feed allowance and optimum dietary nutrient concentration makes the formulation of commercial feeds more difficult for fish than for farm animals.

Because fish are fed in water, feed that is not consumed within a reasonable time not only represents an economic loss, but can reduce the water quality. Therefore, feed allowance, feeding method, and water stability of the feed are factors that the fish culturist must consider but the livestock feeder does not. The culture environment may make valuable nutrient contributions to the fish. For example, most waters contain enough dissolved calcium to provide most of the fish's requirement. For fish that feed low on the food chain, such as shrimp and some tilapias, the pond environment can be a valuable source of protein, energy, and other nutrients.

Fish convert practical feeds into body tissue more efficiently than do farm animals. Cultured catfish can gain approximately 0.84 g of weight per g of practical diet, whereas chickens, the most efficient warmblooded food animal, gain about 0.48 g of weight per g of diet (Table 13.1). The

Table 13.1

Efficiency Utilization of Feed and Dietary Protein and Metabolizable Energy (ME) by Fish, Chicken, and Cattle[a]

	Feed consumption			Efficiency		
Source	Protein (%)	ME (kcal/g)	ME–protein ratio (kcal/g)	Weight gain per g of food consumed (g)	Protein gain per g of protein consumed (g)	ME required per g of protein gain (kcal)
Channel catfish	32	2.7	8.5	0.75	0.36	21
Broiler chicken	18	2.8	16.0	0.48	0.33	43
Beef cattle	11	2.6	24.0	0.13	0.15	167

[a] From Lovell (1989).

reason for the superior food conversion efficiency of fish is that they can economically assimilate diets with higher percentages of protein, apparently because of their lower dietary energy requirement. Fish have a lower energy requirement than terrestrial animals because of their lower maintenance requirement and lower heat increment. Fish, however, do not hold an advantage over monogastric farm animals in protein conversion; as shown in Table 13.1, poultry convert dietary protein to body protein at nearly the same rate as fish. The primary advantage of fish over land animals is the lower energy cost of protein gain rather than the superior food conversion efficiency. The metabolizable energy requirement per g of protein gain is 21 for channel catfish versus 23 for the broiler chicken.

13.2 Channel Catfish

13.2.1. The Industry

Culture of channel catfish (Fig. 13.2) accounts for about two-thirds of the commercial aquacultural production in the United States. The production of farm-raised catfish reached 225,000 tons in 1998 (USDA, 1999). Once considered to have primarily a regional appeal as a food, farm-raised catfish are now in national and international markets. Catfish reach the processing plant alive and are kept alive until they are slaughtered, which takes less than 30 min. Farm-raised catfish are fed grain-based feeds, which give the fish a mild flavor, with the absence of a "fishy" odor. The flesh is mostly white muscle, which is free of intramuscular bones. Nutritionally, an 8.4-g serving of farm-raised catfish contains approximately 140 kcal, 17 g of protein, 9 g of fat, 50 mg of cholesterol, 40 mg of sodium, and a number of essential vitamins and minerals. The delicate flavor, light flesh, high nutritional value, and year-round availability make farm-raised catfish an appealing choice to the food service industry and to consumers.

Channel catfish possess several qualities that make it amenable to culture. The fish normally does not reproduce in culture ponds, it is easy to spawn under hatchery conditions, and it produces a large number of fry, readily accepts a variety of prepared feeds, and tolerates water temperatures from near freezing to 34°C and wide fluctuations in water quality in production ponds. Channel catfish grow rapidly; a 10-g fingerling reaches a harvestable size of 0.5 kg in about 6 months as long as the water temperature remains above 23°C. Also, it converts feed efficiently; feed conversion ratios (feed/grain) of 1.4–1.5 can be achieved.

In the early 1970s, when catfish farming was in its infancy, farmers stocked earthen ponds at rather low densities, ranging from 2500 to 5000 fingerlings

Fig. 13.2

Channel catfish weighing approximately 0.5 kg harvested from a 5-ha pond that constrained approximately 7500 kg of fish per hectare.

per hectare, in the spring and harvested the fish in the fall. The fish were fed a pelleted, concentrated feed, and yields of 1000 to 2000 kg per hectare were typical. Today, yields range from 4000 to 7000 kg per hectare. The increased yields can be attributed to higher stocking densities and to improvements in feeds, feeding practices, water quality management, and disease control. In addition, a multiple-batch cropping system is used in which fish of different sizes and ages are present in the pond simultaneously. Harvest-size fish are removed several times during the year, and ponds are restocked with fingerlings without draining. Figure 13.3 shows catfish that have been collected by seine and are waiting to be loaded onto a truck for transport to the processing plant.

Catfish farming has become a major industry located primarily on the Mississippi River flood plain. A typical farm is several hundred hectares in size, although some may be 1000 ha or more, with individual ponds of 5 to 10 ha. Large farms coupled with the development of specialized feed mills

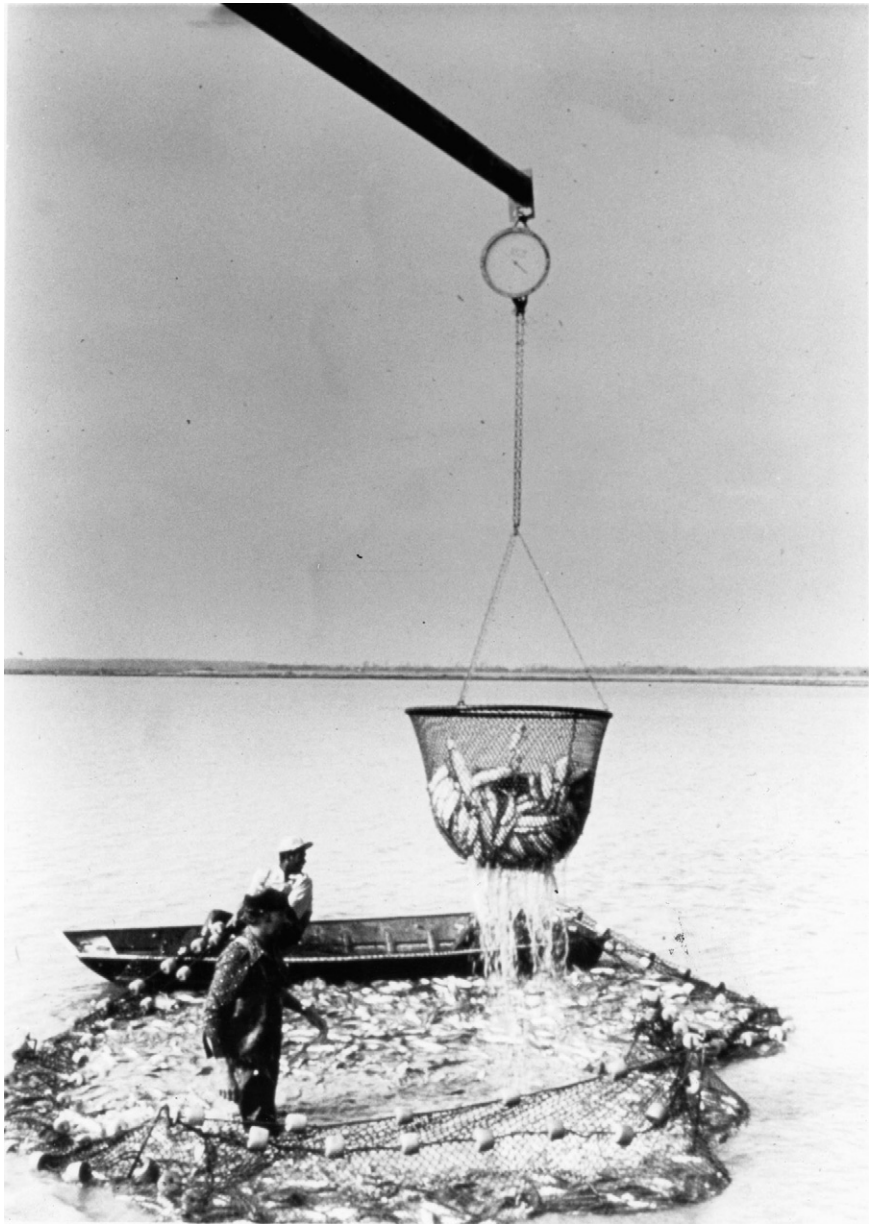

FIG. 13.3

Channel catfish harvested by seine from a 5-ha pond are held in a "live sock" until loaded onto a truck and hauled alive to the processor.

and catfish processing plants have made catfish farming a profitable, stable industry. Ninety-five percent of the catfish produced in the United States is produced in Mississippi, Arkansas, Alabama, and Louisiana in approximately 160,000 ha of catfish ponds (USDA, 1999).

Although nitrogenous and phosphorus compounds excreted from the fish or from uneaten feed are of concern, management of dissolved oxygen is the most critical pond environment problem. During periods of heavy feeding dissolved oxygen levels drop precipitously during the night. Permanent electrical aerators located in the ponds as well as temporary aeration devices powered by tractors are used to provide oxygen in emergencies.

13.2.2. Feeding Practices

Even though catfish have been cultured for many years and considerable research has been conducted on nutrition and feeding of catfish, feeding is far from an exact science. There is considerable variation in feeding practices among commercial catfish farms. Some farmers feed a fixed amount of feed in the pond daily, usually the maximum amount that the pond can safely "metabolize," and have the pond overstocked with fish so no feed will be wasted. Other farmers feed what the fish in each pond will efficiently consume each day, using a floating feed so feeding activity can be observed. However, water quality or fish health problems may cause farmers to restrict the daily feed allowance or to feed less frequently.

13.2.2.1. Fry

Newly hatched catfish fry, which are only about 2.5 mm in total length, are usually held in indoor troughs or tanks for about 10 days before releasing into outdoor nursery ponds. Initially, catfish fry use their yolk sac as an energy and nutrient source. Once the yolk sac is absorbed, at approximately 3 to 5 days after hatching, fry begin to seek food. In the hatchery, fry are fed finely ground, meal-type feeds containing 45 to 50% protein, supplied primarily from fish meal, at a daily rate equal to about 25% of the body weight divided into 8 to 10 feedings. Table 13.2 presents a formula for fry feed.

The best way to ensure good growth and survival of recently stocked fry is to ensure that plenty of natural food is available in the nursery pond when the fish are stocked. Natural foods for channel catfish fry include microcrustaceans, insect larvae, and zooplankton. Even though fry presumably meet their nutrient needs from natural food organisms for the first 3 or 4 weeks, they are fed once or twice daily using finely ground feed at a rate equal to 25 to 50% of the fry biomass. Since the feed is a supplement to natural pond foods, it is not necessary to feed a high-protein feed as used in the hatchery. Fines from 32% grow-out feeds are suitable for catfish fry during this phase.

Table 13.2

Catfish Fry, Fingerling, and Production Feed

Ingredient (%)	Fry feed (50% protein)	Fingerling feed (35% protein)	Production feed	
			32%	28%
Menhaden meal (61%)	60.2	6.0	—	—
Meat/bone/blood (65%)	15.3	6.0	4.0	4.0
Soybean meal (48%)	—	38.8	27.5	25.5
Cottonseed meal (41%)	—	10.0	27.5	10.0
Corn grain	—	16.1	20.1	31.4
Wheat middlings	19.0	20.0	18.0	22.5
Dicalcium phosphate	—	1.0	1.0	1.0
Catfish vitamin mix[a]	Include	Include	Include	Include
Trace mineral mix[a]	Include	Include	Include	Include
Vegetable or fish oil[b]	5.0	2.0	1.5	1.5

[a] Commercial mix that meets the requirements for catfish.
[b] Sprayed on after extrusion to reduce feed dust.

13.2.2.2. Fingerlings

After a few weeks, when the fry reach 2.5 to 5 cm in length and are generally referred to as fingerlings, they will come to the pond surface to accept food. Initially, the small fingerlings are fed once or twice daily to satiation using a crumbled feed or small pellets (3-mm diameter) containing 35% protein (Table 13.2). The feed should contain some fish meal or other animal protein source. Fingerlings are generally fed according to this regimen until they reach about 12 to 15 cm in length, at which time they are stocked in grow-out ponds to be grown as production fish to harvestable size.

13.2.2.3. Production Fish

Catfish grown to harvestable size are typically fed a 28 to 32% floating feed (Table 13.2), with a pellet diameter of approximately 4 to 5 mm. Low-protein feeds (28%) can be used if the fish are fed to satiation. Li and Lovell (1992) found that channel catfish grew maximally when fed 24 or 26% balanced protein feeds, if fed as much as they would consume, but if they were fed to less than satiation, they required higher levels of protein for optimum growth. Because most farmers feed conservatively (underfeed) to avoid waste, a 32% protein feed is considered practical for most operations.

Catfish are fed expanded feeds that are manufactured by extrusion processing, which allows them to float. This type of feed is advantageous because the farmer can see how much the fish are consuming. On most commercial catfish farms the feed is typically blown onto the surface of the water using

pneumatic dispensers mounted on or pulled by vehicles. Feed should be scattered over a large area to provide feeding opportunities for as many fish as possible. It is desirable to feed on all sides of the pond, but this is generally not practical because prevailing winds dictate that feed must be distributed along the upwind side to prevent it from washing ashore.

Typically, catfish producers feed once a day, 7 days a week. Feeding twice a day when the water temperature is above 25°C has been shown to allow for a higher rate of feed consumption and a correspondingly faster growth rate (Lovell, 1979); however, the additional time and management required for multiple daily feedings make the practice unattractive to most farmers. During disease episodes or during extremely hot weather when feeding activity is poor, it may be beneficial to feed every other day or every third day.

Feed allowance is affected by several factors including fish standing crop, fish size, water temperature, and water quality. Water temperature and fish size have a profound affect on feed consumption by channel catfish. Feed consumption increases as the water temperature increases until a temperature of about 32°C is reached and subsequently begins to decrease. As the fish size increases, the feed consumption as a percentage of the body weight decreases and the feed conversion efficiency is reduced (Table 13.3).

When catfish are cultured using a multiple-harvest production system in which several sizes of fish are present in the pond simultaneously, they should be fed to satiation. Offering as much feed as possible, without wasting feed, provides a better opportunity for the smaller, less aggressive fish to get feed. Satiation feeding appears to be particularly important when catfish are fed less frequently than on a daily basis. Although it is recommended that catfish

Table 13.3

Feed Consumption and Feed Conversion for Different Sizes of Channel Catfish[a]

Fish size (g)	Feed consumption (% body weight)	Feed conversion ratio
27	4.0–4.5	1.1–1.2
45	3.5–4.0	1.3–1.4
136	2.5–3.0	1.4–1.6
272	2.0–2.5	1.6–1.8
340	1.5–2.0	1.8–1.9
454	1.3–1.5	1.9–2.0
908	1.1–1.2	2.0–2.2
1362	1.0–1.1	2.2–2.4

[a] At optimum temperature (27–29°C). Adapted from Lovell (1989).

typically be fed as much feed as they will consume, at high standing crops of fish it may be impossible to satiate the fish and maintain the water quality. Feeding rates should not exceed what can be assimilated by organisms in the pond and not require excessive use of aeration or cause toxic concentrations of waste metabolites, such as ammonia. Generally, the long-term daily feed allowance should not exceed 100 to 120 kg per hectare. Overfeeding should definitely be avoided because wasted feed reduces feed efficiency and also contributes to deterioration of the water quality.

The best time of day to feed is still debated, but the point is more or less academic. On large catfish farms, the time at which fish are fed is largely dictated by the logistics required to feed many hectares of ponds in a limited time period. As a result, many catfish farmers start feeding in early morning, as soon as the dissolved oxygen levels begin to increase. Figure 13.4 shows the diurnal variation in dissolved oxygen in typical catfish ponds. Research has shown that there are no significant differences in weight gain, feed consumption, feed conversion, and survival among catfish fed to satiation at 0800, 1600, or 2000 hr (Robinson *et al.,* 1995). There were also no differences in emergency aeration time among treatments. However,

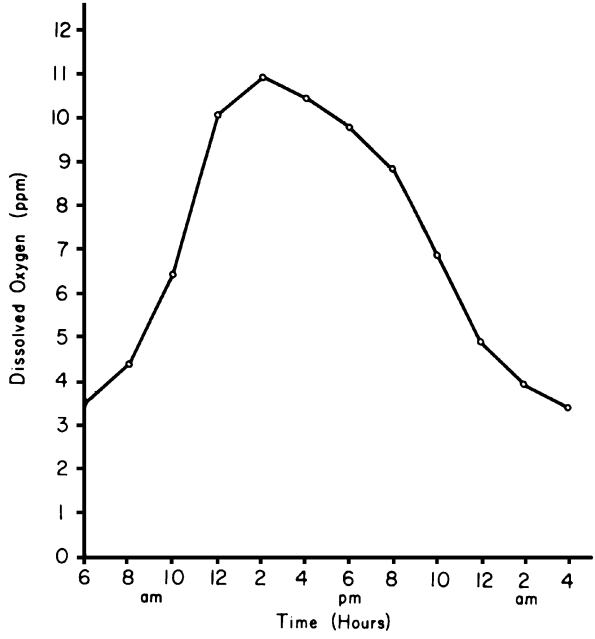

FIG. 13.4

Typical diurnal variation in dissolved oxygen content in intensively stocked catfish ponds.

feeding in late evening or at night in commercial catfish ponds is not recommended because the peak oxygen demand by the fish occurs 6–12 hr after feeding, when dissolved oxygen levels in the pond are low. Generally, it appears most practical, during the warm growing season, to begin feeding in the morning as the dissolved oxygen begins to increase. In cool weather, during fall, winter, and spring, the water temperature is usually higher in the afternoon and fish will feed better.

13.2.2.4. Winter Feeding

Although catfish feed inconsistently at water temperatures below 20°C, selective winter feeding on warmwater days can prevent weight loss and sometimes result in weight gain. A study conducted at Auburn University demonstrated that catfish (0.45 kg) held over winter in ponds without food lost 9% of their body weight, whereas fish fed 1% of their body weight on days when the water temperature was above 12°C increased their body weight by 18%. The amount of gain or loss realized during winter depends on the severity of the winter. Fish will gain or lose more weight during a mild winter than during a cold winter.

The benefits of winter feeding on weight gain are fairly well documented; however, the health aspect of winter feeding is less well defined. Logically one would expect fish fed during the winter to be in better condition and perhaps more resistant to disease-causing organisms than fish that are not fed. However, two studies at Auburn University showed that food-size catfish not fed during the winter were more resistant to challenge from *Edwardsiella ictaluri,* the bacteria causing enteric septicemia, than fish fed over winter. Small (fingerling) catfish were more resistant to *E. ictaluri* infection when fed during the winter.

Many catfish farmers, however, do not feed during the winter months because inclement weather often prevents access to pond levees or because they do not see any benefit to winter feeding. Research at Auburn University has shown that food-size catfish that are not fed during the three coldest winter months (December, January, and February) can make up differences in weight gain compared with fish that are fed during the winter months when satiate feeding is resumed in the spring and summer. If fish are to be marketed during the winter, however, it would appear to be prudent to follow a winter feeding program, particularly during a mild winter. However, if large (food-size) fish are not to be harvested until spring or summer, they do not benefit from being fed in December, January, or February. Fingerlings should be winter fed.

The type of feed that should be fed during winter has not been precisely defined, however, a 25% protein feed will provide sufficient nutrition for overwintering catfish. Many farmers feed a slow-sink feed in cool weather because the fish seem reluctant to feed on the water surface, and wind may

blow floating feed to shore before it is eaten. This feed is processed by extrusion, but its density is higher than that of a floating feed and it sinks slowly.

A recommended guide for winter-weather feeding of catfish in ponds is to feed 0.75 to 1% of their estimated weight when the water temperature at a depth of 1 m (3 ft) is ≥13°C (54°F). Research during two consecutive winters showed that both fingerlings and adult fish in Mississippi and fingerling catfish in Arkansas grew as well on a 25% protein feed as on a 35% protein feed.

13.2.2.5. Feeding Diseased Fish

Feeding diseased fish is difficult because fish that are sick feed poorly, if at all. However, offering medication through the feed is generally the only method available to treat bacterial infections. Two antibiotics, Romet (sulfadimethoxine-ormetroprim; Hoffmann–La Roche, Nutley, NJ) and Terramycin (oxytetracycline; Pfizer, Inc., New York), are registered by the Food and Drug Administration (FDA) to treat bacterial infections of catfish through their incorporation into feeds.

Romet is registered for control of enteric septicemia in catfish (ESC), caused by *Edwardsiella ictaluri,* and has also been shown to be effective in treating motile aeromonad septicemia (MAS), caused by *Aeromonas hydrophila*, and systemic columnaris infections. Romet-medicated feed is fed at a feeding rate sufficient to deliver 2.3 g of antibiotic/45 kg of fish/day for 5 consecutive days. A 3-day withdrawal period is required before fish can be processed.

Terramycin has been shown to be effective in treating MAS and other aeromonad infections, ESC, and systemic columnaris infections. Most catfish feed formulations currently used deliver 2.5 g of antibiotic/45 kg of fish/day when fed at 1% of the body weight/day. Terramycin-medicated feed is fed for 7 to 10 consecutive days. A 21-day withdrawal period is required before fish are slaughtered.

13.2.3. Nutrient Requirements for Catfish Feeds

Energy requirements of catfish were largely neglected in the early stages of catfish feed development, primarily because an imbalance in dietary energy does not appreciably affect the health of the fish. Also, feeds prepared from feedstuffs typically used in catfish feeds, such as soybean meal, corn, and fish meal, are unlikely to be deficient or excessive in energy when protein requirement is met. Based on available information, it appears that a digestible energy (DE) level of 8 to 9 kcal/g of protein is adequate for use in catfish feeds (Lovell, 1989; Robinson and Li, 1996). Thus, a 32% protein feed should contain a digestible energy level of about 2600 to 2800 kcal/kg of diet.

Essential fatty acid requirements (EFA) for catfish and most other warmwater fish have not been precisely defined, but catfish apparently require

small amounts of *n*-3 and *n*-6 fatty acids. It appears that 0.5 to 0.75% highly unsaturated fatty acids, supplied by marine fish oil, will satisfy the *n*-3 EFA requirement (Satoh *et al.,* 1989). The *n*-6 EFA requirement, approximately 0.5%, is usually met through the lipids in the plant ingredients in the feed. Natural pond food organisms may also be a source of EFA.

Marine fish oils may impart a "fishy" flavor to the fish flesh if fed at high levels. Also, there is evidence that when menhaden fish oil is the only lipid source in the diet, levels as low as 2% reduce the survival of catfish exposed to the bacterial pathogen *Edwardsiella ictaluri* (Fracalossi *et al.,* 1994; Li *et al.,* 1994). This is likely caused by the immunosuppressive effect of highly unsaturated *n*-3 fatty acids as has been demonstrated in warmblooded animals. A 1 : 1 combination of marine fish oil and vegetable oil added to the feed at a level of 2% does not affect the flavor or immune response.

Catfish use carbohydrates effectively as an energy source and, thus, do not require as much lipid in their diet as salmonids, which do not use carbohydrates as well for energy. Lipid levels in commercial catfish grow-out feeds rarely exceed 5%. About 3 to 4% of the lipid is inherent in the feed ingredients, with the remaining 1 to 2% being sprayed onto the finished pellets to control feed dust. This allows an energy-to-protein ratio of 8 to 9 kcal/g of protein, which seems to be the optimum for production diets for catfish. Increasing the lipid content of catfish feeds causes a direct increase in the body lipid content of the fish, which can reduce the dress-out yield in processing and flesh quality.

Protein requirements vary for numerous reasons, including water temperature, feed allowance, fish size, amount of nonprotein energy in the diet, protein quality, natural food available, and management practices. However, 28 or 32% protein feeds are typically used for fish during grow-out (Robinson and Li, 1996). Levels as low as 24% have been shown to be adequate for food-size catfish if the fish are fed to satiation (Li and Lovell, 1992). However, under practical conditions, catfish are not usually fed to satiation; therefore, higher protein percentages, perhaps 30 or 32%, are the most profitable levels of protein for production feeds.

Amino acid requirements of channel catfish are presented in Chapter 3. Lysine is generally the limiting amino acid for catfish, and if feeds are formulated to meet the minimum lysine requirement, all other amino acid requirements are met or exceeded if traditional feed ingredients are used. In a practical feed, animo acid requirements are usually met by feeding a mixture of feedstuffs in which soybean meal and a small amount of animal protein source provide most of the protein. Cystine can replace about 60% of the methionine and tyrosine can replace about 50% of the phenylalanine. Research has shown that synthetic amino acids, such as lysine hydrochloride, can be used by catfish when supplemented in a practical feed (Robinson and Li, 1993).

Vitamin requirements of small catfish are presented in Chapter 2. Catfish feeds are generally supplemented with a vitamin premix that contains all essential vitamins in sufficient quantities to meet requirements and to compensate for losses due to feed processing and storage. Although the vitamins inherent in feedstuffs contribute to the nutrition of the catfish, they are not usually considered during feed formulation because their bioavailability is not known. Vitamin losses during storage are not a major factor in the catfish farming industry, where feed is usually not stored for more than 1 week.

Vitamin deficiencies in the diet can reduce the resistance of young channel catfish to disease-causing bacteria. Studies have demonstrated that deficiencies of vitamin C, vitamin E, and folic acid increased mortality and impaired various specific and nonspecific immune responses in catfish challenged with *Edwardsiella ictaluri*.

Mineral requirements of channel catfish are presented in Chapter 5. Phosphorus supplements are added in catfish feeds to provide the 0.3–0.4% biologically available phosphorus that is required. Data from both laboratory and pond studies have shown that dicalcium phosphate and finely ground defluorinated rock phosphate have equal biological value and are approximately 67% bioavailable to catfish. Plant feedstuffs are poor sources of phosphorus because approximately two-thirds of the phosphorus is in the form of phytate, a bound form of phosphorus that is poorly available to fish. Laboratory studies have demonstrated that phytase enzymes can be used in catfish feeds to release phytate phosphorus and make inorganic phosphorus supplementation in the diet unnecessary (Jackson *et al.*, 1996).

Catfish feeds typically contain 5% or less of an animal protein source and, therefore, are supplemented with a trace mineral premix that contains iron, copper, zinc, manganese, selenium, iodine, and, sometimes, cobalt in sufficient amounts to meet or exceed dietary requirements of catfish. There is evidence that supplemental trace minerals are not necessary in catfish feeds containing 15% or more of animal protein sources.

Mineral deficiencies can cause increased sensitivity to bacterial infections in channel catfish. Studies at Auburn University demonstrated that dietary deficiencies of phosphorus, zinc, or selenium caused increased mortality and suppressed various immune responses in catfish fingerlings challenged with *E. ictaluri*.

13.2.4. Effect of Feeds on the Sensory Quality of Processed Catfish

Earthy off-flavor is a serious but controllable problem in pond-raised catfish. However, this type of flavor is caused by phytoplankton in the culture ponds and not by feeds. A study conducted by the USDA Southern Regional Research Center and the USDI Fish Farming Experiment Station

demonstrated that commercial feeds composed of oilseed meals, grains, and animal products generally have little influence on the flavor quality of farm-raised catfish. High levels of dietary marine fish oil will give catfish a "fishy" flavor that is undesirable, but catfish fed feeds containing 2% or less of marine fish oil have no distinct "fishy" flavor.

The preferred color of catfish flesh is white. At high dietary levels, yellow–orange carotenoids (xanthophylls), found in some plant feedstuffs, have been shown to concentrate in catfish, giving the flesh a yellowish coloration that is undesirable (Lovell, 1989). Corn gluten meal is limited as a feed ingredient because of its high concentration of xanthophyll. Corn and corn screenings contain the pigment, but it is present at concentrations that are not problematic. Xanthophyll concentration in catfish feed should not exceed 11 mg kg^{-1}.

13.2.5. Compensatory Growth in Catfish

Compensatory growth is the propensity to "catch up" or compensate for a period of feed deprivation by animals after full feeding is resumed. Research demonstrated that catfish are able to catch up in weight following a 3-week restricted feeding regimen when the fish are returned to full feed. Also, catfish not fed during the winter months of December, January, and February can make up for the weight loss when full feeding resumes the following spring. These studies clearly indicate that catfish exhibit compensatory growth. This is of practical importance because catfish are often not fed or are fed infrequently during the winter, and during summer there are occasions when the fish must be held off feed because of disease or poor water quality. If this period of feed deprivation is not too long, the fish can compensate for the missed feedings by eating more when feeding is resumed. However, at high standing crops and at peak feeding periods, it may be difficult to feed enough feed to realize compensatory growth fully and avoid the negative effects of increased feed input on water quality.

13.2.6. Fish Size and Feed Consumption and Conversion

Research at the Delta Research and Extension Center in Mississippi demonstrated that efficiency of feed conversion decreased significantly as size of catfish increased. This is shown in Table 13.3. Feed consumption also decreases as fish size increases. The minimum size of marketable catfish is approximately 0.35 kg and the optimum size for commercial processing plants is 1 to 1.5 kg. Although the larger fish require more feed for weight gain, the producer is presently not paid a premium for growing the fish to a larger size.

13.3 Salmonids

Salmonid aquaculture is the oldest form of fish rearing in Europe and North America, with records of culture efforts dating back hundreds of years in Europe and nearly a century and a half in North America. For decades, salmon and trout in the United States were raised primarily for fishery enhancement. State and federal government agencies raise salmon in freshwater hatcheries to the stage at which they normally migrate to the sea. The juvenile fish are then released to complete their life cycle in the ocean and return to enhance coastal fishing. Trout, including anadromous rainbow trout (steelhead trout), are also raised to restock depleted lakes and streams and to enhance public fishing. Until 15 years ago, most salmon and trout nutrition research was conducted on fry and fingerling fish and intended to improve juvenile production. However, the rapid growth of the salmon farming industry from the early 1980s, producing 4- to 6-kg fish, has changed the character and focus of salmonid nutrition research. This industry has grown from virtually nothing in the 1970s to an industry now producing over 60% of the salmon consumed worldwide at restaurants and in the home. The main species being farmed are Atlantic salmon and rainbow trout, with Pacific salmon, coho, and chinook constituting a second, much lower tier of production. Arctic char and other species of trout are also raised for food production, but in relatively low numbers.

In North America, the rainbow trout is by far the most extensively cultured salmonid for stocking of public waters for recreational fishing and for food. Over 300 state and federal hatcheries rear trout for fisheries enhancement. The total public production of trout is about 200 million fish (70,000 metric tons) annually. Private trout farms raise an additional 25,000 metric tons each year for food. Pacific salmon are cultured primarily for fisherly enhancement. Pacific states rear and release about 600 million salmon fingerlings, or smolts, each year. British Columbia contributes an additional 400 million.

In Europe, commercial marine net pen culture of Atlantic salmon is a successful, mature industry. Figure 13.5 shows a marketable-size Atlantic salmon raised in a net pen in seawater. Norway is the leader in salmon farming, producing over 250,000 in 1996. Salmon are now the second most important food production crop in Norway. Scotland is the other major producer of farmed salmon in Europe, with Ireland, Iceland, and the Faroe Islands producing lower quantities. Chile has the most rapidly growing salmon and trout farming industries, increasing from virtually nothing in 1986 to over 125,000 metric tons in 1996 (Hardy and Castro, 1994).

FIG. 13.5

An Atlantic salmon weighing 7.5 kg raised in a net pen in coastal waters of Norway.

This rapid growth has affected nearly all aspects of salmon and trout culture, including feeds and nutrition. It has placed unparalleled demands on scientists to provide information on feed ingredients, feed formulations, feed manufacturing processes, and feed quality or efficiency. This, in turn, has resulted in a significant increase in the amount of applied salmonid nutrition research.

13.3.1. Culture Methods

Salmon and trout hatch from large eggs which provide nutriment for the larvae until the feeding and digestive mechanisms are fully functional. When yolk sac absorption is complete, about 30 days posthatch, the fish begin to consume food. At that time they are able to assimilate dry, prepared diets. This is in contrast to the condition with most marine fish, which hatch with very small yolk sacs and require food before the digestive system is fully developed. For reasons that are still unclear, these fish do not assimilate dry diets well and require live foods during early life.

The salmonid fry are first fed a finely textured mash, and as they increase in size, the size of the feed particles increases. The small fry are fed almost constantly, and as their size increases, the feeding frequency decreases. The fry are fed liberally on highly concentrated diets for rapid growth. The crude protein content of starter diets ranges from 36 to 48% and the fat content ranges from 10 to 19%. Salmon for release, however, are fed to a given target size. The feeding rate is adjusted so that the smolts do not get too large, a factor which increases the percentage of fish that return as undersized precocious males.

Generally, trout, including Atlantic salmon, perform best in fresh water, and Pacific salmon best in salt water, however, both groups can be

conditioned to cultures in either area of salinity. All spawn in fresh water. Pacific salmon adjust to seawater quickly, while trout must be acclimated gradually. Most of the rainbow trout cultured are in freshwater raceways fed by constant-temperature, coldwater springs. Figure 13.6 shows a commercial raceway system in the Snake River valley where rainbow trout are raised in fresh water from underground sources.

Salmon and trout reared for market are raised in ponds, tanks, raceways, and net pens (cages). Transfer to sea cages must be done when the fish are undergoing smoltification and are able to osmoregulate in the marine environment. Seawater tolerance occurs at the fry stage in pink and chum salmon, but chinnok, coho, and Atlantic salmon must live in fresh water for months to over a year, depending on the species, stock, and details of rearing, before they can tolerate seawater. Rainbow trout can adapt to seawater after they reach approximately 100 g. After transfer to marine pens, salmon and trout are fed mainly extruded, slowly sinking pelleted feeds until harvest or, in the case of brookstock fish, until voluntary feeding ceases several months before final maturation and spawning.

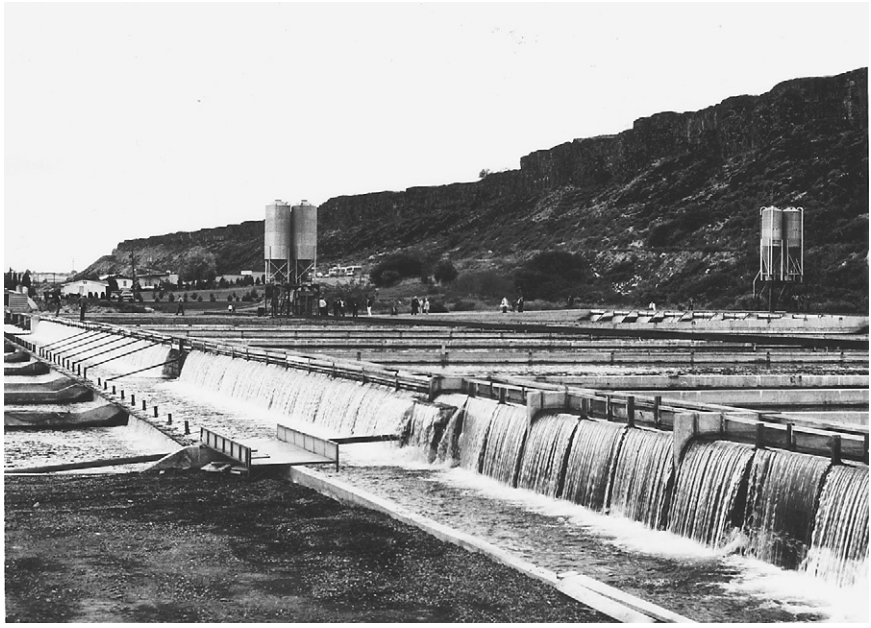

FIG. 13.6

Commercial trout farm in Hagerman, Idaho. Rainbow trout are grown to marketable size in raceways furnished by fresh water from underground sources.

13.3.2. Nutrient Requirements

The dietary requirements for vitamins, minerals, and amino acids in salmonids have been determined in fresh water by feeding small fish in a nonstressed environment. Usually the fish are fed a semipurified diet which contains all the known required nutrients except the one being tested. The dietary level of the nutrient being tested is varied and fish growth or some other physiological parameter is measured and associated with the dietary level of the nutrient at which additional supplementation does not result in increased performance. Little is known about differences in nutrient requirements due to species and size differences, physiological function (such as disease resistance or production), temperature, water salinity, or environmental stresses. Recently information has been obtained on energy and protein levels and sources for practical feeds, especially for Atlantic salmon.

13.3.2.1. Protein and Amino Acids

Salmon and trout require the same 10 amino acids in their diet as other fish and growing warmblooded animals [National Research Council (NRC), 1993]. The quantitative amino acid requirements for coho salmon and rainbow trout are presented in Chapter 3. Amino acid requirements are of extreme economic importance in formulating least-cost fish feeds, thus the minimum dietary level for optimum rate of growth or other responses should be known with as much accuracy as possible.

When the amino acid requirements of salmon and trout are added up, they total only about 15% of the diet. Thus, theoretically, it should be possible to grow salmonids at an acceptable rate on a diet containing 15% essential amino acids and an additional 10% or so nonessential amino acids. In practical feeds, the dietary protein level is usually formulated to be about 50% for swim-up fry and 42% for postjuveniles. The difference between these optimum practical dietary protein levels and the theoretical level of about 25% can be explained by amino acid availability and by the fact that salmonids use amino acids as metabolic energy sources. An additional factor explaining the differences noted above is that dietary amino acids present in excess of the amount needed for tissue protein synthesis are not stored for later use.

The optimum level of protein in commercial salmonid diets is influenced primarily by feed ingredient costs, fish size, dietary energy level, and feed allowance for the fish. For physiological optima, the protein and energy levels should be coordinated; this ratio is reported to be approximately 90 mg of protein/kcal of DE (NRC 1993) for juvenile fish and decreases as the size increases. In production diets for rainbow trout in the United States, 36% protein and 4.0 kcal of DE/g are generally fed. However, in areas in

Europe where feed allowances are restricted, highly concentrated salmon diets are fed, containing up to 50% protein and 5 to 6 kcal of DE/g. The concentrated feeds contain vary high levels of lipid (over 20%) to keep the protein-to-energy ratio in balance.

13.3.2.2. Energy

Salmon and trout are very efficient at using dietary proteins and lipids for energy, but they assimilate carbohydrates very poorly. Lipids are generally a less expensive source of energy for salmonids than are protein and carbohydrate sources, when compared on a metabolizable energy (ME) basis. Raw starch is poorly digested by trout but cooked starch and glucose have higher DE and ME values. While DE and ME appear to be satisfactory indications of productive energy for fats and proteins for salmonids, they are less reliable for carbohydrates. Glucose is over 90% absorbed by rainbow trout, but digestible glucose has a relatively poor protein-sparing effect compared to digestible lipid. High dietary levels of digestible carbohydrate in salmon and trout feeds increase liver glycogen deposition, reduce consumption, and reduce the growth rate.

Salmon and trout raised in hatcheries and net pens have much higher body contents of lipid, in both muscle and visceral stores, than their wild counterparts. High-lipid diets (above 20%) apparently are not detrimental to the fish. In fact, a high body lipid content has been associated with higher fish survival after release from hatcheries. The extremely high fat content of large Atlantic salmon fed high-fat diets, which may reach 30% of the flesh weight, may affect the consumer response in some markets.

13.3.2.3. Essential Fatty Acids

Salmon and trout require 1–2% *n*-3 fatty acids in the diet to prevent essential fatty acid deficiency signs (Castell *et al.*, 1972). These signs can be prevented by any of the *n*-3 fatty acids, although growth rates are reportedly higher when long-chained, highly unsaturated fatty acids of the *n*-3 group, such as C22 : 5 and C22 : 6, are fed. A level of 5% or more of marine fish oil in the diet usually provides a sufficient dietary level of *n*-3 fatty acids for salmonids.

13.3.2.4. Vitamins

Salmon and trout require 15 vitamins in their diet to ensure good growth and optimal health. The quantitative dietary vitamin requirements to prevent overt deficiency signs in salmon and trout fry and fingerlings under constant laboratory conditions have been established and are presented in Chapter 2. However, requirements for fish of various sizes, interaction with other nutrients, broodfish, and various environmental and stress conditions have not been determined. The National Research Council (NRC) vitamin requirements for salmonids do not allow for processing and storage losses.

Because the amount and availability of vitamins in feedstuffs are highly variable, practical diets are supplemented with all the vitamins, usually in amounts in excess of those recommended by the NRC. Overt signs of vitamin deficiency in practical salmon and trout culture are not found when good-quality, fresh commercial feeds are used. Biotin and inositol are usually available in practical feedstuffs in quantities sufficient to preclude their supplementation in commercial feeds.

Production of overt signs of most vitamin deficiencies requires long periods, especially in postjuvenile fish. This, and the fact that practical feed ingredients contain most vitamins except vitamin C, makes it unlikely that fish will develop vitamin deficiency signs quickly when the source of commercial feed is changed. Vitamin C deficiency might be the exception.

13.3.2.5. Minerals

Salmonids, like all animals, require dietary sources of minerals, which they utilize for structural purposes, for osmoregulation, and as cofactors in metabolic reactions. Salmonid dietary requirements for several minerals are presented in Chapter 5. In fresh water, calcium dissolved in the water can supply much of the fish's needs, and in seawater, several other minerals which are dissolved at high concentrations can be a major source of mineral requirements. Other minerals must come from the diet. Feeds which contain a significant amount of fish meal, as do most commercial salmonid feeds, should contain levels of essential elements sufficient to satisfy the requirements for growth. Some dietary components, such as phytic acid in soybean meal and high-ash fish meal, can reduce the availability of certain divalent cations, such as zinc, to the fish, resulting in signs of deficiency. Such diets are overfortified with zinc to prevent deficiency.

Restrictions on phosphorus levels in salmon and trout hatchery effluents and increasing demand on the finite world supplies of fish meal are stimulating efforts to replace a portion of the fish meal in salmonid feeds. This trend, coupled with the conversion to high-energy feeds, may require salmon and trout feeds to be supplemented with essential minerals. At present, salmon and trout feeds are routinely supplemented with several essential microelements (Cu, I, Mn, Se, Zn), mainly to guarantee that the fish receive sufficient amounts should the microelements in the diet have a reduced bioavailability because of complexation with high concentrations of phytic acid and/or calcium phosphate (bone ash).

13.3.3. Feed Formulation

Prior to pelleted feeds, salmonids were fed wet feeds made primarily from beef liver, other slaughterhouse by-products, and various fish or plant products that were available to the hatchery (Hardy, 1989). As the demand

for fresh ingredients outstripped the supply, fresh ingredients were extended by combining them with dry feed mixtures. The development of the Oregon moist pellet (OMP) was a natural evolutionary step in the production of salmonid diets made from wet-mix/dry-mix combinations. The major improvements over earlier feeds were the pasteurization of the fresh ingredients and the pelleting and freezing of the wet/dry mix for shipment to hatcheries from a feed plant. This eliminated the transmission of disease from the feed and the need for each hatchery to prepare its own feed. Eventually, other feed types evolved to the point where the performance of juvenile salmon fed such feeds was equal to that of juvenile salmon fed the OMP, and the OMP was replaced by dry or other semimoist feeds. In trout farming, dry pelleted feeds have been used since the industry was first developed. In Pacific salmon hatchery production, pelleted feeds based on open formulas, such as the Abernathy diet, have replaced the OMP (Hardy, 1991). Several feed manufacturers continue to produce semimoist feeds (12–25% moisture) which contain mold inhibitors and preservatives, thus permitting extended storage without freezing. These feeds are commonly used as starter feeds for Pacific salmon.

When commercial Atlantic salmon farming began in Europe, high-protein, moist feeds were used. The ratio of wet material to dry mix in the moist feeds was about 60 : 40, and these feeds were usually fed within 2 or 3 days of production. Today, nearly all feeds used in commercial salmon farming are made by cooking extrusion. Cooking extrusion was adopted over compression steam-pelleting for four reasons: (1) it produced a lower pellet density, reducing the sinking rate; (2) higher levels of fish oil could be top-dressed onto extruded pellets; (3) cooking extrusion gelatinized the starch in salmon feeds, thus increasing the water stability and digestibility; and (4) extruded pellets are less likely to break during shipping and handling. Today, relatively low levels of starch are added to salmon and trout feeds because salmonids cannot tolerate high levels of available carbohydrates. A primary function of carbohydrates is as a binding agent in expanded (extruded) feeds.

The typical salmon or trout diet consists of fish meal, other high-protein plant or animal sources, fish oil, grain-derived products for binding, and micronutrient premixes (Table 13.4). Diet formulations for salmonids are very high in protein, fat, and DE compared to other animal feeds, and this limits the use of many common feed ingredients, namely, grains, used in conventional animal feeds. Another factor affecting feed ingredient choice is that undigested feed is excreted into the aqueous environment and becomes a pollutant. Restrictions on the concentration of suspended solids, and, in many regions, phosphorus, in aquaculture effluents dictate that feeds be produced from highly digestible ingredients. Ingredients which contain relatively high amounts of crude fiber or which are low in protein and

Table 13.4

Model Diets for Salmonids

Ingredient	Percentage				
	Moist pellet[a]	Fry, dry[b]	Juvenile, dry[c]	Grower, rainbow trout[d]	Concentrated grower, Atlantic salmon[e]
Herring meal	47.5	5.8	55.0	20.0	65.4
Wheat flour or middlings	—	11.22	9.5	20.0	8.0
Wheat germ meal	19.9	—	—	—	—
Dried whey	4.0	—	7.7	7.0	—
Cottonseed meal	—	—	—	—	—
Soybean meal	—	—	—	12.0	—
Corn gluten meal	—	—	—	17.0	
Blood meal	—	2.5	2.5	9.0	—
Poultry meal	—	12.6	12.57	—	—
Vitamin mix	1.5	1.5	1.5	1.0	1.5
Trace mineral mix	0.1	0.05	0.1	1.0	0.1
Herring oil	7.0	12.0	9.0	13.0	25.0
Wet fish waste	20.0	—	—	—	—
Binder	—	2.0	2.0	—	—
Mold inhibiter	—	0.13	0.13	—	—
Carophyll Pink	—	—	—	—	50 mg/kg
Composition					
Crude protein	35.0	48.0	45.0	41.0	50.0
Crude fat	10.0	18.0	15.0	14.0	30.0

[a] Oregon moist pellet.
[b] Abernathy S9 mash.
[c] Abernathy A2-2 starter.
[d] Ontario MNR-89G
[e] Based on guidelines of Torrissen *et al.* (1995).

metabolizable energy cannot be included in salmonid feeds. Thus, corn and other whole grains, the primary ingredients in feeds for domestic animals, are not used. Ground wheat is used sparingly, at levels of between 7 and 12%, as a binder. This trend in commercial salmon feeds has been toward increasing the level of fish oil, so that over the last 20 years, the lipid content of salmon feeds has increased from approximately 15 to more than 30% (Torrissen *et al.,* 1995). Over the same period, sources in salmon feeds decreased, so that by the mid-1990s, salmon feeds were comprised of fish meal, fish oil, ground wheat or some gelatinized grain by-product, and micronutrient premixes. There was no room in these feed formulations for any other ingredient, particularly if it contained less protein than premium-grade fish meal.

Feed conversion ratios decreased during this period from 1.8–2.0 to about 1.0 or less. Protein retention values increased from about 20 to nearly 50%.

An important reason for these changes to more concentrated feeds in northern Europe is to comply with regulations devised to limit the levels of phosphorus and other wastes in hatchery effluents and waters surrounding net pens. The approach by regulators has been to restrict the amount of feed an individual farmer can purchase to raise a fixed amount of fish. To remain profitable, farmers have had to use more efficient feeds, which in turn has resulted in the changes in feed formulation and ingredient selection described above. In North America, regulators have taken a different approach, simply limiting hatchery effluent levels of various materials and letting fish culturists and the salmonid feed industry use their ingenuity to meet target effluent levels.

In addition to ingredient limitations caused by the high dietary protein and lipid levels in salmon feeds and by pollution concerns, the presence of antinutritional factors and compounds that reduce palatability have restricted the use of feed ingredients, mainly those derived from oilseeds. Fry and juvenile Pacific salmon find diets containing even low levels of soybean meal unpalatable, while trout appear to tolerate much higher dietary levels (Bureau *et al.,* 1996). Older salmon and rainbow trout will consume diets in which fish meal is completely replaced by soybean meal, but the performance of fish is improved if some fish meal remains in the feed (Watanabe and Pongmaneerat, 1993). Cottonseed meal can be fed at levels of up to 15% in salmon and trout grower feeds but not broodstock feeds. Higher levels are not recommended due to the gossypol content of cottonseed meal. Canola meal contains compounds which impair thyroid function, so salmon diets should contain no more than 15% and trout diets should be restricted to 25%. High-ash (>15%) fish meal, meat and bone meal, and poultry byproduct meal are generally restricted in salmon and trout feed formulations because of concerns over high phosphorus levels in the hatchery or farm effluents.

Modifications to salmon and trout feeds to influence sensory attributes of farmed products have been limited mainly to carotenoid supplementation for color enhancement of skin and flesh. Salmonids cannot synthesize carotenoid pigments; they obtain these pigments from their prey. In nature, these pigments are synthesized by algae and obtained through the food chain. Early salmon feeds contained carotenoid pigments derived from natural sources, mainly crustacea such as shrimp, crab, and krill. Today, nearly all carotenoid pigment supplementation to salmon and trout feeds is from Carophyll Pink, which is gelatin-encapsulated astaxanthin, the pigment found in crustacea and wild solmonids. Because the astaxanthin in Carophyll Pink is produced by chemical synthesis, it contains a mixture of isomers that

differs from the mixture of isomers in astaxanthin from natural sources. The ratio of astaxanthin isomers can be used to identify wild or farmed salmonids. Recently, astaxanthin was shown to be essential in the diet for normal development of Atlantic salmon fry which were offspring of females reared throughout their life cycle on feeds devoid of carotenoid pigments.

Salmon and trout feeds can be modified to alter the lipid level and fatty acid composition, which can influence the aroma, taste, fresh-product shelf life, and frozen storage stability of products of salmon farming. Increasing the lipid level of the feed increases the lipid storage levels in muscle of salmon and trout. For example, wild trout typically contain about 4–5% lipid in muscle tissue; however, feeding high-lipid diets to rainbow trout can result in muscle lipid levels in excess of 20%.

The tissue fatty acid composition of fish generally reflects the fatty acid profile of lipid sources used in the feed. Fillets of Pacific and Atlantic salmon fed feeds containing soybean oil in place of fish oil have higher levels of linoleic acid and lower levels of ω-3 fatty acids than do salmon fed diets containing fish oil (Hardy *et al.,* 1987, 1991). Rainbow trout fed feeds containing sunflower oil in place of fish oil have much higher levels of oleic acid than control fish and score higher in taste tests (Skonberg *et al.,* 1993). In general, consumers in North America prefer fish that do not smell or taste fishy, and rainbow trout fed feeds in which fish oil is replaced with plant oils are less fishy than those fed feeds containing fish oil. Skonberg *et al.* (1993) reported that fillets of fish fed diets containing sunflower oil had lower concentrations of polyunsaturated fatty acids and were resistant to lipid oxidation after thawing compared to fillets from fish fed feeds containing fish oil.

Supplementing rainbow trout feeds with α-tocopherol during the last few months of rearing greatly increases the concentration of α-tocopherol in the tissues (Boggio *et al.,* 1985), which, in theory, should delay the onset of lipid oxidation in fresh or frozen fish products. However, Boggio *et al.* (1985) were unable to detect any difference in degree of lipid oxidation between high- and low-α-tocopherol fillets after 10 months of frozen storage.

Low-polluting diets are formulated to reduce the organic matter and phosphorus levels in hatchery farm effluent by reducing the levels of indigestible material and unavailable phosphorus in the diet. Reducing phosphorus levels in the hatchery and farm effluents involves careful formulation to match the available phosphorus concentration in the feed to the requirements of the fish and reduce the amount of phosphorus in the feed that cannot be digested by the fish. An additional reduction in the amount of phosphorus generated by salmon and trout farming can be made by feeding a phosphorus deficient diet during the final grow-out period of production (Lellis *et al.,* 1998). Salmonids maintain a body reserve of phosphorus in their hard tissues (bone, skin, fins, scales) from which they can withdraw

phosphorus to maintain plasma levels and maintain other critical functions when the dietary intake is below their requirement (Hardy *et al.*, 1991; Skonberg, 1997). Salmon and trout can continue growing normally when fed phosphorus-deficient feeds for some time. Eventually, the fish use up their body reserves, become aneroxic, and stop growing. If the fish are harvested before they use up their body reserves, they suffer no ill effects, and if they are fed a phosphorus-sufficient feed, their body reserves are restored.

13.3.4. Feeding Practices

How fish are fed affects the profitability of salmon and trout farming and, also, the extent to which salmon and trout farming affect the aquatic environment. Feeding level, feeding frequency, feed particle size, and feed delivery system affect fish growth, feed conversion, uniformity of fish size, cost of fish production, and the amount of waste that must be captured before it leaves the farm and pollutes the environment. Of the feeding practices, feeding level is the most important variable influencing fish growth and feed conversion values. As the feeding rate increases above the maintenance level, the feeding level at which fish neither gain nor lose weight (about 1% of the fish biomass), salmon and trout initially gain weight in a more or less linear fashion. Then as the feeding level approaches the maximum amount the fish can consume, the efficiency of feed conversion starts to diminish and weight gain no longer increases in direct proportion to feeding level. The optimum feeding rate is an economic consideration, although feeding to near-satiation may not be the most efficient physiologically, that level of fish production is usually the most profitable, provided that there is no wasted feed.

Salmon and trout are fed by hand, automatic feeders, and demand feeders. Hand-feeding a series of discrete meals is common with fry and fingerling rearing, but once fish are transferred to larger growing areas, the labor costs are too high. In some situations, fish in raceways may be fed using mechanical blowers that distribute a large amount of feed from a truck or tractor, but most salmon and trout farms now use automatic or demand feeders to reduce labor costs. Figure 13.7 shows an automatic feeder that periodically blows a prescribed amount of feed into a trout raceway. Modern salmon farms often use automatic feeders controlled by computers. The use of feedback devices, such as underwater cameras and ultrasonic detectors, that measure uneaten feed falling to the bottom of net cages, helps to optimize the amount of feed delivered by the automatic feeders. In the United States, many trout farms use demand feeders, which are simple mechanical devices that deliver a small amount of feed in response to an action by the fish. Demand feeders consist of a feed hopper that tapers to a conical bottom with a narrow opening that allows feed to flow onto a movable platform. A

FIG. 13.7

An automatic feeder which blows feed periodically into a trout raceway.

rod extends from the platform into the water, and when fish bump the rod, feed falls into the water.

In many cases the fish are fed using automatic feeders which release a prescribed amount of feed over 24 hr. This makes it necessary to have an accurate estimate of expected feed intake at a given water temperature and fish size. A number of feed allowance equations and tables have been developed for Atlantic salmon (Austreng *et al.*, 1987), Pacific salmon (Buterbaugh and Willoughby, 1967), and rainbow trout which take into account factors such as fish size, temperature, anticipated feed conversion, and desired rate of weight gain.

Feed particle size should be increased as fish grow, and feeding charts such as Table 13.5 provide guidelines for selecting appropriate feed particle and pellet sizes during salmon and trout production cycles. Feeding a correct particle or pellet size is important because, if a pellet is too small, feed will be wasted. If a particle or pellet is too large, the fish will have to break up the feed before swallowing, also increasing the feed waste.

Feeding frequency is another operational variable in salmon and trout rearing. First-feeding fry require almost-constant feeding, while grow-out fish are usually fed heavily once or twice a day. This point of optimizing

Table 13.5

Particle Sizes of Feeds for Various Sizes of Salmon[a]

Fish size (g)	Particle diameter (mm)
<0.5	<0.6 (starter meal)
0.5–2	0.6–0.85 (crumbles)
2–5	0.85–1.2 (crumbles)
5–10	1.2–2 (crumbles)
10–20	2–3.2
20–100	3.2 (pellet)
100–500	4.8 (pellet)
>500 and broodfish	6.5 (pellet)

[a] From Ziegler Bros., Inc., Gardners; PA.

the feeding frequency for fry and fingerlings is to ensure that aggressive fish within a tank, raceway, or pen are not the only fish consuming feed at each feeding. In other words, the feeding frequency must be linked to the amount fed to make sure that all fish have an opportunity to feed. This prevents excessive size disparity within the group or wasted feed and water pollution associated with a single or a few large feedings.

13.4 Tilapias

Tilapias are grouped into two genera: *Tilapia,* which are macrophagous and substrate-spawners, and *Oreochromis,* which are microphagous and mouth-breeders (Trewavas, 1982). About 70 species have been identified in these two genera, however, only 2 *Tilapia* species, *T. rendalli* and *T. zillii,* and 3 *Oreochromis* species, *O. mossambicus, O. niloticus,* and *O. aureus,* have been used widely in practical culture. The common name referred to fish belonging to both genera is tilapia. The most popular culture species is *Oreochromis niloticus,* the Nile tilapia, with a natural dark color or red- or gold-pigmented skin. Figure 13.8 shows a Nile tilapia with light red skin.

Tilapia are tropical fish and do not survive temperatures below approximately 10°C. They have desirable qualities for culture in the tropics in that they grow rapidly, use natural aquatic foods effectively, accept a variety of supplemental feeds, reproduce readily under managed conditions, and are tolerant to a range of water-quality conditions. Some of the cultured species have been shown to survive dissolved oxygen concentrations of 0.1 mg/liter and tolerate nonionized ammonia concentrations of 2.4 mg/liter. Although indigenous to fresh water, tilapias are euryhaline and able to grow well in saline water if properly acclimated. Their activity and feeding become reduced below 20°C and feeding stops at about 16°C.

FIG. 13.8

Genetically improved light-skin (red) *Oreochromis niloticus* grown in an intensive recirculating tank system and fed a nutritionally complete feed.

13.4.1. Culture Practices

Most tilapias are able to reproduce at 5 to 6 months of age and can spawn every 6 to 8 weeks at water temperatures of between 25 and 32°C. The total number of eggs produced per spawning is low and differs among species and fish of different sizes. The mouth-brooding (*Oreochromis*) species lay fewer eggs than the substrate spawners (*Tilapia*). A large-size *Tilapia* species can lay as many as 7000 eggs per spawning, whereas large *Oreochromis* brooders seldom produce more than 2000 eggs.

Breeding of tilapias can be done in earthen ponds, nets, or tanks. Mature fish are stocked at a ratio of two to five females to one male. At water temperatures of 25 to 30°C, fry can be found in about 10 to 14 days after the breeders are stocked. The fry should be removed from spawning facilities at regular intervals, usually weekly or biweekly. The fry collected from the spawning areas are usually transferred to nursery ponds or tanks for rearing to a size suitable for stocking into production units.

The early reproductive capability, high frequency of breeding, and high rate of larval survival often create problems of overpopulation in ponds, which can result in most of the fish not reaching marketable size. To

overcome this problem, it is necessary to suppress reproduction or practice monosex culture. In monosex culture, males are preferred because they have a faster growth rate than females. This can be managed by manual sexing, hybridization, or sex reversal of genotypic females with the use of hormones. Manual sexing, though labor intensive, can be done by selecting the males after the fingerlings have reached 20 to 50 g and have well-developed sexual parts. Hybridization between certain species of tilapia can produce a high percentage of males (85–100%). However, a disadvantage of this technique is the difficulty of maintaining pure stocks that produce a high percentage of males. Sex reversal to produce a monosex male population can be accomplished by administering androgenic hormones during the early larval stage, usually by incorporating hormones into the diet. Methyltestosterone or ethynyltestosterone is incorporated at a concentration of 30 to 60 mg kg^{-1} of diet and fry are fed at 10 to 12% of their body weight per day, divided into three or four feedings, from first feeding for approximately 4 weeks. This method of monosexing of tilapias for culture is widely used commercially and is practiced in most countries in the world. It has not been approved for use in the United States, but application for its use has been filed with the FDA.

Pond culture is the most commonly used system in tropical and subtropical regions. In many parts of the world, where commercial diets are unavailable or expensive, only manures (animal excreta or compost) or inorganic fertilizers are added to the pond. At low stocking densities, where natural food constitutes an important source of nutrients, supplementary feeding with locally available, inexpensive feed materials, such as rice bran, copra meal, brewery waste, coffee pulp, and similar materials, can increase production appreciably. As the stocking rate increases, the natural food becomes less significant and better-quality supplemental feeds are needed. The growing period may last from 3 to 6 months, depending on the preferred market size and management practice. Yields of 300 to 1200 kg ha^{-1} have been obtained with only fertilization in ponds.

Intensive culture of tilapia has gained popularity in recent years because of the good market demand for tilapia fillets in the United States and other industrialized countries. Fish are stocked at very high densities in earthen ponds or raceways with flowing water and aeration and fed with high-quality pelleted feeds. All-male seed stocks are produced in hatcheries, grown to fingerling or submarket size (10 to 50 g) in nursery ponds, and finished to harvest size (0.05 to 1.0 kg) in production ponds. In tropical countries, where favorable temperatures allow year-round production, two or more crops of marketable fish can be harvested in a year. Standing crops of 10,000 to 15,000 kg ha^{-1} are found on commercial farms; however, water exchange and aeration are required.

Under intensive production, nutritionally complete feeds are generally used. Because tilapias, especially those of a small size, can feed effectively on plankton and other natural pond foods, nutritionally complete feeds are probably not necessary in many instances. However, because of the uncertainty of the conditions under which a nutrient is not needed in the diet, nutritionally balanced diets are usually fed in intensively stocked culture systems.

In temperate regions, as in the United States, temperature-controlled recirculating raceway systems are used to grow tilapias. The production units (raceways) are in an enclosed area, such as a greenhouse, to minimize heat loss. The water flows through biofilters to remove organic and inorganic wastes and to replenish oxygen (liquid oxygen is often diffused into the water). Off-flavor is often a problem in recirculating culture systems; however, the flavor of the fish can be corrected by holding the fish for 3 to 5 days in clean water that has not be recirculated.

13.4.2. Nutrient Requirements

Tilapias require the same 10 essential amino acids as other fish and land animals: are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The quantitative requirements for these essential amino acids for growth of young Nile tilapias are presented in Chapter 3. The requirement for the sulfur amino acids, methionine and cystine, can presumably be met by either methionine alone or a combination of methionine and cystine. Dietary cystine can substitute up to 50% of the total sulfur amino acid requirement for *O. mossambicus, O. niloticus,* and, presumably, other tilapias. As for other warmwater fish, lysine is generally the first limiting amino acid in most practical feeds, followed by sulfur amino acids.

Fish meal is a better protein source than plant protein for tilapia. However, soybean meal is a high-quality protein for these species (Jackson *et al.,* 1982). When the 10 essential amino acids were individually added to an all-soybean protein diet, only histidine, isoleucine, phenylalanine, and valine increased the growth of *O. aureus.* However, supplementation of the limiting essential amino acids is not required when soybean protein is supplemented with a small amount of fish meal (Viola and Arieli, 1983).

Tilapias digest the protein of fish meal and meat and bone meal as well as do channel catfish. However, the digestibility of the protein in cereal grains and oilseed meals is higher for Nile tilapias than for channel catfish (Popma, 1982). The digestibility of protein of some common feed ingredients for *O. niloticus* is given in Table 13.6.

Many studies have been reported on protein requirement of tilapias. Most have involved small fish. This is unfortunate because most of the commercial

Table 13.6

Digestibility Coefficients of Feedstuffs Used in Commercial Feeds for *Oreochromis niloticus*[a]

Feedstuff	Percentage digestibility			
	Protein	Fat	Carbohydrate	Gross energy
Fish meal	84.8	97.8	—	87.4
Meat and bone meal (high grade)	77.7	—	—	68.7
Soybean meal	94.4	—	53.5	72.5
Corn	83.8	89.9	45.4	55.5
Uncooked				
Uncooked, mixed with fish meal	—	—	65.4	—
Cooked	78.6	—	72.2	67.8
Wheat	89.6	84.9	60.8	65.3
Wheat bran	70.7	—	—	—
Alfalfa meal	65.7	—	27.7	22.9
Coffee pulp	29.2	—	—	11.4

[a] From Popma (1982).

feed is consumed by large fish. Data on protein requirements for fish from 1 to 50 g in size have ranged from 30 to 50%, varying with protein quality, dietary energy level, feeding rate, natural food, and fish size. Optimum protein levels reported for larger fish have ranged from 25 to 45%. A consensus of experimental data indicates that, without natural foods, small fish should be fed approximately 36% balanced crude protein, and grow-out fish 30 to 32% protein, similar to channel catfish. In the presence of natural pond food, 25 to 30% protein feeds may suffice.

The dietary protein-to-energy ratio required for maximum growth decreases with increasing size of tilapias. Winfree and Stickney (1981) found that small *O. aureus* grew best when the diet contained a digestible energy/protein (DE/P) ratio of 8.2 to 9.4 kcal g^{-1} of protein. Kubaryk (1980) reported that small *O. niloticus* grew maximally when the DE/P ratio was 8.3 kcal g^{-1} for a 36% protein diet. He also found that as the DE content of the diet increased, food consumption decreased, but the amount of protein in the diet did not affect the consumption rate. Tilapias digest the gross energy in commercial feedstuffs relatively well as shown in Table 13.6. They do not digest highly fibrous feedstuffs, such as alfalfa meal and coffee pulp, well for energy needs. They digest carbohydrates in feedstuffs relatively well, much better than salmonids do. Fats or proteins are more digestible to tilapias than are carbohydrates.

Tilapias appear to have a dietary requirement for fatty acids of the linoleic (*n*-6) family. Supplementation of tilapia diets with vegetable oils (soybean or corn oil), rich in 18 : 2 *n*-6, has produced better performance than marine

fish oils high in 20 : 5 and 22 : 6 *n*-3 fatty acids (Takeuchi *et al.,* 1983). The optimum dietary level of *n*-6 fatty acid has been estimated to be about 0.05 to 1%. Deficiency signs observed in fish fed diets deficient in essential fatty acids were poor appetite, retarded growth, and fatty livers. Tilapias do not tolerate as high a level of dietary fat as do salmonids. A dietary lipid level in excess of 12% depressed the growth of juvenile *O. aureus* × *O. niloticus* hybrids (Jauncey and Ross, 1982).

Tilapias can obtain a significant amount of their vitamin needs from the environment when cultured in ponds, even at high densities. Vitamin supplements are often deleted from practical feeds for tilapias cultured under low-fish density conditions in ponds. In intensive raceway systems, where no natural food organisms are present, supplemental vitamins must be added to commercial feeds.

Metabolically, tilapias appear to have vitamin requirements similar to those of other warmwater species. They show the classical vitamin C deficiency signs when deprived of the vitamin in the absence of natural foods. Lovell and Limsuwan (1982) showed that *O. niloticus* produced vitamin B_{12} in their intestinal tract through bacterial synthesis and did not require the vitamin in their diet. They found, however, that young channel catfish fed the same basal diet required supplemental vitamin B_{12} for maximum growth. Other B-complex vitamins apparently are synthesized by the intestinal microorganisms. Until definitive information is available on the vitamins that are produced in sufficient quantities in the intestine, a complete vitamin supplement should be included in tilapia feeds that are fed in cultures where natural food is absent or limited. Some of the dietary vitamin requirements of tilapias are presented in Chapter 2.

Tilapias probably require the same minerals as other fish species for tissue formation, for metabolism, and to maintain osmotic balance between the body fluid and the water. Like other finfish, they probably get a significant amount of calcium from the water. Although there is a lack of information on the mineral requirements of tilapias, it is likely that their quantitative requirements are similar to those of other warmwater finfish species. Phosphorus and trace mineral supplements designed for channel catfish have been used successfully in tilapia feeds.

13.4.3. Feeds and Feeding

13.4.3.1. Natural Foods

Fish of the genus *Tilapia* are macrophyte feeders, that is, the adults feed mainly on filamentous algae and higher aquatic plants. Figure 13.9 shows a *T. zillii* grown in a pond and fed only plant leaves. Tilapias of the genus *Oreochromis* are microphagous; their feeding regime consists notably of phytoplankton, zooplankton, detritus, and benthic organisms. Species

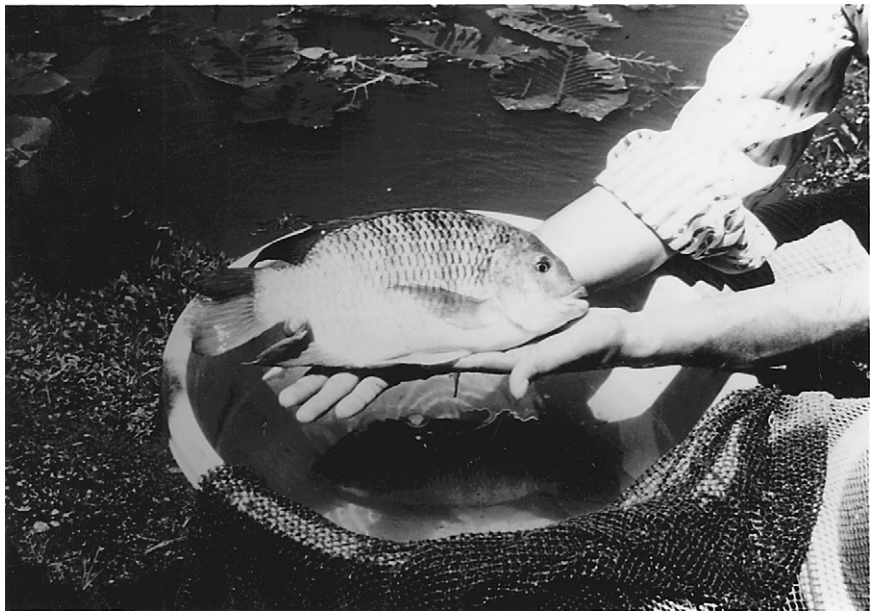

FIG. 13.9

Tilapia zillii, a macrophyte feeder, grown to marketable size without processed feed. The fish was fed plant leaves, which can be seen in the background in the culture pond.

of the genus *Oreochromis* are primarily omnivores. However, there is a great deal of overlap among the diet compositions of various species of tilapias. Bacteria, protozoans, and microcrustaceans attached to detrital particles are important sources of protein, lipids, and vitamins. Some algae are relatively high in protein and energy with a good digestibility.

Most tilapias have short and widely spaced gill rakers but are efficient in ingesting phytoplankton, even *Nannochloris,* a solitary coccoid green alga measuring less than 5 μm in diameter. The collecting processes of the minute food particles involve entrapment of algae in mucus secreted by mucous glands in the mouth and filtration by microbranchiospines present on the outermost gill arches (Fryer and Illes, 1972).

Most tilapia culture in the world is in ponds, where natural food organisms can supply substantial amounts of nutrients required by the fish. Schroeder (1983) used stable carbon isotope analyses of the fish and the food sources and found that natural foods contributed 50 to 70% of the growth of tilapias in ponds receiving supplemental feeds. Stomach analysis showed that up to 50% of the stomach contents of tilapias are natural food in the intensively fed pond cultures, indicating that the natural pond productivity contributed

a substantial amount of nutrients. The size of this contribution to the fish's nutrient requirements for maximum growth will depend upon the pond productivity and the fish density in the pond.

13.4.3.2. Practical Feeds

Commercial pond feeds for tilapias usually contain 25 to 28% protein. Natural pond foods contribute a significant amount of protein, so this level is assumed to be high enough. However, few pond studies have been conducted to compare various diet formulations for extensive or semiintensive culture of tilapias. A 25% protein pellet composed of 15% fish meal, 20% soybean meal, 20% ground wheat, and 45% ground sorghum has been used successfully in this type of production in Israel. The importance of micronutrient supplementation in pond feeds for tilapias is not well known. Due to the extreme variation in the culture practices used, formulation of practical feeds to supplement the nutrient contribution of the natural food efficiently is practically impossible.

In intensive cultures, such as raceways or cages, where tilapias rely solely on the prepared feeds as a source of nutrients, a nutritionally complete feed containing all essential nutrients is fed. The protein content of cage or raceway feeds is usually 32%; however, there have been relatively few experiments on the protein allowance for production feeds for tilapias. In recirculating systems, where the overhead costs of operating the systems are high and unretained organic and inorganic matter is objectionable, highly concentrated feeds may be desirable. Some recirculating culture operators use 36% protein feeds, with energy balanced with protein, and claim that the additional growth rate, compared to that with a 32% protein feed, is economically beneficial. Model formulas for pond and raceway feeds for tilapias are listed in Table 13.7.

Tilapias accept a variety of feeds, in nonpelleted form and in sinking and floating pellets. The crude feedstuffs, such as rice bran and brewery wastes, are offered in nonpelleted form, whereas the compounded diets are most often processed into pellets. Studies have shown that tilapias can utilize meal-type feeds effectively, however, they obviously do not consume the nonpelleted feeds efficiently. High-quality feeds should be processed into pellets to minimize waste.

Feeds in meal or crumble forms are used for fry and fingerlings. These are made by first pelleting or extruding the feed mixture and then reducing the particles to size by crumbling. Usually only one size pellet is used for feeding tilapias to the marketable size of 500 g. The most common pellet size is approximately 3 to 4 mm in diameter and 6 to 10 mm in length. Tilapias seem to prefer smaller pellets than do channel catfish and salmonids of comparable size. They tend to chew the pellets rather than swallow them immediately as do most finfish species.

Table 13.7

Model Tilapia Feeds for Ponds and Raceways[a]

Ingredient	Percentage		
		Raceways	
	Ponds (26% protein)	32% protein	36% protein
Soybean meal	38.3	48.5	50.8
Wheat middlings	4.0	20.0	18.0
Fish meal	4.0	6.0	12.0
Corn	50.8	22.6	16.5
Dicalcium phosphate	1.0	1.0	0.8
Plant oil (sprayed) on pellet surface	1.5	1.5	1.5
Vitamin mix[b]	0.2	0.2	0.2
Trace mineral mix[b]	0.2	0.2	0.2

[a] Adapted from Lovell (1989).

[b] Vitamin and mineral allowances for channel catfish given in Chapter 9 will be sufficient. Vitamin and trace mineral supplements are not necessary in pond feeds if the fish are small and the density is low.

Feeding rates for tilapias are affected by the species, size, energy level in the diet, water quality, feeding frequency, and availability of natural foods. For example, *T. randali* consume more feed than *O. niloticus* of comparable age (Balarin and Haller, 1982). As with other fish, feed consumption rate is inversely related to fish size. Tilapias, like many other cultured fish species, benefit from multiple daily feedings. Kubaryk (1980) found that *O. niloticus* grew faster when fed four times daily than when fed two times but did not

Table 13.8

Feeding Rates and Frequencies for Various Sizes of Tilapias[a]

Size	Daily feeding (% of fish weight)	Times fed daily
2 days old to 1 g	30–10	8
1–5 g	10–6	6
5–20 g	6–4	4
20–100 g	4–3	3 or 4
>100 g	3	3

[a] At 28°C. Adapted from Jauncey and Ross (1982), Coche (1982), and Kubaryk (1980).

grow faster when fed eight times. Small fish should be fed more often than larger fish. The feeding frequency is decreased as the fish increase in size. Table 13.8 lists feeding rates and frequencies for various sizes of Nile tilapias for maximum growth.

13.5 Penaeid Shrimp

Until about two decades ago, all shrimp supplied to the world markets was harvested from the oceans. Presently, the ocean fisheries for shrimp are at the near-maximum sustainable yield. Shrimp catches from the sea are unpredictable due to uncontrollable natural phenomena. Also, pollution and other human activities have disrupted the ecology of shrimp nursery grounds in many areas. Energy requirements for harvesting ocean shrimp are high, which makes the consumer price of the product high. Therefore, the increase in the world demand for shrimp is dependent on an increase in their production through aquaculture. In 1986, pond-raised shrimp represented an estimated 6 to 8% of the world shrimp supply. By 1996, according to the Food and Agriculture Organization (FAO) of the United Nations, farmed shrimp accounted for 43% of the estimated world shrimp consumption.

The development of modern shrimp farming technology began in the 1930s, when Japanese shrimp farmers first successfully spawned and reared larvae of *Penaeus japonicus* in captivity. Since then technology has been developed for mass production of marine shrimp larvae. Within the past 20 years, several shrimp species in the Penaeid family, such as *Metapenaeus ensis, Penaeus japonicus, P. monodon, P. indicus, P. mergiensis, P. aztecus, P. setiferus, P. schmitti, P. chinensis* (also known as *P. orientalis*), *P. penicillatus, P. stylirostris,* and *P. vannamei,* have been matured and spawned, and their larvae reared in captivity. Figure 13.10 shows a harvestable-size *P. japonicus* from a culture pond in Southeast Asia.

As shrimp farming has expanded, the production methods have shifted from the traditional method of trapping and holding wild shrimp which enter coastal ponds through the incoming tidal water exchange to intensive systems where 24,000 kg ha^{-1} is produced per crop. Natural food constitutes an important source of nutrients for extensive culture, whereas artificial feeds are the primary source of nutrients for semiintensive and intensive practices. In semiintensive and intensive culture systems, feed represents up to 50% of the total variable costs. Thus, the use of least-cost, nutritionally balanced feeds and good feeding practices are fundamental to successful shrimp farming.

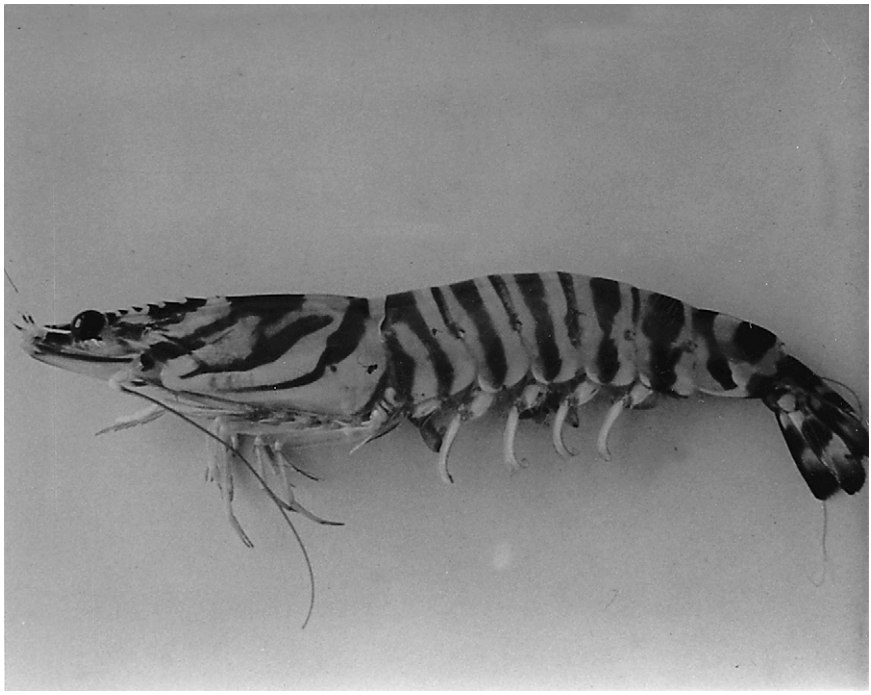

FIG. 13.10

The marine shrimp *Penoeus japonicus* was the first species grown in captivity. It is a popular culture species in Japan and other subtropical areas of Asia.

13.5.1. Culture Methods

The first limiting factor in the culture of penaeid shrimp is the availability of seed stock, or small shrimp for stocking in production ponds. These can come from the wild or from hatcheries. Wild sources are highly unpredictable and fluctuate considerably in quantity, size, and species. Hatchery production of seed stock requires taking spawners, or gravid females, from the ocean or production ponds and allowing them to spawn in hatcheries. The eggs hatch into nauplii, which, after five or six moltings, metamorphose into protozoea. The nauplii do not eat but are nourished from nutrients in the yolk sac.

The protozoeal larvae need exogenous nutrients and are fed primarily with planktonic diatoms such as *Skeletonema* sp., *Tetraselnius* sp., and *Chaetoceros* sp. Food must be in suspension in the culture tank at all times because zoeal larvae have no reflex to search for food but must wait until, by chance, suitable food particles come into contact with the mouth.

Within 4 to 5 days the zoea molt two or three times before transforming into myses. The mysis larvae are fed mainly with *Artemia* nauplii or zooplankton in addition to phytoplankton. The myses metamorphose to postlarvae after three moltings which last 3 to 4 days. During the first 5 days in the postlarval stage, they are fed primarily *Artemia.* Artificial feeds and minced fish flesh are substituted for part of the live foods as the larvae gradually acquire the habit of living and feeding on the bottom of the tanks. The larvae are stocked in nursery or production ponds 14 to 20 days after hatching.

In earthen nursery ponds, the most common stocking rates are from 50 to 200 postlarvae/m^2. The postlarvae are fed two to four times daily with dry diets and/or fresh foods such as chopped fish. Postlarvae in ponds are usually fed a dry, meal-type diet, initially at a rate of 125%, then decreasing to 10% of their body weight per day.

The methods of shrimp culture may generally be classified into three categories: extensive, semiintensive, and intensive techniques. Extensive culture is characterized a low stocking density, usually lower than 2.5 postlarvae or juveniles/m^2. Natural stocking with postlarvae coming in during high tides is still being practiced in some Asian countries. Supplementary feeding is seldom practiced and the shrimp depend mainly on natural foods available in the ponds. Organic and inorganic fertilizers are used to increase the productivity of natural foods. Water management is done through tidal fluctuation. The yields obtained generally range from 150 to 500 kg/ha/crop.

Most of the world's supply of farmed shrimp is produced in earthen ponds by semi intensive management techniques. In semiintensive culture operations the stocking rates per square meter vary from 3 to 15 juveniles, or up to twice this number if early postlarvae are stocked. Commercial feeds are given as supplements to the natural foods. Fertilizers are often applied initially to enhance the growth of the natural food organisms. Water is pumped through the ponds at a rate of 2 to 10% of the pond volume daily when the pond receives feed. Generally, the yields range from 600 to 2000 kg/ha/crop, with 2 to 2.5 crops per year. The average shrimp weight at harvest varies from 16 to 36 g, depending upon the stocking density, pond environment, and management. The price of shrimp is directly related to their size. Figure 13.11 shows shrimp being harvested from large (20-ha) semiintensive ponds in Central America.

In areas where land for pond construction is expensive or unavailable and when the price of shrimp is high, intensive shrimp culture practices may be feasible. Intensive culture operations require sophisticated management techniques, high rates of aeration and water exchange, and nutritionally complete diets. Shrimp are stocked in production units, which may be tanks or ponds, at rates of 20 to 40/m^2. The artificial feed is essentially the sole source of nutrients for the shrimp. Production generally ranges from 2000

FIG. 13.11

Shrimp are harvested at the outflow gate of a 20-ha semiintensive pond on the Pacific coast of Honduras. The production was approximately 1500 kg/ha. The pond will yield another harvest of similar size the same year.

to 9000 kg/ha/crop, but in intensive tank culture as practiced in Japan, production can reach levels as high as 24,000 kg/ha/crop.

13.5.2. Nutrient Requirements and Sources

Shrimp require the same 10 essential amino acids as do finfish and terrestrial animals. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine have been found to be essential for *Penaeus japonicus, P. azteus, Palaemon serratus,* and *P. monodon.* However, the quantitative requirements for all of the essential amino acids have not been determined. In the absence of information on quantitative amino acid requirements, the essential amino acid profile of the protein in an animal can be used. Table 13.9 lists the essential amino acid contents of *P. japonicus* and *P. vannamei* muscle and, also, of squid, which is a high-quality protein for shrimp. A shrimp diet with an essential amino acid profile similar to that of shrimp muscle, clam, or squid would likely provide good growth in the fed shrimp.

Table 13.9

Essential Amino Acid Content of Proteins from Shrimp Muscle (*Penaeus japonicus* and *P. vannamei*) Clam, Squid, and Whole Egg[a]

	Protein source (%)					
Amino acid	*P. japonicus*[b]	*P. vannamei*	Clam[b]	Squid	Casein	Whole egg[b]
Arginine	7.46	8.54	4.50	5.40	3.30	5.45
Histidine	1.66	1.86	1.27	1.50	2.65	1.71
Isoleucine	2.89	3.40	2.00	2.88	4.50	3.46
Leucine	7.04	6.28	4.01	5.79	8.76	6.47
Lysine	7.24	6.97	4.68	5.52	7.34	5.45
Methionine	2.92	2.48	1.70	2.30	2.51	3.01
Phenylalanine	3.90	3.39	2.13	2.86	4.75	4.15
Threonine	3.62	2.69	2.81	3.28	3.77	3.73
Tryptophan	0.52	1.27	0.51	0.72	1.21	3.76
Valine	2.87	3.38	2.18	2.66	5.83	3.76

[a] Adapted from Lovell (1989).
[b] From Deshimaru (1982).

The optimum dietary protein level for growth of penaeid shrimp has been reported to range from 28 to 60%. These values differ due to species, size, protein quality, level of nonprotein energy, physical quality of pellet, palatability of diet, feeding rate, water quality, and availability of natural food organisms. Most of the quantitative protein requirements were determined with small shrimp in tanks or aquaria in the absence of natural aquatic foods. Shrimp grown to harvestable size in ponds likely require less protein in their feed than the higher values reported in the literature.

The protein percentage in commercial feeds fed in intensive culture systems is usually 35% or higher. That in semiintensive culture feeds varies, generally from 25 to 35%. Research at the Enrique Ensenot Marine Laboratory in Panama showed that 25% protein feeds were as productive as higher-protein feeds in fertilized semiintensive culture ponds containing five shrimp per square meter. Similar results were obtained in a cooperative study between Auburn University and Granjas Marinas Farm in Honduras.

Numerous studies have been conducted to evaluate the nutritional values of various protein sources. Fish meal is a high-quality source for finfish but seems to have a lower nutritional value for shrimp, especially when fed as the sole protein source. This has been reported for several species. Shigueno (1975) has suggested that this may be due to a shortage of phenylalanine and the basic amino acids (arginine, histidine, and lysine) in some fish meals. An important role of fish meal in shrimp feeds is to enhance palatability.

Soybean meal, the most commonly used plant protein in feeds for warmwater fish, has been found to be a relatively good protein source for shrimp. It provided better growth of *P. duorarum* than fish meal, shrimp meal, casein, or corn gluten meal (Sick and Andrews, 1973). Soybean meal at levels of 20 to 50% of the diet has been suggested as a replacement for much of the fish meal, shrimp meal, squid meal, or their combination in practical diets for shrimp. Lim and Dominy (1990), however, suggested that, if pellet palatability and water stability can be maintained, the level of soybean meal in the diets of juvenile *P. vannamei* can be increased up to 56%.

Cottonseed meal has been shown to be relatively palatable to shrimp. However, cottonseed meal (0.41% free gossypol) should not be included at more than 26%, or 1100 ppm of free gossypol, in diets of juvenile *P. vannamie*. Also, cottonseed meal is very low in lysine and, for this reason, is not an equal substitute for soybean meal.

Attempts to supplement amino acid-deficient diets with crystalline amino acids have not been successful with shrimp, in contrast to results with channel catfish and farm animals. Deshimaru (1982) showed that the rate of incorporation of free radioactive arginine into muscle protein was less than 1%, compared to the incorporation of 90% of protein-bound arginine. Mai *et al.* (1988) showed that juvenile *P. orientalis* did not absorb free methionine and lysine simultaneously with the protein-bound amino acids. The inability of shrimp to utilize free amino acids as substitutes for protein-bound amino acids is probably due in part to differences in the rate of absorption of free and protein-bound amino acids.

Providing the optimum amount of energy in the diet is important because a deficiency in nonprotein energy means that part of the protein will be used for energy. Inclusion of an appropriate amount of carbohydrates and lipids in the diets of several shrimp species lowered the protein requirement without reducing the growth performance. Excess energy in the diet, however, can limit feed consumption, thereby reducing the intake of protein and other nutrients. Sedgwick (1979) found that the amount of feed consumed by *P. merguiensis* is regulated by the dietary energy level irrespective of the protein content. Relatively little is known about the energy requirements of shrimp. However, the optimum energy-to-protein ratio for of shrimp seems to be similar to that of finfish, 9–11 kcal/g protein. Small *P. merguiensis* had maximum growth with a 39.5% protein diet having a gross energy value of 4.42 kcal/g (Sedwick, 1979). The crude protein-to-gross energy ratio in this study was approximately 90 mg/kcal or 11 kg/g. A practical diet made with conventional ingredients and containing 25 to 35% protein and 4 to 5% fat would have a favorable energy-to-protein ratio.

Shrimp appear to utilize carbohydrates and lipids as dietary energy sources to spare protein. They utilize protein well as an energy source, however, studies have shown that diets with too high a ratio of protein-to-nonprotein

energy depress the growth rate. Lipids are required in the diets of shrimp not only for their caloric value but also as sources of essential fatty acids, fat-soluble vitamins, sterols, and phospholipids necessary for normal growth.

Feeding experiments have shown that linoleic (*n*-6)- and linolenic (*n*-3)-series fatty acids are dietary essentials for *P. japonicus, P. indicus, Palaemon serratus, P. stylirostris,* and *P. vannamei.* Shrimp appear to have a limited ability to desaturate and elongate 18 : 3 *n*-3 to 20 : 5 *n*-3 and 22 : 6 *n*-3 fatty acids, which are the biologically active fatty acids. The optimum dietary level of highly unsaturated *n*-3 fatty acids, 20 : 5 *n*-3 and 22 : 6 *n*-3, for shrimp has been determined to range from 0.5 to 1.0%, while the optimum level of the *n*-6 series of fatty acids is estimated to be approximately 0.5%. Diets containing 0.5% *n*-6 and 0.5% *n*-3 fatty acids have provided for maximum growth of several shrimp species.

Although dietary lipids have a sparing effect on the utilization of protein, shrimp do not tolerate as high a dietary lipid level as do salmonid fish. Several studies using different lipid sources or combinations have suggested that a lipid level in excess of 10% tends to depress growth.

Crustaceans do not synthesize sterols from acetate or mevalonate as do finfish, and therefore they require a dietary source. Cholesterol is the major sterol found in crustaceans and is a precursor of sex hormones, molting hormones, and a constituent of the hypodermis in crustaceans. In addition to the essential fatty acids and sterols, marine shrimp seem also to have a dietary requirement for phospholipids, such as lecithin. The optimum dietary levels of phospholipids for shrimp range from 1 to 3% depending on the species, life stages, and source and nature of phospholipids and, possibly, other dietary nutrients. Lecithin from soybeans is an economical and nutritionally suitable source of phospholipids for shrimp.

Among the 15 vitamins which have been identified as essential for finfish, 14 have been demonstrated through research to be dietary essentials for shrimp. Dietary requirements for several of the fat-and water-soluble vitamins are discussed in Chapter 2. The dietary levels of various vitamins reported for shrimp are considerably higher than those for finfish. Whether or not shrimp have a metabolic requirement for such high levels of these nutrients or whether a substantial quantity is lost into the water during ingestion by the shrimp is unknown. Moreover, the information available is confined to the postlarval or early juvenile stage cultured in the laboratory under well-controlled environmental conditions. Thus, in the absence of clear-cut information on the vitamin requirements of shrimp, the vitamin allowances listed in Table 13.10 are offered as guidelines for nutritionally balanced shrimp feeds (Chhorn Lim, United States Department of Agriculture, Diseases and Parasites Laboratory, Auburn, AL).

Shrimp respond to dietary additions of phosphorus, but in seawater they apparently are able to absorb sufficient magnesium and calcium from the

Table 13.10

Recommended Vitamin Allowances for Supplemental and Complete Practical Feeds for Shrimp[a]

Vitamin	Amount per kilogram diet	
	Supplemental	Complete
Vitamin A	2000 IU	4000 IU
Vitamin D	1000 IU	2000 IU
Vitamin E	50 mg	100 mg
Vitamin K	10 mg	20 mg
Thiamin	20 mg	50 mg
Riboflavin	10 mg	30 mg
Pyridoxine	30 mg	60 mg
Pantothenic acid	30 mg	80 mg
Niacin	30 mg	80 mg
Biotin	0	2 mg
Folic acid	2 mg	5 mg
Vitamin B_{12}	0.01 mg	0.05mg
Inositol	50 mg	200 mg
Vitamin C (stable form)	100 mg	200 mg
Choline chloride	500 mg	1500 mg

[a] From Dr. Chhorn Lim, United States Department of Agriculture, Fish Diseases and Parasites Laboratory, Auburn, AL.

water. Supplementation of shrimp diets with the trace minerals, iron, copper, zinc, selenium, and manganese, improves growth (Deshimaru and Yone, 1978). Lim has recommended the mineral allowances listed in Table 13.11 for practical feeds for shrimp.

13.5.3. Feeds and Feeding

13.5.3.1. Natural Foods

Adult penaeid shrimp are regarded as omnivorous scavengers that feed on a variety of benthic organisms and detritus, but they cannot be placed in any one trophic level because they are generally opportunistic feeders. In the zoea and mysis stages, larvae feed on free-swimming plankton. Postlarvae, being strictly demersal, are detritivores. The feeding habit of juveniles is initially omnivorous and then changes to carnivorous, and they prey mainly on slowly moving microinvertebrates.

Highly productive shrimp diets for intensive culture systems have been developed. These diets, however, are probably not the most economical in semiintensive pond systems, where most shrimps are commercially produced, because shrimp are omnivorous scavengers which actively feed on a

Table 13.11

Recommended Mineral Allowances for Nutritonally Complete Shrimp Diets[a]

Mineral	Amount per kilogram dry diet
Macromineral (g)	
Calcium	10.0
Phosphorus	10.0
Potassium	6.0
Magnesium	0.4
Micromineral (mg)	
Manganese	40.0
Zinc	33.0 or 200.0
Iron	60.0
Copper	32.0
Iodine	5.0
Selenium	0.4
Cobalt	0.4

[a] From Dr. Chhorn Lim (1999), United States Department of Agriculture, Fish Diseases and Parasites Laboratory, Auburn, AL.

variety of aquatic organisms. Under pond conditions, the primary source of natural foods for shrimp is the thin aerobic layer of the pond bottom, which consists of living and dead algae and plankton, small crustaceans and mollusks, bacteria, detritus, and other benthic organisms such as polychaetes and annelids. Although shrimp obtain a significant quantity of nutrients from the pond, the amount or percentage of their daily requirement from this source is difficult to estimate. Shrimp yields of up to 500 kg/ha/crop are obtained in ponds without feeding. Because of the strong propensity of shrimp to consume natural food and great variations in the availability of food under various culture environments and management conditions, formulation of least-cost feeds for semiintensive pond cultures is difficult.

13.5.3.2. Feeding Behavior

Shrimp find their foods mainly by chemosensory mechanisms rather than vision. The chemoreceptors are concentrated on the anterior appendages, antennae, and antennules. Once the scent is detected, the shrimps move toward the food and rapidly seize it with their chelate pereiopods. The food is chewed to a fine particulate size before being swallowed. The ingested food passes through the esophagus and enters the anterior chamber of the proventriculus (foregut), where it is reduced further to a semifluid state mechanically and by digestive enzymes. The fluid passes into the posterior

chamber and, finally, into the tubules of the hepatopancreas for further digestion and absorption. The coarser particulates pass directly to the midgut, where there is some digestion and which is an important site of nutrient absorption. The undigested portions of food enter the hindgut, which serves mainly as a region for the compaction and transportation of fecal material.

13.5.3.3. Practical Feeds

Commercially processed feeds are the primary source of nutrients offered to shrimp in nurseries and semiintensive and intensive grow-out operations. However, fresh tissue, such as trash fish, clam, and squid, is often fed to broodfish, whereas live foods, such as rotifers, *Artemia,* and algae, are major sources of food fed to shrimp larvae.

Although research information is available on the basic nutrient requirements of several shrimp species, there is a scarcity of research data on recommendations for pond feeds. Because the culture environment makes a valuable contribution to the nutrient requirements of shrimp, cost-effective feeds for the various culture systems and management practices are difficult to design. The necessity of supplementing various pond feeds with all nutrients, such as vitamins and essential lipids, has not been established. Most commercial feeds contain a vitamin premix. Generally, higher-protein diets are fed during early postlarval stages and juvenile stages, and the protein percentage decreases during the grow-out period. Examples of formulas for practical shrimp diets are listed in Table 13.12.

13.5.3.4. Feed Processing

Due to the benthic feeding behavior of shrimp, practical commercial feeds should be processed into sinking pellets. Sizes of the pellets vary depending on the size of the shrimp. Crumbles are used during postlarval stages and pellets are fed from the juvenile stage through the marketable size. Recommended pellet diameters for various sizes of shrimp are 1–2 mm for 0.5- to 2-g shrimp, 2 mm for 2- to 5-g shrimp, 3 mm for 5- to 10-g shrimp, and 4 mm for 10-g shrimp and above. Shrimp are selective and slow eaters, thus, shrimp pellets should remain stable in the water for several hours until consumed.

Two manufacturing processes, extrusion and steam pelleting, are commonly used to produce shrimp feeds. Extrusion processing is widely used to produce floating pellets for finfish, however, sinking feeds can also be produced with a cooker extruder by reducing the expansion rate, thus yielding a denser pellet. The extruded pellets usually have a good water stability due to the high level of starch gelatinization. However, most shrimp feeds are processed with specially designed compression pellet mills equipped with steam injectors and a steam-jacketed preconditioner to allow for extra cooking of the feed mix.

Table 13.12

Model Diets for Salmonids

Ingredient	Moist pellet[a]	Fry diet[b]	Juvenile diet[c]	Grower diet, rainbow trout[d]	Concentrated grower diet, Atlantic salmon[e]
Herring meal	47.5	5.8	55.0	20.0	65.4
Wheat flour or midds	—	11.22	9.5	20.0	8.0
Wheat germ meal	19.9	—	—	—	—
Dried whey	4.0	—	7.7	7.0	—
Soybean meal	—	—	—	12.0	—
Corn gluten meal	—	—	—	17.0	—
Blood meal	—	2.5	2.5	9.0	—
Poultry meal	—	12.6	12.57	—	—
Vitamin mix	1.5	1.5	1.5	1.0	1.5
Trace mineral mix	0.1	0.05	0.1	1.0	0.1
Herring oil	7.0	12.0	9.0	13.0	25.0
Wet fish waste	20.0	—	—	—	—
Binder	—	2.0	2.0	—	—
Mold inhibiter	—	0.13	0.13	—	—
Carophyll pink	—	—	—	—	50 mg/kg
Composition					
Crude protein,%	35.0	48.0	45.0	41.0	50.0
Crude fat, %	10.0	18.0	15.0	14.0	30.0

[a] Oregon moist pellet
[b] Abernathy S9 mash
[c] Abernathy A2-2 starter
[d] Ontario MNR-89G
[e] Based on guidelines of Torrissen *et al.* 1995.

Various substances, either natural, modified or synthetic, are used as binding agents for steam-pelleted shrimp feeds. Wheat gluten, high-gluten wheat flour, tuber and cereal starches, and/or their combination are the most commonly used natural binders in shrimp pellets. Commercial binders, such as lignin sulfonate and bentonite, which are commonly used for pelleted livestock feeds, have been found to be less effective for shrimp feeds that require a long duration in water. Organic hydrocolloids, such as carboxymethyl cellulose, alginate, and gums, have been used successfully in laboratory-prepared diets but their use in commercial feeds is limited due to high costs. Synthetic binders such as Aqua-firm 1A and Aqua-firm 2A (produced by Agresearch, Inc., Joliet, IL) are marketed for use in shrimp feeds.

Attractants are often used in shrimp feeds to increase feeding activity and feed consumption. Various substances, such as amino acids, fatty acid,

and extracts of fish, shrimp, squid, mussel, and clam, have been shown to stimulate the feeding response in shrimp. These studies have been primarily under laboratory conditions and the value of attractants under pond feeding conditions is not clearly established. Fish meal is generally considered to be an attractive ingredient in shrimp and finfish feeds.

13.5.3.5. Feeding Practices

Because shrimp are slow feeders, satiation feeding is difficult and probably impractical to attain. Some producers feed according to a prescribed schedule throughout the growing season based on the initial stocking rate experience. To feed more efficiently, some producers will try to estimate the feeding activity in the pond. They may place feeding trays in the pond, and the amount of feed remaining in the tray an hour or two after feeding will determine if the feed allowance for the following day will be increased or decreased. Some producers collect a handful of bottom mud from the feeding area and look for uneaten feed or foul odor of decomposing feed. Experienced producers can observe the swimming behavior of shrimp along the

FIG. 13.12

Shrimp in large semiintensive ponds are being fed from a boat. Unlike most finfish, shrimp do not move great distances to feed, thus the diet must be distributed over a large area of the pond.

shoreline an hour or two after feeding and determine if the shrimp were fed enough. Feeding tables have been prepared by feed companies and various agencies for various environments; these may be used as feeding guidelines if the biomass of shrimp in the pond can be estimated. The amount of feed offered should not exceed the capacity of the system to assimilate the waste products and maintain a sufficient level of dissolved oxygen.

Because shrimp eat slowly and more or less continuously, multiple daily feedings are desirable. Under laboratory conditions, the optimum feeding frequency for *P. monodon* juveniles was three times per day (Lim and Pascual, 1979). Disintegration of the feed and loss of water soluble nutrients can be minimized through multiple daily feedings rather than once-daily feeding. Unlike fish, shrimp are territorial and do not swim great distances to feed. Thus, it is important to distribute the feed uniformly over the pond or in areas where the shrimp are located. For small ponds feeding may be done by hand-broadcasting from the pond bank. In large ponds, feeds are distributed from boats (Fig. 13.12), tractor-mounted feed blowers, or airplanes.

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14

Nutrient Flow and Retention

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14.1 Introduction

Fish nutrient metabolism is the sum total of the biochemical reactions in life processes. Nutrient metabolism pathways outlined in classical biochemistry texts such as those by Lehninger (1979), West *et al.* (1966), and Kaneko (1989) for terrestrial animals also apply to poikilothermic animals such as fish. Fish species vary from carnivores to omnivores to herbivores living in a wide range of water temperatures, from near 0 to near 40°C, with corresponding abilities to utilize carbohydrate, protein, and lipid sources for energy and metabolites for anabolic tissue processes. The main nutritional difference between terrestrial and aquatic animals involves the ability to exploit plants as food sources. Terrestrial animals exploit the ability of plants to convert the sun's energy into leaves and seeds, either directly or indirectly through the food chain. Some fish utilize aquatic plants directly, but most exploit plankton, both phytoplankton and zooplankton, again either directly or indirectly through the food chain. There are no fish equivalents of ruminant animals. Thus, fish exhibit subtle differences in genetic isozymes associated with their habitat, but in general, cellular metabolism among fish species is similar. In this chapter, the major nutrient pathways are outlined, and the emphasis is on differences among fish species and between fish and terrestrial animals.

Most fish live in a heat sink and their metabolism rates are governed by the water temperature. As a result, most fish have adapted enzyme systems to poikilothermic environments and their "genetic isozymes," while very similar in structure, combine with the same general substrates to accomplish the digestion, absorption, and subsequent biochemical reactions necessary for energy generation and metabolite incorporation into cell functions. Different groups of fish have different abilities to digest and use complex polysaccharides as energy sources but are well adapted to use proteins and lipids as a preferred energy source. Warmwater freshwater fish generally can use more carbohydrate in the diet and herbivorous fish have well-developed systems to utilize plant material. All fish still need to metabolize proteins for amino acid sources for anabolism and growth. As a result, all fish studied have well-developed proteolytic enzyme systems and some have both acidic and basic tryptic enzymes, in contrast to terrestrial animals. Another feature is the presence of active chitinase in many species with the ability to digest and utilize the 2-aminoglucose polymer present in the exoskeletons of insects and crustaceans. Lipid utilization is well developed in most fish, although certain cyprinids have a limited ability to use high-lipid diets without generating liver toxicity and obesity. Fish, living in water, also have the advantage of direct contact with a good solvent for absorption of

excretions. Therefore, fish eliminate most of the nitrogen resulting from amino acid catabolism and other nitrogenous metabolites into the water as ammonia from the gills. This is an energy-sparing system that increases the amount of metabolic energy that fish derive from protein metabolism. As a result, fish are more efficient in protein utilization than are homeothermic terrestrial animals, which must eliminate nitrogenous waste as urea or uric acid. More specific information on the above principles can be found in the chapters on bioenergetics (Chapter 1), proteins (Chapter 3), lipids (Chapter 4), and digestive physiology (Chapter 7) in this book.

14.2 Carbohydrate Metabolism

Digestibility of polysaccharides is poorly developed, although present, in carnivorous fish. Salmonids and most marine species use protein and lipid as the major energy and anabolic intermediates for growth. Cowey (1989) summarized the amylase, pepsin, and trypsin activities in carp and yellowtail, showing a 10-fold increase in amylase activity in the warmwater carp versus the marine yellowtail, little, if any, pepsin activity in the neutral stomach of the carp, but comparable tryptic activity in both species.

Glycogen is still the major carbohydrate storage form in the liver and muscle of fish, and even during long periods of starvation or migration of salmonids, little loss of liver or muscle glycogen occurs (French *et al.,* 1983). This is evidence for gluconeogenesis from amino acids catabolized during the starvation period.

14.3 Glycolysis

The classical Emden–Meyerhof pathway of glucose metabolism after the formation of glucose 1-phosphate exists in fish. After glucose 6-phosphate is formed, it is converted into fructose 1,6-diphosphate, which is split into two molecules of glyceraldehyde 3-phosphate and then condensed into two molecules of pyruvate which can enter the tricaboxylic acid cycle (TCA) as acetyl-coenzyme A (acetyl-CoA). All enzymes of the cycle have been identified in fish tissues (Tarr, 1972) (Fig. 14.1).

This pathway allows conversion of glucose into acetyl-CoA and subsequent generation of the high-energy bonds in adenosine triphosphate (ATP) from adenosine diphosphate (ADP). The net result from 1 mol of glucose is 36 mol of ATP. Each mole of ATP hydrolyzed in the cell yields 7 kcal of energy for anabolic and catabolic reactions. The biological efficiency of the

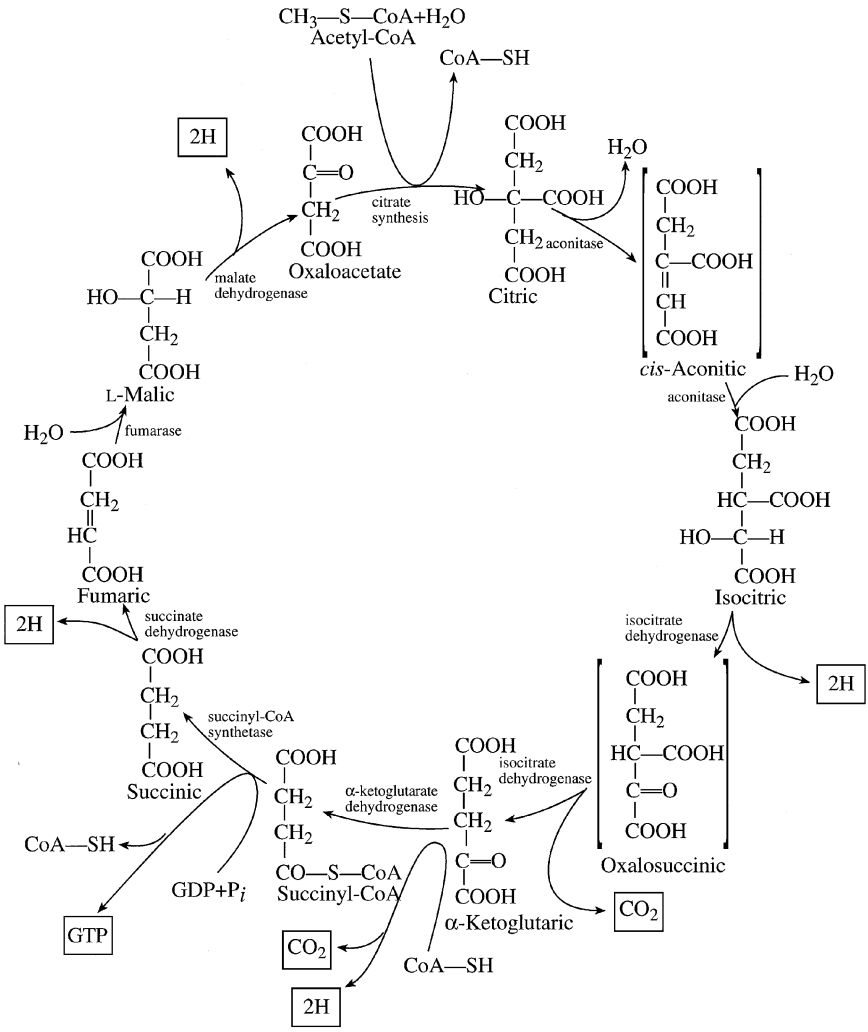

FIG. 14.1

Major enzymes in glycolysis.

aerobic metabolism of glucose can be calculated from the bond energies involved.

$$C_6H_{12}O_3 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 686 \text{ kcal}$$

$$\text{Enzymatic glycolysis} \longrightarrow 36 \text{ ATP} \times 7 \text{ kcal} \longrightarrow 252 \text{ kcal}$$

$$\text{Theoretical biological efficiency} \longrightarrow 38\%$$

Under anaerobic conditions in the muscle of fish, the glucose is converted into lactate and only 2 mol of ATP per mole is available. The lactate also requires considerable time to be eliminated from the muscle with adequate oxygen in the water environment. Tarr (1972) listed each of the enzymes involved in glycolysis and the references for each demonstrated in fish tissues. Cowey and Walton (1989) discussed the irreversible reactions and probable regulatory factors in glycolysis and gluconeogenesis and, also, the range of glycolytic enzymes in trout (Walton and Cowey, 1982). Other specific enzymes in different fish tissues were more recently identified and are included in the discussions in Chapters 1 through 5 of this book.

14.4 Carbohydrate Synthesis

Carbohydrate synthesis is a very energy-expensive process in fish tissue. Two moles of pyruvate are needed for the synthesis of 1 mol of glucose. This uses six high-energy phosphate bonds, but anaerobic conversion of glucose into pyruvate yields only 2 mol of ATP. Gluconeogenesis does occur in fish, however, especially during extended periods of starvation or migration when muscle and liver glycogen must supply the necessary blood glucose for life processes.

14.5 Pentose Phosphate Pathway

The pentose phosphate pathway is a minor path for synthesis of ribose molecules and niacin adenine diphosphate (NADP). The pathway in fish has been described by Walton and Cowey (1982):

$$\text{D-Glucose 6-phosphate} + 2\ \text{NADP} + H_2O$$
$$\longrightarrow \text{D-ribose 5-phosphate} + CO_2 + 2\ \text{NADPH} + 2\ H^+$$

The reducing equivalents may be used for lipid synthesis. High-carbohydrate diets in catfish induced increased levels of glucose-6-phosphate dehydrogenase in the liver, indicating stimulated lipogenesis (Likimandi and Wilson, 1982). For more information on lipogenesis see Chapter 4.

14.6 Glycogenolysis

Glycogen in liver and muscle is the available glucose storage unit in fish as in terrestrial animals. Glycogenolysis is one source of glucose, but glycogen

maintenance and hydrolysis to meet metabolic needs are critical to fish health. Glycogenolysis does not appear to be the major source of liver or muscle glucose when adequate dietary levels of precursors are present, but it may be temperature dependent and an emergency strategy before glucose is made available from the diet or by gluconeogensis from amino acids from digested protein (Renaud and Moon, 1980; Siebert, 1985; Cowey and Walton, 1989).

14.7 Diet and Carbohydrate Metabolism

Dietary balances may affect carbohydrate metabolism. Cowey *et al.* (1977) showed that trout with a 60% protein diet and no digestible carbohydrate had significantly higher gluconeogenesis than trout fed a low-protein diet with 50% carbohydrate present. Fasting fish also had a high gluconeogenesis rate, comparable to that of fish on a high-protein diet. Two other glycolytic enzymes measured also showed an elevated activity, similar to that in fish fed the high-protein diet. Buhler and Halver (1961) showed that salmon would utilize simple hexoses readily and disaccharides well, but polysaccharides were poorly used in the cold waters that salmon occupy. Digestible energy values were calculated to be as follows.

Raw starch:	1.5–2 kcal/g
Cooked starch:	2.5–3 kcal
Dextrin:	3–3.5 kcal
Glucose:	4.0 kcal
Sucrose:	4.0 kcal
Cellulose:	0.1 kcal

Digestible energy values for protein and lipids were calculated by Smith (1989) to be as follows.

Digested protein:	5.0 kcal/g
Digested lipid:	8.0 kcal/g

14.8 Lipid Metabolism

An extensive discussion on lipid metabolism can be found in Chapter 4. Simple fats are digested by lipases, releasing fatty acids and glycerol. The fatty acids are degraded 2 carbon units at a time in classical β-oxidation steps,

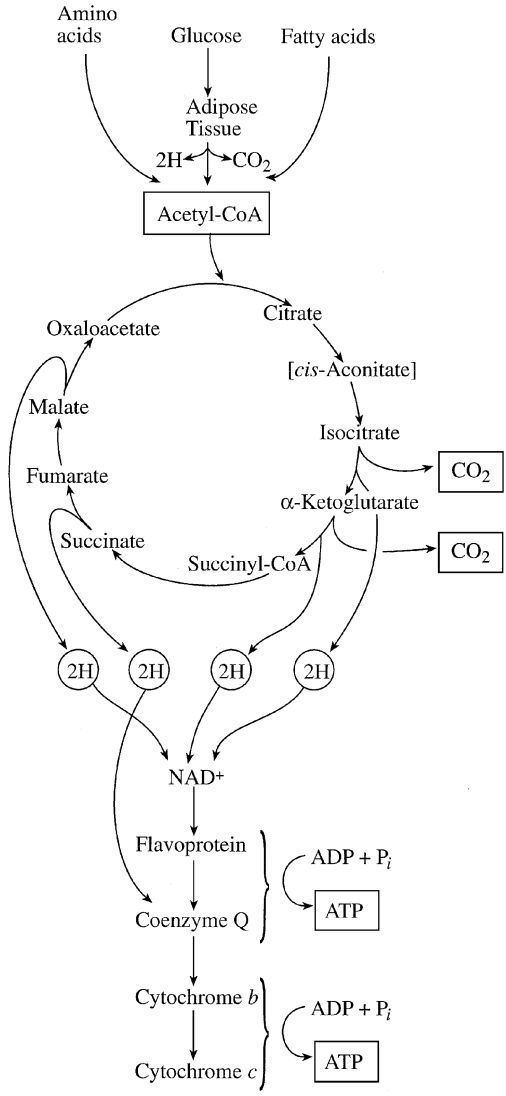

FIG. 14.2

Nutrient flow and ATP production.

yielding acetyl-CoA for transport into the TCA cycle to generate high-energy phosphate bonds in ATP. The primary oxidation sequence is as follows:

(1) Fatty acid plus ATP and S–CoA forms fatty acid–S–CoA.
(2) Dehydration forms β-dehydro fatty acid–CoA.
(3) Hydration forms β-hydroxy fatty acid–CoA.
(4) Oxidation forms β-keto fatty acid–CoA.
(5) The lysis by CoA–SH forms new fatty acid–CoA and acetyl–CoA.

Then further β-oxidation in the steps above is needed to yield more acetyl-CoA. Each cycle reduces the carbon chain of the fatty acid by two carbons.

As an example, palmitic acid (C_{16}) forms palmityl-CoA and yields eight acetyl-CoA molecules. In the process, seven reduced flavin adenine dinucleotide (FADH) and seven reduced diphosphopyridine nucleotide (DPNH) molecules are generated. The net energy gain is then as follows.

(a) Palmitic acid to palmityl-CoA ----------→ −2 ATP
(b) 7 Palmityl-CA yields 8 acetyl-CoA
  (i) 7 FADH to 7FP (7 × 2) ----------→ +14 ATP
  (ii) 7 DPNH to 7 DPN (7 × 3) ----------→ +21 ATP
(c) 8 Acetyl-CoA to 16 CO_2 + 8 HOH
  + 8 CoASH (8 × 12) ----------→ +96 ATP

Estimated biological yield: 129 ATP × 7 kcal = 903 kcal

But complete oxidation of palmitic acid to CO_2 and H_2O is 2340 kcal. Hence the biological efficiency is 903/2340, or 39%. The biological efficiency of fatty acid cellular oxidation is thus approximately the same as that for digestible carbohydrates or lipids and, as shown later, for proteins as well.

Several vitamins are involved in the various steps of oxidation: pantothenic acid in CoA in step (1); riboflavin as flavin adenine diphosphate in step (2); biotin and ascorbic acid in step (3); riboflavin, niacin, and pyridoxine in step (4); pantothenic acid again in step (5); and lipoic acid during the TCA cycle that follows utilization of the "active acetate" to generate ATP for cell metabolic energy demands (see Fig. 14.2).

14.9 Odd-Chain-Length Fatty Acid Oxidation

Odd-Chain-Length fatty acids are present in small amounts in fish skin slime and on exoskeleton surfaces of insects and crustaceans. These serve as bactericidal agents when degraded by β-oxidation to three-carbon propionic acid, which cannot be degraded by most bacteria. Metabolism of these fatty acids involves removing one carbon atom from the fatty acid and thus generating an even-chain-length fatty acid that can then be used by β-oxidation for energy production. Specific enzymes for demethylation similar to those

found in terrestrial animals have been identified in fish (Nicolaides and Laves, 1958; Gurr and Harwood, 1991).

14.10 Electron Transfer Cascade

In oxidation–reduction reactions electrons are transferred from the electron donor to an oxidant or electron acceptor. There are four main classes of these enzymes.

(1) Pyridine dehydrogenases catalyze reversible transfer of electrons from the substrate to niacin adenine dinucleotide (NAD) or NAD phosphate to form NADH or NADPH.
(2) The flavin dehydrogenases contain flavin mononucleotide (FMN) or dinucleotide (FAD). The most important flavin oxidases are succinic dehydrogenase and NADH dehydrogenases.
(3) Iron–sulfur proteins have from two to eight atoms of iron and a comparable number of acid-labile sulfur atoms where the iron atoms undergo Fe^{2+} to Fe^{3+} changes as electrons are transferred.
(4) Cytochromes in series transfer electrons from the flavoproteins to oxygen. These contain iron–porphyrin complexes and can undergo reversible Fe^{2+} to Fe^{3+} reactions. Electron transport from NADH to oxygen involves a decrease in free energy, which can be conserved by phosphorylation of ADP to ATP to yield the high-energy bonds readily available as an energy source for anabolism and catabolism in tissues. Copper-containing cytochromes also occur in the pathway, with the copper available for electron transfer from the Cu^{+} to the Cu^{2+} state. Molecular oxygen can be used in the system also to hydroxylate organic compounds. Dioxygenases insert two oxygen atoms into the substrate and monooxygenases insert only one atom of oxygen. Cytochrome P450 is a common active cofactor in enzymatic hydroxylations. The schematic cascade of oxidative phosphorylation was succinctly illustrated by West *et al.* (1966) in their textbook of biochemistry for medical students.

14.11 Amino Acid Metabolism

Fish and all animals need a constant source of amino acids for tissue protein synthesis and for synthesis of other compounds associated with metabolism, including hormones, neurotransmitters, purines, and metabolic enzymes. Amino acids are also catabolized to supply metabolic energy. Amino acids for these purposes are supplied by the diet and, also, by turnover of amino acids in tissue proteins, which occurs constantly. Free amino acid levels in tissues are relatively low; most amino acids in the body are

components of tissue proteins. Thus, the need for a constant supply of amino acids for metabolic needs is a reality of life. Compared to rats, fish appear to be more dependent on dietary amino acids to supply metabolic needs. Cowey and Luquet (1983) estimated that catabolism of tissue proteins supplies about 50% of the amino acids used in metabolism; whereas in rats this figure approaches 70% (Millward *et al.,* 1976). Additional details are reported in Chapter 6.

As mentioned earlier, fish excrete nitrogen from amino acid catabolism mainly as ammonia, rather than as urea or uric acid, as is the case in mammals or birds, respectively. Synthesis of urea and uric acid from ammonia consumes energy, thus mammals and birds derive less metabolic energy from amino acid catabolism than do fish. This special facility of fish to utilize amino acids as energy sources may explain why it is difficult to feed diets under laboratory conditions that yield much more than 55% protein (nitrogen) retention, whereas in other animals, nitrogen retention under ideal conditions is higher (National Research Council, 1974). Ammonia represents 70–90% of the total nitrogenous wastes in fish (Mommsen and Walsh, 1992), with 5–15% excreted as urea, depending on the species of fish (Dosdat *et al.,* 1996). Between 80 and 90% of all nitrogen excretion in fish occurs through the gills.

Amino acid catabolism occurs mainly in the liver as a part of nutrient metabolism via deamination reactions, i.e., aminotranferase and deaminase reactions in the cytosol and mitochondria, respectively. Each amino acid has a specific route of catabolism involving enzyme-catalyzed reactions with specific end products. This subject has not been studied extensively in fish; it is assumed that pathways and reactions identified in animals also exist in fish. Cowey and Walton (1989) summarized the enzymes and catabolic pathways of amino acid degradation for each amino acid, and this information still holds today. The first step in the catabolism of amino acids is the removal of the amino group (transdeamination) to intermediate compounds that can be further metabolized in the TCA cycle to yield energy or used as substrates for synthesis of other compounds. The amino group, containing nitrogen, is transferred to α-ketoglutarate to form glutamic acid. This reaction involves cytoplasmic transaminases. The deaminated product can then be metabolized through oxidative pathways to yield energy. The net biological efficiency of converting protein to energy is about 40%.

Glutamic acid is then transported into mitochondria, is deaminated by glutamate dehydrogenase, and the resulting NH_4^+ leaves the mitochondria (Walsh, 1997). It is hypothesized that NH_4^+ participates in an ammonia "shuttle" involving cytoplasmic glutamine synthetase to produce glutamine, which is then transported into the mitochondria, where it is converted to NH_3. This proposed mechanism exists in the kidney of elasmobranches and aids in the establishment of a chemiosmotic gradient needed for ATP

production. More detailed descriptions of specific amino acid metabolism can be found in Chapter 6.

Nearly all nitrogen excretion in fish occurs in the gills. The amino group (NH_2) removed from glutamic acid in the liver yields α-ketoglutarate and NH_3, which is then carried by the circulatory system to the gills, where it is excreted by direct diffusion from blood to water, direct diffusion of NH_3, and/or functional Na^+/NH_4^+ exchange. Which mechanism is dominant depends on whether the fish involved is a marine or a freshwater species. In freshwater species, NH_3 diffusion is the main mechanism of excretion. Most teleost fish are ammoniotelic, yet a portion of their nitrogen excretion is in the form of urea. Elasmobranchs, coelacanths, and several other teleost fish living in specialized environments are ureotelic, and the majority of their nitrogen excretion is as urea (Walsh, 1997). Elasmobranchs and coelacanths are ureosmotic fish and can maintain osmoregulatory balance by maintaining a high level of urea in their tissues. In spiny dogfish (*Squalus acanthias*) more than 90% of nitrogen excretion is as urea excreted by the gills (Wood *et al.,* 1995). Urea is much less toxic than ammonia, but at the levels found in some ureosmotic fish, urea can precipitate proteins. Fish counteract this by having high concentrations of trimethylamine oxide (TMAO), which counteracts the effects of urea and stabilizes proteins (Somero, 1986).

Because ammonia is toxic, it must be excreted relatively rapidly to prevent tissue accumulation. The plasma ammonia concentration is related to the protein intake (Kaushik, 1980; Jobling, 1981; Kikuchi *et al.,* 1991) and begins to rise 3–8 hr following a meal, depending on the species of fish, protein intake, and quality of protein in the meal (Dosdat *et al.,* 1996). Freshwater species (trout) had higher levels of plasma nitrogen following a meal (6.5 mg/liter) compared to marine species (sea bass, sea bream, and turbot), which were uniformily lower (3.5 mg/liter). In contrast, plasma urea concentrations in marine species were not related to dietary protein intake and were seven to eight times higher in marine species than in freshwater species (44–59 vs 6.5–7 mg/liter, respectively). Maximum nitrogen excretion occurred 3–5 hr following a meal in small fish (ca. 10 g), compared to 5–8 hr following a meal in large fish (100 g). Dosdat *et al.* (1996) reported that between 30 and 38% of ingested nitrogen was excreted within 24 hr in trout, sea bass, and sea bream, but for turbot, the value was only 21% of ingested nitrogen.

14.12 Effect of Diet on Intermediary Metabolism

The dietary lysine intake influences the fractional rates of protein synthesis and degradation in liver and muscle of rainbow trout (Garzon *et al.,* 1994). Fish fed lysine-deficient diets exhibited increased rates of protein

degradation without significant changes in protein synthesis rates in the liver, while in the muscle lysine deficiency caused significant increases in both the protein degradation and the protein synthesis rates. Other published studies confirm that various amino acid transferases exhibit a higher activity in fish fed high-protein diets compared to fish fed low-protein diets but this effect is not uniform (summarized by Cowey and Walton, 1989). There is little effect of dietary protein intake on the activities of amino acid-catabolizing enzymes.

14.13 Measuring Protein Accretion and Degradation

New and sensitive methods have been developed to measure protein synthesis and degradation rates in tissues using a flooding dose of [^{3}H]phenylalanine, administered by injection (Garlick *et al.*, 1980). Using this approach, researchers have developed a number of insights into the metabolic processes associated with protein accretion in tissues. As summarized by Houlihan (1991), "It is well established that the amount of protein synthesized exceeds the amount retained as growth." This amount varies with feed (protein) intake, fish size, and species and also varies among tissues. Carter *et al.* (1993a) found a relationship among protein intake, protein synthesis, and protein retention efficiency (protein gain/protein intake rate) in grass carp, but the efficiency of protein growth was determined by the rate of protein degradation. Faster-growing fish had lower rates of protein degradation, higher retention of synthesized protein, higher RNA activity, and a lower capacity for synthesis and variable rates of protein synthesis. Houlihan *et al.* (1988) reported that fractional rates of protein synthesis differed among tissues, with liver > gills > intestine > spleen > ventricle > stomach > gonads > white muscle in cod. White muscle in cod had the highest protein retention efficiency and accounted for 40% of the total body protein accretion per day, an observation also reported by Facuconneau *et al.* (1995) for rainbow trout. Houlihan *et al.* (1986) ranked rainbow trout tissues in terms of fractional protein synthesis and degradation rates as follows: gills > ventricle > red muscle > white muscle. However, the ranking based upon total amounts of protein synthesized per day was as follows: white muscle = gills > red muscle > ventricle. They reported that 76% of the protein synthesized in white muscle appeared as growth, compared to only 4% for gills. Recently, the possibility of estimating protein (muscle) synthesis rates in fish by measuring tissue levels of mRNA associated with myosin synthesis has been introduced (Overturf and Hardy, 2001). If a similar method can be developed to measure protein (muscle) degradation rates, this may

lead to a deeper understanding of the factors that control protein accretion efficiency and provide a tool on which to base diet formulation, genetic selection of fish for more rapid and efficiency growth, and other beneficial applications.

14.14 Intake and Metabolism

Feed intake also determines protein synthesis rates, with fractional rates of protein synthesis increasing from 5 to 11 times in larval herring when feeding was restored after a period of fasting (Houlihan *et al.*, 1995). Carter *et al.* (1993b) found that differences in growth of individual Atlantic salmon were not correlated with the feed intake, digestive capacity, or feeding hierarchy rank but, rather, with the efficiency of protein retention, i. e., differences among fish in synthesis retention of protein, again related to lower rates of protein degradation. These authors concluded that greater use of recycled amino acids for protein synthesis and growth, rather than for oxidation and excretion, enabled some salmon to retain protein more efficiently. In flounder, individual differences in growth efficiency were related to differences in protein turnover, and fast-growing fish had lower rates of both protein synthesis and protein degradation (Carter *et al.*, 1998).

14.15 Sexual Maturity and Metabolism

Protein metabolism in fish differs during sexual maturation compared to that during growth stages of life history. Martin *et al.* (1993) found that there is considerable protein turnover and repartitioning of amino acids during starvation and sexual maturity in Atlantic salmon. The ovary obviously makes the largest contribution to the energy and amino acid demands of the fish during this period. Most of the amino acids needed for maturation of the ovary originate in white muscle and are made available as a result of protein degradation.

Recently, the technique used to determine the information reported above has been extended to the use of stable isotopes, mainly ^{15}N, using the same flooding dose technique used with radioactive isotopes of amino acids (Owen *et al.*, 1999). The advantage of this approach is that it can be used in the field or in situations where the use of radioactive isotopes is limited.

An outline of nutrient flow, retention, and metabolism in fish is shown in Fig. 14.3.

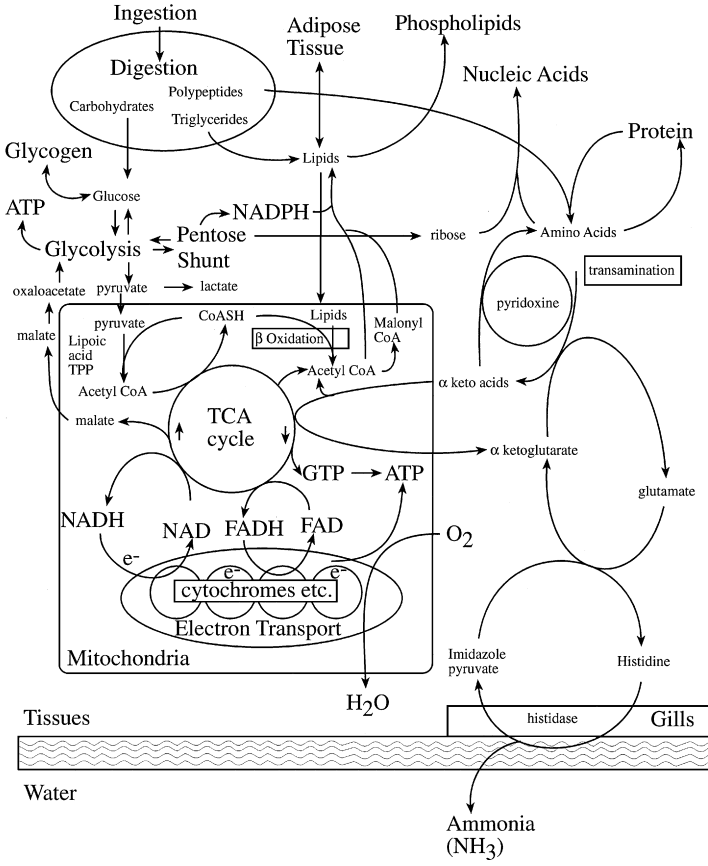

FIG. 14.3

Nutrient flow in fish.

14.16 Prospects for Improvement of Protein Retention Efficiency

As discussed above, protein retention efficiency is determined by a number of endogenous and exogenous factors, including the feed intake, dietary protein and energy levels, dietary amino acid leves, dietary amino acid bioavailability, life history stage, and genetically controlled rate of protein turnover (degradation). Protein (nitrogen) retention in farmed fish has improved greatly over the past decade in species that tolerate high dietary

lipid levels, such as Atlantic salmon. In the late 1980s, the average protein retention in farmed Atlantic salmon was 22–25%, with 75% or more of ingested nitrogen excreted. By 2000, protein retention had increased to 45% or more, the result of changes in dietary energy levels (up to 35% lipid) and improvements in protein quality. Further increases are possible in Atlantic salmon, but such increases are limited by metabolic reality. In other farmed fish species, considerable opportunities for improvement of protein (nitrogen) retention exist, but not all species tolerate high lipid diets as well as do Atlantic salmon.

Improvements of the protein retention efficiency in farmed fish are needed to reduce the environmental impacts of aquaculture and, also, to make more efficient use of dietary protein, the most expensive component of diet formulations for fish. Perhaps the greatest potential for improving protein retention lies in the selection of broodstock having higher protein retention rates due to lower rates of protein degradation, but opportunities to improve protein retention through the formulation of "ideal proteins" and by increasing dietary energy levels also need to be explored.

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Appendix

Several typical diets are listed here and in Chapter 9, for salmon and trout. These formulas, together with some constraints and specifications, are included to serve as guidelines for type formulas for fish diets. These formulas have been selected from many that are currently being fed to salmon, trout, carp, catfish, and several other important commercial species of fish, but these are not specific feed mixtures recommended by the authors or editors for any one specific fish or environment. Dietary requirements for the different fish species vary with the fish size, water temperature, water chemistry, fish activity, and final purpose of the fish husbandry program. Therefore it is recommended that appropriate rations be formulated from the more complete list of nutrients present in potential fish food ingredients included in the National Academy of Science–National Research Council bulletins, "Nutrient Requirements of Coldwater Fishes" (1981), "Nutrient Requirements of Warmwater Fishes and Shellfish" (1983), and "Nutrient Requirements of Fish" (1993).

Table A.1

Typical Formula and Specifications for Moist Salmon Pellets[a]

Ingredient	Percentage
Herring meal	28
Dried whey	5
Wheat germ meal	remainder
Corn distillers' solubles	4
Cottonseed meal	15
Pasteurized wet fish	30.0
Trace mineral premix	0.1
Vitamin and antioxidant mix	1.5
Fish oil	6.0–6.75
Choline chloride (70% liquid)	0.5 N
	100

[a] Detailed specifications for the processing of the Oregon Moist Pellet are necessary for the manufacture of a satisfactory feed. These are:

(1) The dry meal ingredients should be passed through a 40-mesh screen, 0.015-inch opening (0.42 mm).
(2) The wet fish ingredients should be ground through a grinder plate with holes not larger than $^5/_{64}$ inch or through a disintegrator providing that the particles are $^5/_{64}$ inch (2.0 mm) or smaller.
(3) The wet ingredients are added to the dry ingredients in a mixer and blended until homogenous and the desired degree of plasticity necessary to extrude satisfactory pellets is obtained.
(4) The mix should not stand more than 4 hr before pelleting.
(5) Bellets should be passed over a grader before bagging to separate oversized particles and "fines."
(6) Pellets should be packed in 50-pound (23 kg) net containers and within 1 hr placed in a freezer and quick frozen at a temperature of at least −30°C.
(7) Pellets may be transferred from the freezer to a storage when they have reached a temperature of −9°C and will not be received by a hatchery until they have reached a temperature of −18°C.

Table A.2

High-Protein Dry Diet for Salmon Fingerlings[a]

	%
Herring meal (70% + protein, TBA-40)	46.5%
Dried whey product (15% + protein)	5.0%
Wheat germ meal (19% + protein, 8% lipid)	5.0%
Poultry by-product meal	1.5%
Feather meal	10.0%
Blood flour	2.5%
Legnon sulfonate	2.0%
Trace mineral premix	0.1%
L-ascorbyl-2-polyphosphate	0.2%
Choline chloride	0.58%
Wheat middlings	16.62%
Fish oil (+0.01% BHA + BHT)	9.0%
Vitamin mix	1.0%
Starter granule: 6% oil added	
$^2/_{64}$-inch granule: 4% oil added	

Abernathy vitamin mixture:

Thiamin mononitrate	3117 mg
Riboflavin	3527 mg
Pyridoxine hydrochloride	2504 mg
Niacin	14.704 mg
d-Pantothenic acid	7668 mg
d-Biotin	39.7 mg
Folic acid	849 mg
DL-α-Tocopherol acetate	33510 IU
Vitamin A (palmotate or acetate)	440,000 IU
Vitamin D (cholecalciterol)	29,398 IU
Vitamin K (menadione sodium bisulfate)	1858 mg
Vitamin B_{12}	3.97 mg
Wheat middlings	remainder
	1000 g

[a] Abernathy salmon diet AB-2 (92). See Chapter 9, Table 9.2.

Table A.3

Typical Formula and Specifications for Trout Pellets[a]

A. Formula SD7[b]

Ingredient	Percentage
1. West coast Canadian or Alaskan herring meal, maximum fat 10.5%, minimum protein 70%, immediate past season.	45.0
(a) Lecithin (blended with fish meal).	
2. Toasted, defatted soy flour, maximum fat 0.5%, minimum protein 50%.	15.0
3. Steam-dried brewer's yeast, minimum protein 40%, minimum fat 0.7%, maximum fiber 3.0%.	5.0
Fish oil.	10.0
4. Condensed fish solubles, dried on wheat middlings (equivalent to 100% condensed fish solubles).	5.0
5. Soluble dried blood flour.	5.0
6. Wheat standard middlings.	9.35
7. Dried whey.	5.0
8. Fish solubles, condensed 50% protein	5.0
9. Vitamin premix No. 30	0.4
10. Choline chloride, 50%	0.2
11. Mineral premix[c]	0.05

[a] National Fish Hatchery diet specifications, U.S. Fish and Wildlife service.

[b] Starter and No. 1 granule sizes only.

[c] Mineral premix (9/kg): $ZnSO_4$, 185; $FeSO_4 \cdot 7H_2O$, 50; $CuSO_4$, 3.85; $MnSO_4$, 207; KIO_3, 0.84; inert carrier, 553.

Table A.3 *(Continued)*

B. Vitamin Premix No. 30[a]

Ingredient	Guaranteed potency per kg premix (mg)
D-Calcium pantothenate	26.4
Pyridoxine	7700
Riboflavin	13.2
Niacin	55
Folic acid	2200
Thiamin	8800
Biotin	880
Vitamin B_{12}	5.5
Menadione sodium bisulfite	2750
Vitamin E, from α-tocopherol acetate (*d* or *dl*) in beadlet form	88,000 IU
Vitamin D_3 activity	110,000 IU
Vitamin A activity (from vitamin A palmitate in gelatin beadlets)	1,650,000 USP
BHT antioxidant	1760
Ascorbic acid[b]	6,600

[a] U.S. Fish and Wildlife Service.

[b] These items will not be part of a packaged premix but shall be blended with one or more cereal ingredients and added to the mixture at the time of manufacture of feed.

Table A.3 *(Continued)*

C. Formula PR-6[a]

Ingredient	Percentage
1. West coast Canadian or Alaskan herring meal, maximum fat 10.5%, minimum protein 70%, immediate past season.	35.0
2. Wheat standard middlings, 13% minimum protein, 9.5% maximum fiber.	13.3
3. Soybean meal, solvent extracted, toasted, and dehulled, minimum protein 50%.	20.0
4. Steam-dried brewer's yeast, minimum protein 40%, minimum fat 0.7%, maximum fiber 3.0%.	5.0
5. Delactosed whey, minimum protein 16%, maximum sugar 50%.	10.0
6. Dehydrated alfalfa meal, 17% protein, reground pellets.	3.0
7. Trace mineral premix[b]	0.1
8. Vitamin Premix No. 30[b]	0.4
9. Fish oil	5.0
10. Choline chloride, 50%	0.2
11. Fermentation solubles	8.0

[a] No. 2 and larger granules and all pellet sizes.
[b] See specifications on previous tables.

Table A.4

A. Pond Fish Formula[a,b,c]

Ingredient	Amount per ton (pounds)
Fish meal, menhaden, minimum protein 60%,	240
or herring meal, Canadian or Alaskan, minimum protein 70%	200
Blood meal, minimum protein 80%	100
Feather meal, guaranteed digestibility 80% (Note: blood meal and feather meal may be used interchangeably)	100
Soybean meal, solvent, toasted, dehulled, 50% protein	400
Dried distillers' solubles or dried fermentation solubles	
(a) If menhaden fish meal is used	160
(b) If herring meal is used	200
Rice bran, 12% protein, 12% fat, 12% fiber or better	700
Rice mill dust or other organic dust passing a U.S. No. 80 mesh (Note: Wheat shorts, wheat middlings, cereal grains, vegetable oil, or fish body oil, and a pellet binder may be used for rice by-products)	200
Dehydrated alfalfa, reground 17% protein pellets	70
Mineralized, iodized salt	20
Vitamin Premix (see additional specification for composition)	10
	2000

[a] Guaranteed analysis of fish feed formula No. 1: crude protein, more than 32.00%; animal protein, more than 15.00%; crude fiber less than 12.00%; crude fat, more than 5.00%.

[b] Physical properties and processing specifications: Fry feed or fish starter meal—100% to pass through 595-μm opening or a U.S. No. 30 sieve; No. 2 pellet—$\frac{1}{8}$ inch diameter × $\frac{1}{8}$ inch long; No. 3 pellet—$\frac{1}{8}$ inch diameter × $\frac{1}{2} - \frac{3}{4}$ inch long; No. 4 pellet—$\frac{3}{16}$ inch diameter × $\frac{1}{4} - \frac{1}{2}$ inch long; No. 5 pellet—$\frac{1}{4}$ inch diameter × $\frac{1}{4} - \frac{1}{2}$ inch long.

Pelleted feed shall be retained on a $\frac{1}{8}$-inch-mesh screen when immersed in water for 10 min, such that only 10% of the original weight is loss. This specification may be met by grinding the formula through a 1/8-inch screen after mising, using high-pressure, high-quality (dry) steam to condition soft feed before pelleting, cooling rapidly, and handling without undue breakage. No more than 4% fines should be present in bagged pellets.

[c] Courtesy of W. H. Hastings, personal communication.

Table A.4 *(Continued)*

B. Pond Fish Vitamin Premix[a]

Ingredient	Guaranteed potency per ton of feed
Vitamin A activity (from palmitate in gelatin beadlets)	5,000,000 IU
Vitamin D_3 activity	1,000,000 IU
α-Tocopherol acetate (*d* or *dl* in beadlet form)	20 g
Menadione sodium bisulfite	20 g
Choline chloride	1,000 g
Niacin	50 g
Riboflavin	10 g
Pyridoxine	5 g
Thiamin	5 g
D-Calcium pantothenate	20 g
Biotin	200 mg
Folic acid	1,000 mg
Vitamin B_{12}	20 mg
BHT antioxidant	10 g
or ethoxyquin	136 g

[a] Premix on finely ground meal carrier.

Table A.5

Essential Amino Acid (EAA) Index and First Limiting Amino Acid of Fish Feeds and Dietary Ingredients, Based on Chinook Salmon Requirements[a]

Diet or dietary ingredient	EAA score	First limiting amino acid
Whole egg (commercial)	90.0	Tryptophan
Salmon egg	85.4	Tryptophan
Casein	81.7	Arginine
Pasteurized salmon carcass	77.7	Tryptophan
Autolyzed salmon carcass	77.6	Tryptophan
Herring meal	76.0	Tryptophan
Commercial trout feed	75.9	Tryptophan
Commercial salmon feed	75.5	Tryptophan
Dried skim milk	75.5	Tryptophan
Drackett soybean protein	75.2	Methionine
Salmon viscera	74.1	Tryptophan
Commercial trout feed (imported)	74.1	Tryptophan
Experimental diet	72.8	Tryptophan
Fresh turbot	71.9	Tryptophan
Soybean meal	71.0	Methionine
Meat scraps	70.8	Tryptophan
Commercial trout feed	68.9	Tryptophan
Oregon moist pellet	68.9	Tryptophan
McNenny No. 31 feed	68.3	Tryptophan
Commercial trout feed	67.5	Tryptophan
Salmon meal	67.4	Isoleucine
Tuna viscera	66.4	Tryptophan
Sesame meal	66.3	Lysine
Brewer's yeast	66.3	Methionine
Wheat germ meal	61.8	Tryptophan
Distillers' solubles	59.3	Tryptophan
Cottonseed meal	59.1	Tryptophan
Wheat middlings	56.2	Isoleucine
Shrimp meal	50.4	Tryptophan
Crab solubles	43.8	Tryptophan

[a] Courtesy of W. E. Shanks, personal communication.

Table A.6

Digestibility Coefficients and Digestible Energy Values for Rainbow Trout and Channel Catfish (All Values on a Dry Weight Basis)[a]

Line No.	Feed name	INFIC Feed Number	Digestion coefficients, trout (t) or catfish (c)			Energy, rainbow trout		Energy, catfish
			Protein (%)	Fat (%)	Cho (%)	DE (kcal/kg)	ME (kcal/kg)	DE (kcal/kg)
001	Alfalfa meal 17% protein	1-00-023	61t	51t	—	513	859	670
002	Algae, *Chlorella vulgaris* grnd, dehyd	5-20-658	—	—	—	—	—	—
003	Algae, *Spirulina* spp. whole meal	5-19-931	—	—	—	—	—	—
004	Bacteria, methanol, whole, dehyd	5-28-531	—	—	—	—	—	—
005	Barley, grain	4-00-530	81t	—	—	—	—	—
006	Bean, mung seeds	5-01-185	—	—	—	—	—	—
007	Bean, winged seeds, heat processed	—	—	—	—	—	—	—
008	Blood, spray dehyd (50%)	5-00-381	86t	—	—	4612	4004	—
009	Brewer's, grains dehyd	5-02-141	—	—	—	—	2390	—
010	Casein	5-01-162	99t	—	—	—	3910	—
011	Cashew, kernels meal, mech extr	5-26-221	—	—	—	—	—	—
012	Cassava (tapioca) starch	4-25-390	—	—	—	—	—	—
013	Chick-pea (garbanzo) seeds	5-01-218	—	—	—	—	—	—
014	Coconut, meats meal, mech extr	5-01-572	—	—	—	—	—	—
015	Corn, dent yellow grain	4-02-935	60c	76c	62c	—	1500	1100
016	Corn, distillers' solubles, dehy	5-02-844	72t	—	59t	2436	2283	—
017	Corn, gluten meal	5-02-900	83t	—	—	3712	3297	—
018	Corn, gluten meal 60% protein	5-09-318	91t	93t	—	4682	4435	—
019	Corn, starch	4-02-889	—	—	—	—	—	—
020	Cotton, seeds, meal mech extr 41% protein	5-01-617	—	—	—	—	—	—
021	Cotton, seeds, meal solv extr 41% protein	5-01-621	80c	87c	17c	—	2080	2550
022	Cotton, seeds w/o hulls, prepressed solv extr 50% protein	5-07-874	76t	—	—	2660	2468	—
023	Cowpea, seeds	5-01-661	—	—	—	—	—	—

Table A.6 (*Continued*)

Line No.	Feed name	INFIC Feed Number	Digestion coefficients, trout (t) or catfish (c)			Energy, rainbow trout		Energy, catfish
			Protein (%)	Fat (%)	Cho (%)	DE (kcal/kg)	ME (kcal/kg)	DE (kcal/kg)
024	Crab, process residue meal (crab meal)	5-01-663	—	—	—	—	2960	—
025	Crab, whole dehyd	—	72t	—	—	3878	3214	—
026	Fats and oils, fat, beef	—	—	—	—	—	—	—
027	Fats and oils, fat, poultry	4-00-409	—	—	—	—	—	—
028	Fats and oils, fat, swine	4-04-790	—	—	—	—	—	—
029	Fats and oils, liver oil, pollack	4-20-954	—	—	—	—	—	—
030	Fats and oils, liver oil, cod	—	—	87t	—	—	—	—
031	Fats and oils, oil, canola (rapeseed)	—	—	—	—	—	—	—
032	Fats and oils, oil, capelin	—	—	85t	—	—	—	—
033	Fats and oils, oil, coconut	4-09-320	—	—	—	—	—	—
034	Fats and oils, oil, corn	—	—	—	—	—	—	—
035	Fats and oils, oil, fish	7-01-965	—	97c	—	—	—	—
036	Fats and oils, oil, herring	—	—	—	—	—	—	—
037	Fats and oils, oil, linseed	—	—	—	—	—	—	—
038	Fats and oils, oil, menhaden	—	—	—	—	—	—	8830
039	Fats and oils, oil, safflower	—	—	—	—	—	—	—
040	Fats and oils, oil, soybean	4-07-983	—	89t	—	—	—	8930
041	Fish, protein hydrolyzate	—	—	—	—	—	—	—
042	Fish, solubles condensed	5-01-969	—	—	—	—	—	—
043	Fish, solubles dehyd	5-01-971	69t	—	—	3684	3345	—
044	Fish, anchovy meal mech extr	5-01-985	85t	—	—	4570	4020	—
045	Fish, carp meal mech extr	5-09-831	—	—	—	—	—	—
046	Fish, catfish meal mech extr	5-09-835	—	—	—	—	—	—
047	Fish, hake meal mech extr	5-02-025	81t	—	—	—	3436	—
048	Fish, herring meal mech extr	5-02-000	87t	94t	—	4717	4133	—
049	Fish, menhaden meal mech extr	5-02-012	—	—	—	—	—	3900
050	Fish, pollock meal mech extr	—	—	—	—	—	—	—
051	Fish, salmon meal mech extr	5-02-012	83t	—	—	4019	3570	—
052	Fish, shark meal mech extr	5-02-018	—	—	—	—	—	—

(continues)

Table A.6 (*Continued*)

Line No.	Feed name	INFIC Feed Number	Digestion coefficients, trout (t) or catfish (c)			Energy, rainbow trout		Energy, catfish
			Protein (%)	Fat (%)	Cho (%)	DE (kcal/kg)	ME (kcal/kg)	DE (kcal/kg)
053	Fish, tuna meal mech extr	5-02-023	—	—	—	—	—	—
054	Fish, white meal mech extr	5-02-025	95t	—	—	3490	2974	—
055	Flax, seeds solv extr grnd	5-02-048	77t	—	—	3262	2934	—
056	Gelatin	5-14-503	—	—	—	—	4050a	—
057	Krill, *Euphausia pacifica* whole, meal	5-16-423	—	—	—	—	—	—
058	Lentil, seeds	5-02-506	—	—	—	—	—	—
059	Linseed, meal	5-02-045	—	—	—	—	—	—
060	Meat, meal, rendered	5-00-385	70t	—	—	3389	3237	—
061	Meat, with bone meal, rendered	5-00-388	75c	77c	—	—	2660	3470
062	Millet, grain	4-03-098	—	—	—	—	—	—
063	Molasses, beet, dehyd	4-00-668	91t	—	—	—	4074	—
064	Molasses, sugarcane, dehyd	4-04-695	—	—	—	—	—	—
065	Oats, grain	4-03-309	—	—	—	—	—	—
066	Pea, seeds	5-03-598	—	—	—	—	—	—
067	Peanut, kernels meal mech extr	5-03-649	—	—	—	—	—	—
068	Peanut, kernels meal solv extr	5-03-650	—	—	—	—	—	—
069	Potato, starch	4-20-962	—	—	55c	—	—	—
070	Poultry, by-product meal, rendered	5-03-798	66t	—	—	3719	3160	—
071	Poultry, feathers hydrolyzed	5-03-795	74c	68t	—	—	2880	3410
072	Rape, seeds solv extr grnd	5-03-871	76t	—	—	2994	2711	—
073	Rape, seeds solv dehulled grnd	5-08-135	—	—	—	2994	2711	—
074	Rice, bran with germs	4-03-928	71c	—	—	—	—	—
075	Rice, grain, grnd	4-03-938	—	—	—	—	—	—
076	Rye, grain, grnd	4-04-047	—	—	—	—	—	—
077	Safflower, seeds solv extr grnd	5-08-499	—	—	—	—	—	—
078	Sesame, seeds meal mech extr	5-04-220	—	—	—	—	—	—

Table A.6 (*Continued*)

Line No.	Feed name	INFIC Feed Number	Digestion coefficients, trout (t) or catfish (c)			Energy, rainbow trout		Energy, catfish
			Protein (%)	Fat (%)	Cho (%)	DE (kcal/kg)	ME (kcal/kg)	DE (kcal/kg)
079	Sesame, seeds meal solv extr	5-09-906	—	—	—	—	—	—
080	Sorghum, gluten meal	4-04-388	—	—	—	—	—	—
081	Sorghum, grain grnd	5-04-329	—	—	—	—	—	—
082	Shrimp, process residue meal (shrimp meal)	5-04-226	—	—	—	—	—	—
083	Soybean, seeds full fat, steam cooked	5-04-597	80t	—	—	4232	3929	—
084	Soybean, seeds meal mech extr	5-04-600	84t	—	—	—	2952	—
085	Soybean, seeds meal solv extr	5-04-604	—	—	—	—	—	2580
086	Soybean, seeds, without hulls meal mech extr	5-04-612	83t	—	—	3260	2885	—
087	Sunflower, seeds meal mech extr	5-04-738	—	—	—	—	—	—
088	Sunflower, seeds meal solv extr	5-04-739	77t	—	—	—	2,567	—
089	Wheat, *Triticum aestivum,* bran	4-05-190	—	—	—	—	—	—
090	Wheat, flour by-product less than 1.5 fiber	4-05-199	65t	—	—	1804	1509	—
091	Wheat, flour by-product less than 7% fiber	4-05-201	72c	90c	—	—	—	—
092	Wheat, flour by-product less than 9.5 fiber (middlings)	4-05-205	68t	20t	—	2442	2237	—
093	Wheat, germ grnd	5-05-218	77t	—	—	3034	2759	—
094	Wheat, grain hard red winter	4-05-268	82c	95c	59c	—	—	2550
095	Wheat, grain soft red winter	4-05-294	—	—	—	—	—	—
096	Wheat, mill run less than 9.5% fiber	4-05-206	—	—	—	—	—	—
097	Whey, dehyd (cattle)	4-01-182	80t	—	—	2700	2384	—
098	Whey, low-lactose dehyd (cattle)	4-01-186	63t	—	—	2638	2279	—
099	Yeast, brewer's dehyd, grnd	7-05-527	85t	—	—	3787	2922	—
100	Yeast, Torula dehyd, grnd	7-05-534	82t	—	—	3678	3362	—

[a] Adapted from INFIC tables.

Table A.7

Amino Acid Content of Feedstuffs (All Values on Dry Weight Basis)[a]

Line No.	Feed name	INFIC Feed number	Crude protein (%)	Arg (%)	Gly (%)	His (%)	Iso (%)	Leu (%)	Lys (%)	Met (%)	Cys (%)	Phe (%)	Tyr (%)	Ser (%)	Thr (%)	Try (%)	Val (%)
001	Alfalfa meal 17% protein	1-00-023	18.9	0.84	0.91	0.36	0.88	1.39	0.93	0.29	0.31	0.87	0.59	0.77	0.77	0.37	0.96
002	Algae, *Chlorella vulgaris* grnd, dehyd	5-20-658	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
003	Algae, *Spirulina* spp. whole meal	5-19-931	65.0	—	—	—	3.90	5.20	3.00	0.91	—	3.25	—	—	3.00	0.91	4.22
004	Bacteria, methanol, whole, dehyd	5-28-531	80.7	3.83	—	—	—	—	5.00	1.94	—	—	—	—	3.83	1.11	—
005	Barley, grain	4-00-530	13.0	0.60	—	0.30	0.60	0.90	0.60	0.20	0.20	0.70	0.40	—	0.40	0.20	0.70
006	Bean, mung seeds, heat processed	5-08-185	23.5	3.06	—	0.93	1.46	2.08	3.39	0.69	0.10	1.58	0.66	—	1.31	0.68	1.59
007	Bean, winged seeds, heat processed	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
008	Blood, spray dehyd (50%)	5-00-381	93.0	3.88	4.14	5.59	0.98	11.86	8.04	0.95	0.78	6.36	2.44	3.82	3.93	1.13	8.13
009	Brewers, grains dehyd	5-02-141	29.4	1.38	1.18	0.56	1.68	2.70	0.95	0.50	0.38	1.56	1.30	1.42	1.01	0.40	1.75
010	Casein	5-01-162	92.7	3.85	1.77	2.86	6.32	9.71	7.88	3.10	0.34	5.31	5.41	6.03	4.32	1.19	7.40
011	Cashew, kernels meal, mech extr	5-26-221	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

012	Cassava (tapioca) starch	4-25-390	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
013	Chick-pea (garbanzo) seeds	5-01-218	23.3	3.17	1.40	0.87	1.52	2.88	2.39	0.43	—	2.09	1.02	1.93	1.28	—	1.58
014	Coconut, meats meal, mech extr	5-01-572	21.2	2.49	1.13	0.52	1.32	1.73	0.71	0.41	0.23	0.94	0.45	—	0.70	0.27	1.25
015	Corn, dent yellow grain	4-02-935	10.9	0.48	0.42	0.29	0.39	1.37	0.28	0.19	0.25	0.54	0.43	0.57	0.40	0.09	0.50
016	Corn, distillers' solubles, dehyd	5-02-844	29.8	1.20	—	0.66	1.31	2.76	0.99	0.52	0.42	1.36	0.99	—	1.08	0.31	1.46
017	Corn, gluten meal	5-02-900	47.1	1.54	—	1.10	2.53	8.35	0.88	1.10	0.66	3.19	1.10	—	1.54	0.22	2.42
018	Corn, gluten meal 60% protein	5-09-318	67.5	2.31	2.29	1.55	2.89	11.58	1.14	1.97	1.12	4.48	3.67	3.89	2.50	0.33	3.45
019	Corn, starch	4-02-889	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
020	Cotton, seeds, meal mech extr 41% protein	5-01-617	44.3	4.51	2.06	1.15	1.56	2.50	1.73	0.62	0.78	2.35	1.01	1.84	1.44	0.57	2.05
021	Cotton, seeds, meal solv extr 41% protein	5-01-621	45.2	4.62	2.17	1.21	1.67	2.56	1.86	0.64	0.85	2.46	1.13	1.92	1.52	0.61	2.06
022	Cotton, seeds w/o hulls, prepressed solv extr 50% protein	5-07-874	54.0	5.2	3.03	1.30	1.59	2.45	1.82	0.81	1.13	2.81	0.87	—	1.78	0.67	2.32
023	Cowpea, seeds	5-01-661	26.3	1.70	—	0.79	1.24	2.58	2.10	0.50	—	1.46	1.24	—	0.90	0.30	1.35
024	Crab, process residue meal (crab meal)	5-01-663	34.8	1.80	1.89	0.53	1.26	1.67	1.50	0.57	0.26	1.26	1.26	1.50	1.09	0.32	1.59

(*continues*)

Table A.7 *(Continued)*

Line No.	Feed name	INFIC Feed number	Crude protein (%)	Arg (%)	Gly (%)	His (%)	Iso (%)	Leu (%)	Lys (%)	Met (%)	Cys (%)	Phe (%)	Tyr (%)	Ser (%)	Thr (%)	Try (%)	Val (%)
025	Crab, whole dehyd	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
026	Fats and oils, fat, beef	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
027	Fats and oils, fat, poultry	4-00-409	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
028	Fats and oils, fat, swine	4-04-790	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
029	Fats and oil, liver oil, pollack	4-20-954	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
030	Fats and oils, liver oil, cod	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
031	Fats and oils, oil, canola (rapeseed)	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
032	Fats and oils, oil, capelin	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
033	Fats and oils, oil, coconut	4-09-320	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
034	Fats and oils, oil, corn	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
035	Fats and oils, oil, fish	7-01-965	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
036	Fats and oils, oil, herring	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
037	Fats and oils, oil, linseed	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
038	Fats and oils, oil, menhaden	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

039	Fats and oils, oil, safflower	—	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
040	Fats and oils, oil, soybean	4-07-983	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
041	Fish, protein hydrolysate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
042	Fish, solubles condensed	5-01-969	65.3	3.25	7.68	2.85	2.06	3.72	3.71	1.42	0.54	2.04	0.87	2.05	1.73	0.68	2.43
043	Fish, solubles dehyd	5-01-971	69.2	3.29	6.20	2.26	2.21	3.21	3.79	1.27	0.66	1.65	0.92	2.19	1.46	0.64	2.26
044	Fish, anchovy meal mech extr	5-01-985	71.2	4.11	4.01	1.76	3.38	5.43	5.49	2.16	0.66	3.03	2.44	2.63	3.00	0.82	3.81
045	Fish, carp meal mech extr	5-09-831	74.4	4.64	—	1.46	2.67	4.98	5.75	1.71	—	2.60	1.99	—	2.97	—	2.87
046	Fish, catfish meal mech extr	5-09-835	55.3	4.45	—	1.21	1.84	3.55	4.03	1.10	—	1.93	1.35	—	2.32	—	2.47
047	Fish, hake meal mech extr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
048	Fish, herring meal mech extr	5-02-000	78.3	5.02	4.80	1.80	3.41	5.64	5.83	2.27	0.81	2.94	2.39	2.88	3.16	0.83	4.68
049	Fish, menhaden mea mech extr	5-02-012	66.7	4.09	4.57	1.58	3.15	4.89	5.15	1.91	0.61	2.69	2.12	2.43	2.73	0.71	3.52
050	Fish, Pollock meal mech extr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
051	Fish, Salmon meal mech extr	5-02-012	68.7	5.59	—	—	—	—	8.17	1.72	0.75	—	—	—	—	0.54	—
052	Fish, shark meal mech extr	5-02-018	73.0	—	—	—	—	—	—	0.88	—	—	—	—	—	—	—

(*continues*)

Table A.7 *(Continued)*

Line No.	Feed name	INFIC Feed number	Crude protein (%)	Arg (%)	Gly (%)	His (%)	Iso (%)	Leu (%)	Lys (%)	Met (%)	Cys (%)	Phe (%)	Tyr (%)	Ser (%)	Thr (%)	Try (%)	Val (%)
053	Fish, tuna meal mech extr	5-02-023	63.6	3.69	4.41	1.89	2.64	4.09	4.54	1.58	0.50	2.32	1.82	2.25	2.49	0.62	2.98
054	Fish, white meal mech extr	5-02-025	68.2	4.41	4.84	1.47	2.98	4.78	4.96	1.84	0.82	2.50	2.00	3.35	2.82	0.73	3.31
055	Flax, seeds solv extr grnd	5-02-048	38.6	3.54	—	—	—	—	1.33	0.66	0.73	—	—	—	—	0.62	—
056	Gelatin	5-14-503	97.4	7.75	21.48	0.85	1.54	3.24	3.95	0.81	0.15	1.99	0.58	3.45	1.96	0.05	2.33
057	Krill, *Euphausia pacifica* whole, meal	5-16-423	33.3	—	—	—	—	—	—	—	—	—	—	—	—	—	
058	Lentil, seeds	5-02-506	26.3	2.10	1.00	0.57	1.02	1.78	1.80	0.16	—	1.13	0.78	1.18	0.89	—	1.13
059	Linseed, meal	5-02-045	32.8	3.16	1.80	0.71	1.66	2.11	1.21	1.68	0.61	1.68	1.75	2.09	1.32	0.57	2.02
060	Meat, meal, rendered	5-00-385	54.8	3.84	6.71	1.02	1.86	3.40	3.45	0.75	0.70	1.94	1.02	2.30	1.75	0.37	2.68
061	Meat, with bone meal, rendered	5-00-388	54.1	3.75	6.93	1.04	1.76	3.29	3.11	0.70	0.53	1.83	0.85	1.94	1.77	0.32	2.63
062	Millet, grain	4-03-098	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
063	Molasses, beet, dehyd	4-00-668	0.30	—	—	—	—	—	—	—	—	—	—	—	—	—	—
064	Molasses, sugarcane, dehyd	4-04-695	10.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—
065	Oats, grain	4-03-309	13.3	0.79	0.52	0.21	0.49	0.91	0.44	0.19	0.22	0.58	0.52	0.50	0.40	0.17	0.63
066	Pea, seeds	5-03-598	24.7	1.54	—	0.79	1.21	1.98	1.76	0.34	0.19	1.43	—	—	1.03	0.26	1.43
067	Peanut, kernels meal mech extr	5-03-649	52.0	5.46	2.59	1.17	1.83	3.26	1.62	0.53	0.81	2.53	1.79	1.56	1.34	0.51	2.24
068	Peanut, kernels meal solv extr	5-03-650	52.3	4.95	2.56	1.03	1.91	2.94	1.93	0.46	0.79	2.22	1.65	3.37	1.26	0.52	2.04

069	Potato, starch	4-20-962	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
070	Poultry, by-product meal, rendered	5-03-798	62.8	4.03	5.80	1.08	2.54	4.28	3.10	1.13	0.98	1.97	1.01	2.81	2.08	0.50	3.06
071	Poultry, feathers hydrolyzed	5-03-795	91.3	7.58	6.92	1.06	4.37	7.46	2.49	0.59	3.48	3.28	2.49	9.96	4.27	0.56	6.97
072	Rape, seeds solv extr grnd	5-03-871	40.6	2.26	1.97	1.09	1.48	2.74	2.18	0.78	0.33	1.55	0.87	1.72	1.72	0.47	1.96
073	Rape, seeds solv dehulled grnd	5-08-135	44.0	2.63	2.30	1.29	1.72	3.20	2.54	0.91	—	1.82	1.00	—	2.01	0.57	2.30
074	Rice, bran with germs	4-03-928	14.1	0.79	0.88	0.25	0.51	0.77	0.54	0.26	0.11	0.49	0.76	0.85	0.47	0.11	0.76
075	Rice, grain, grnd	4-03-938	8.9	0.64	0.62	0.15	0.34	0.63	0.30	0.18	0.13	0.37	0.60	0.50	0.27	0.12	0.50
076	Rye, grain, grnd	4-04-047	13.8	0.61	0.56	0.29	0.53	0.80	0.48	0.19	0.21	0.64	0.30	0.60	0.41	0.13	0.64
077	Safflower, seeds mech extr grnd	5-08-449	45.7	3.47	2.89	—	—	—	1.50	0.80	0.58	—	—	—	—	0.58	—
078	Sesame, seeds meal mech extr	5-04-220	51.5	5.16	—	1.18	2.26	3.66	1.40	1.51	0.65	2.37	2.15	—	1.72	0.84	2.58
079	Sesame, seeds extr	5-04-221	45.1	4.61	4.29	1.18	1.71	3.00	1.29	0.64	2.14	2.14	—	—	1.71	0.64	2.36
080	Shrimp, process residue meal (shimp meal)	5-04-226	44.2	2.79	—	1.07	1.86	2.98	2.41	0.91	0.66	1.76	1.47	—	1.58	0.40	2.03
081	Sorghum, gluten meal	5-04-329	46.2	1.33	—	0.89	2.53	8.21	0.89	0.67	—	2.89	—	—	1.55	0.44	2.77

(*continues*)

Table A.7 *(Continued)*

Line No.	Feed name	INFIC Feed number	Crude protein (%)	Arg (%)	Gly (%)	His (%)	Iso (%)	Leu (%)	Lys (%)	Met (%)	Cys (%)	Phe (%)	Tyr (%)	Ser (%)	Thr (%)	Try (%)	Val (%)
082	Sorghum, grain grnd	4-04-383	12.4	0.43	0.38	0.26	0.50	1.60	0.28	0.15	0.22	0.62	0.46	0.55	0.40	0.12	0.58
083	Soybean, seeds full fat, steam cooked	5-04-597	42.2	3.11	2.22	1.12	2.42	2.85	2.67	0.60	0.61	2.33	1.36	2.41	1.88	0.58	2.24
084	Soybean, seeds meal mech extr	5-04-600	47.7	3.41	2.64	1.26	2.92	4.02	3.10	0.72	0.63	2.45	1.72	2.23	1.92	0.68	2.53
085	Soybean, seeds meal solv extr	5-04-604	49.9	3.38	2.03	1.19	2.27	3.65	2.99	0.58	0.83	2.36	1.48	2.36	1.85	0.71	2.25
086	Soybean, seeds, without hulls meal mech extr	5-04-612	55.1	4.07	2.68	1.35	2.73	4.14	3.52	0.79	0.83	2.71	1.86	2.76	2.15	0.77	2.82
087	Sunflower, seeds meal mech extr	5-04-738	44.1	4.52	—	1.18	2.58	3.23	2.15	1.72	0.86	2.58	—	—	1.72	0.65	2.58
088	Sunflower, seeds meal solv extr	5-04-739	49.8	4.75	3.03	1.32	2.42	4.12	2.06	1.25	0.79	2.54	1.49	2.37	2.07	0.65	2.80
089	Wheat, *Triticum aestivum,* bran	4-05-190	17.1	1.09	0.97	0.44	0.57	1.03	0.65	0.22	0.36	0.62	0.48	0.77	0.51	0.28	0.78
090	Wheat, flour by-product less than 1.5% fiber	4-05-199	13.4	0.49	0.51	0.28	0.53	0.99	0.28	0.21	0.35	0.69	0.39	0.68	0.37	0.14	0.57

091	Wheat, flour by-product less than 7% fiber	4-05-201	18.6	1.34	1.09	0.51	0.66	1.23	0.89	0.31	0.41	0.76	0.53	0.87	0.68	0.24	0.93
092	Wheat, flour by-product less than 9.5% fiber (middlings)	4-05-205	18.4	1.03	0.57	0.43	0.75	1.21	0.76	0.20	0.24	0.72	0.45	0.82	0.61	0.22	0.85
093	Wheat, germ grnd	5-05-218	29.1	1.78	—	0.56	1.33	1.22	1.78	0.33	0.56	0.89	—	—	0.89	0.33	1.22
094	Wheat, grain hard red winter	4-05-268	14.4	0.73	0.65	0.34	0.58	1.00	0.41	0.24	0.36	0.71	0.49	0.67	0.42	0.19	0.67
095	Wheat, grain soft red winter	4-05-294	13.0	0.73	0.62	0.36	0.51	1.02	0.41	0.24	0.41	0.72	0.43	0.73	0.44	0.30	0.65
096	Wheat, mill run less than 9.5% fiber	4-05-206	17.2	1.04	0.59	0.44	0.78	1.33	0.64	0.37	0.26	—	0.56	—	0.56	0.23	0.93
097	Whey, dehyd (cattle)	4-01-182	14.2	0.36	0.53	0.18	0.84	1.26	1.00	0.20	0.32	0.37	0.26	0.50	0.96	0.19	0.73
098	Whey, low-lactose dehyd (cattle)	4-01-186	17.9	0.64	0.77	0.29	1.03	1.65	1.50	0.43	0.46	0.59	0.49	0.63	1.01	0.29	0.93
099	Yeast, Brewer's dehyd, grnd	7-05-527	46.9	2.35	1.87	1.17	2.37	3.45	3.33	0.79	0.53	1.96	1.60	—	2.27	0.55	2.52
100	Yeast, torula dehyd, grnd	7-05-534	52.7	2.83	2.85	1.42	3.06	3.78	4.01	0.83	0.65	3.06	2.14	2.96	2.83	0.56	3.17

[a] Adapted from INFIC tables.

Table A.8

The Recommended Amount of Food to Feed Rainbow Trout per Day[a,b]

Water temperature (°F)	Number of fish per pound[c]										
	>2542 (<1)	2542–304 (1–2)	304–88.3 (2–3)	88.3–37.8 (3–4)	37.8–19.7 (4–5)	19.7–11.6 (5–6)	11.6–7.35 (6–7)	7.35–4.94 (7–8)	4.94–3.47 (8–9)	3.47–2.53 (9–10)	<2.53 (>10)
36	5.3	4.4	3.5	2.6	2.0	1.6	1.3	1.1	1.0	0.9	0.8
37	5.5	4.6	3.7	2.8	2.1	1.7	1.4	1.2	1.0	0.9	0.8
38	5.8	4.8	3.9	2.9	2.2	1.7	1.4	1.2	1.1	1.0	0.9
39	6.0	5.0	4.0	3.0	2.3	1.8	1.5	1.3	1.1	1.0	0.9
40	6.3	5.2	4.2	3.1	2.4	1.9	1.6	1.4	1.2	1.0	1.0
41	6.6	5.5	4.4	3.3	2.5	2.0	1.7	1.4	1.2	1.1	1.0
42	6.9	5.7	4.6	3.5	2.6	2.1	1.7	1.5	1.3	1.1	1.0
43	7.2	6.0	4.8	3.6	2.7	2.2	1.8	1.5	1.4	1.2	1.1
44	7.5	6.2	5.0	3.8	2.8	2.3	1.9	1.6	1.4	1.3	1.1
45	7.9	6.5	5.3	4.0	3.0	2.4	2.0	1.7	1.5	1.3	1.2
46	8.2	6.7	5.5	4.1	3.1	2.5	2.1	1.8	1.5	1.4	1.2
47	8.6	7.1	5.8	4.3	3.2	2.6	2.2	1.8	1.6	1.4	1.3
48	9.0	7.5	6.0	4.5	3.4	2.7	2.3	1.9	1.7	1.5	1.3
49	9.4	7.8	6.3	4.7	3.5	2.8	2.4	2.0	1.8	1.5	1.4
50	9.9	8.1	6.5	4.9	3.7	2.9	2.5	2.1	1.9	1.6	1.5
51	10.3	8.5	6.8	5.1	3.8	3.1	2.6	2.2	1.9	1.7	1.5
52	10.7	8.9	7.1	5.3	4.0	3.2	2.7	2.3	2.0	1.8	1.6
53	11.2	9.3	7.5	5.6	4.2	3.4	2.8	2.4	2.1	1.9	1.7
54	11.6	9.7	.8	5.8	4.4	3.5	2.9	2.5	2.2	1.9	1.8
55	12.2	10.1	8.2	6.1	4.6	3.7	3.0	2.6	2.3	2.0	1.8
56	12.7	10.5	8.5	6.4	4.8	3.8	3.2	2.7	2.4	2.1	1.9
57	13.4	11.0	8.9	6.7	5.0	4.0	3.3	2.8	2.5	2.2	2.0
58	14.0	11.5	9.3	6.9	5.2	4.2	3.5	3.0	2.6	2.3	2.1
59	14.5	12.0	9.7	7.2	5.4	4.4	3.6	3.1	2.7	2.4	2.2
60	15.1	12.6	10.1	7.6	5.7	4.6	3.8	3.2	2.8	2.5	2.3

[a] Values in percentage of body weight for different size groups held in water of different temperatures.

[b] From Deuel *et al.* (1952).

[c] Values in parentheses indicate the approximate size in inches.

Table A.9

Feeding Chart for Salmon Expressed as the Percentage of Body Weight To Be Fed per Day[a,b]

Average water temperature (°F)	Number of fish per pound						
	Over 2500	2500–1400	1400–300	300–150	150–90	90–40	40–10
40	8.1	7.1	6.1	5.1	4.1	3.0	2.0
41	8.4	7.4	6.3	5.2	4.2	3.2	2.1
42	8.7	7.6	6.5	5.4	4.4	3.3	2.2
43	9.0	7.9	6.8	5.6	4.6	3.5	2.3
44	9.3	8.2	7.0	5.8	4.8	3.6	2.4
45	9.6	8.5	7.3	6.1	5.1	3.8	2.5
46	9.9	8.7	7.6	6.3	5.3	4.0	2.6
47	10.3	9.1	7.9	6.5	5.5	4.1	2.8
48	10.7	9.5	8.2	6.8	5.8	4.3	2.9
49	11.0	9.8	8.5	7.0	6.0	4.5	3.0
50	11.4	10.2	8.9	7.3	6.3	4.7	3.2
51	11.9	10.6	9.2	7.6	6.6	4.9	3.3
52	12.4	11.0	9.6	7.9	6.9	5.2	3.5
53	12.8	11.4	10.0	8.2	7.2	5.4	3.6
54	13.4	11.9	10.4	8.5	7.5	5.6	3.8
55	13.8	12.3	10.8	8.9	7.9	5.9	3.9
56	14.4	12.8	11.2	9.2	8.2	6.1	4.1
57	15.0	13.3	11.6	9.6	8.6	6.4	4.3
58	15.5	13.9	12.2	10.0	9.0	6.7	4.5
59	16.0	14.3	12.6	10.4	9.4	7.0	4.7
60	16.7	15.0	13.2	10.8	9.8	7.3	4.9
61	17.4	15.6	13.8	11.2	10.2	7.6	5.1
62	18.1	16.2	14.4	11.6	10.6	7.9	5.3

[a] From Burrows *et al.* (1951).
[b] Using moist diets.

Table A.10

Some Typical Diets for Trout and Salmon[a]

Mix weight	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)
Fresh ingredients										
Beef liver	33.3	33.3	12.5	12.5	12.5	12.5	25	25	—	15
Hog liver	33.3	33.3	—	—	—	—	—	—	—	—
Beef or pork spleen	—	33.3	12.5	12.5	12.5	12.5	25	25	—	35
Salmon viscera	33.3	—	—	—	—	—	—	—	—	—
Turbot	—	—	—	—	—	—	—	—	20	—
Tuna viscera	—	—	—	—	—	—	—	—	20	—
Dry ingredients										
Cottonseed meal	—	—	25	25	25	—	12	12	26.4	11
Wheat middlings	—	—	—	—	—	—	12	12	—	11
What germ meal	—	—	—	—	—	—	—	—	3.6	—
Crab solubles	—	—	—	—	—	—	—	—	5.4	—
Distillers' solubles	—	—	—	—	—	—	—	12	2.4	11
Corn oil	—	—	—	—	—	—	—	—	1.8	—
Dried brewer's yeast	—	—	—	—	—	—	—	—	—	2
Cod liver oil	—	—	—	—	—	—	—	—	—	2
Fish meal	—	—	25	—	—	25	12	12	18.0	11
Salmon egg meal	—	—	—	25	—	25	—	—	—	—
Meal meal	—	—	—	—	25	—	—	—	—	—
Dried skim milk	—	—	25	25	25	25	12	—	—	—
Salt	2	2	—	—	—	—	2	2	—	2
Vitamins, etc.	—	—	—	—	—	—	—	—	2.4	—

[a] Adapted from R. E. Burrows, personal communication, and W. E. Pearson, Unilever, Bedford, England, Tech. Rep., 1968.

Table A.11

Some Typical Pellet Mixtures[a]

Mix weight	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)	11 (%)	12 (%)	13 (%)	14 (%)	15 (%)	16 (%)	17 (%)	18 (%)	19 (%)	20 (%)
Cottonseed meal	15	23	20	15	15	5			15	24	23	23	19	24	10	10				
Soya bean meal							10	16					19				5	8	5	4.5
Groundnut meal																	5	8	5	
Wheat middlings	20	24	20	22	19	7	20	16	25	24	24	24		24	10					14
Wheat bran																10				
Wheat pollards																20				
Maize															7		20	20	15	10
Rye flour													13				15	16	15	
Barley																	19.5	19.5	19.5	
Fish meal	16	38	21	38	24	24	31	28	40	16	38	36	10	16	30	20	10	8	10	10
Meat meal							10	6								10	10	8	10	
Liver meal	15						5	10											4	10
Blood meal																1.5	4	3		
Dried skim milk	10	5	10	5.5	1.5	3.5			7	11	5	5	10	11	15	15				10
Condensed fish solubles		3						0.2			3	1.5				2				5
Dried distillers' solubles	10		15		21	21		6		11		1.5		11	10					
Whey powder							10	5									4	3	4	
Yeast	10	5	8	10	10	10	5	5	10	10	5	7	15	10	10		4	3	4	4
Alfalfa meal			2																	6
Molasses			2																5	
Wheat germ oil															2					
Cod liver oil	2		2	3	3	3	2	2	3				2		2					
Lard/animal fat													6							
Seaweed meal								3												4
Beet pulp								1.5												
Limestone flour																2.5	1	1	1	
Bone meal				5	5	5										0.85			1	

(*continues*)

Table A.11 (*Continued*)

Mix weight	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)	11 (%)	12 (%)	13 (%)	14 (%)	15 (%)	16 (%)	17 (%)	18 (%)	19 (%)	20 (%)
Cellulose flour						20														
Oat bran																				14
Shrimp meal																				7
Salt	2	2					3.8	1			4	2	2	1	2	3				0.5
Gelatin														5						
Carboxymethyl cellulose															2					
Vitamins	+	+	+	1.5	1.5	1.5	0.2	0.5		+	+			+		5.15	1.5	1.5	1.5	
Dicalcium phosphate																	1	1		1
Vitamins[b]																				
Vitamin																				
A (MIU)	1	1	1				0.002	0.005								3.6				8
D (MIU)	0.4	0.4	0.4				0.00036	0.0009								0.36				1.6
E (g)	1	1	1	150	150	150	60	150	150	150										20
K (g)	1	1	1	20	20	20			20	20										
B_{12} (g)				0.04	0.04	0.04	0.02	0.05	0.04	0.04										
Thiamine (g)				20	20	20	90	225	20	20						2				
Riboflavin (g)	20	20	20	66	66	66	90	225	66	66						4.2				
Pantothenic acid (g)	32	32	32	110	110	110	50	125	110	110						8				
Niacin (g)	90	90	90	300	300	300	100	250	300	300						4				
Pyridoxine (g)	5.6	5.6	5.5	20	20	20	20	50	20	20						2.3				
p-Amino benzoic acid (g)				140	140	140			140	140										
Inositol (g)				500	500	500			500	500										
Choline (g)	100	100	100	3000	3000	3000	250	635	3000	3000						145				
Folic acid (g)				5.6	5.6	5.6	3	7.5	5.6	5.6										
Biotin (g)				4	4	4	0.6	1.5	4	4										
Ascorbic acid (g)				340	340	340	200	500	340	340				1000						300

[a] Adapted from W. E. Pearson, Unilever, Bedford, England, Tech. Rep., 1968, and R. E. Burrows, personal communication.
[b] Vitamins added per ton of food.

Table A.12

Vitamin and Mineral Premixes Used in Aquaculture Diets[a]

A. Vitamin and Mineral Mix No. 1 (Warmwater Fish)[b]

Ingredient	mg/kg of dry diet
Vitamin A	6,000 I.U.
D_5	1,000 I.U.
E	60
K	12
C	240
B_1	24
B_2	24
Pantothenic acid	60
Niacin	120
Vitamin B_6	24
Biotin	0.24
Folic acid	6
Choline Chloride	540
Vitamin B_{12}	0.24
FE	50
Cu	3
Mn	20
Zn	30
I	0.1
Co	0.01
Se	0.1

[a] From FAO manual on Feed and Feeding of Fish and Shrimp (New, 1987).

[b] Source: Chow, 1982 FI:DP/IND/75/031 FAO, Rome. The FAO Manual also lists 43 dietary formula or feedstuff mixes which have been used to rear Coldwater and Warmwater fish and Shrimp.

Table A.12 *(Continued)*

B. Vitamin and Mineral Mix No. 2 (Rabbit Fish, Sea Bass, and Grouper[a]

Ingredient	mg/kg of dry diet
Thiamin HCl	40
Vitamin B_2	40
Pyridoxine	40
Nicotinic Acid	150
Calcium Pantothenate	100
Folic Acid	5
Biotin	1
Vitamin B_{12}	0.02
Inositol	800
Choline Chloride	3500
Sodium Ascorbate	2000
Vitamin E	200
Vitamin K	80
Vitamin A	5000 I.U.
Vitamin D	1000 I.U.
Zn	40
Mn	20
Cu	4
I	0.80
Co	0.12

[a] Source: Kanazawa, 1984, FI:DP/MAL/77/008 FAO, Rome.

Table A.12 *(Continued)*

C. Vitamin Mix No. 4 (Marine Shrimp)[a]

Ingredient	mg/kg of dry diet
Thiamin HCl	120
Vitamin B_2	40
Pyridoxine HCl	120
Nicotinic Acid	150
Calcium Pantothenic	100
Folic Acid	5
Biotin	1
Vitamin B_{12}	0.02
Inositol	4000
Choline Chloride	1200
Sodium Ascorbate (Vit. C)	5000
Vitamin E	200
Vitamin K	40
Vitamin A	5000 I.U.
Vitamin D	1000 I.U.

[a] Source: Kanazawa, 1984: FI:DP/MAL/77/008 FAO Rome; sterol source not indicated (ed.).

Table A.12 *(Continued)*

D. Vitamin Mix No. 6 (Catfish, Siluras sp.)[a]

Ingredient	mg/kg of dry diet
Vitamin A	800 I.U.
Vitamin D_3	400 I.U.
Vitamin E	8
Vitamin K (Menadione)	4
Vitamin C	80
Biotin	0.4
Choline	1200
Folic Acid	2
Niacin	100
Pantothenic Acid	16
Pyridoxine	4
Vitamin B_2	8
Vitamin B_1	4
Inositol	12
Vitamin B_{12}	0.008
BHT	60
or Ethoxyquin	40

[a] Source: Halver, 1982, FI:DP/HUN/79/001 FAO, Rome.

Table A.12 *(Continued)*

E. Vitamin Mix No. 11 (Marine Percoidae)[a]

Ingredient	mg/kg of dry diet
Vitamin A	6000 I.U.
Vitamin B_1	20
Vitamin B_2	20
Vitamin B_6	20
Vitamin B_{12}	0.02
Folic Acid	5
Inositol	600
Niacin	150
Pantothenic Acid	50
Vitamin C	200
Choline	2000
Vitamin D_3	2500 I.U.
Vitamin E	200
Biotin	1
Vitamin K	10

[a] Source: New, 1986, *Kuwait Bull. Mar. Sci.* **1,** 75–151.

Table A.12 *(Continued)*

F. Vitamin Mix No. 12 (Sea Bass, Grouper, and Siganids)[a]

Ingredient	mg/kg of dry diet
Vitamin A	5000 I.U.
Vitamin D	1000 I.U.
Vitamin E (Tocopherol)	
Vitamin B_1 (Thiamin HCl)	120
Vitamin B_2	40
Vitamin B_6 (Pyridoxine HCl)	120
Vitamin B_{12}	0.02
Folic Acid	5
Inositol	800
Niacin	150
Calcium Pantothenate	100
Vitamin C (Sodium ascorbate)	1000
Choline chloride	1200
Biotin	1
Vitamin K (Menadione)	40

[a] Source: Meyers, 1987 FE:DP/INS/81/008 FAO, Rome.

Table A.12 *(Continued)*

G. Vitamin/Trace Element Mix No. 13 (Common Carp)[a]

Ingredient	mg/kg pelleted feed
Vitamin A	8000 I.U.
Vitamin D	900 I.U.
Vitamin E	
Vitamin K	4
Vitamin B_2	3.6
Niacin	20
Choline Chloride	160
Pantothenic Acid	7
Vitamin B_6	0.2
Vitamin B_{12}	0.005
Mn	70
Zn	60
Fe	20
Cu	2
I	1
Co	0.2

[a] Source: Viola and Arieli, 1982, *Bamidgeh* **34,** 39–46.

Table A.13

Scientific Names of Aquaculture Species[a]

Common name	Scientific name
Anabantids	
Climbing perch	*Anabas testudibneus*
Gourami	*Osphronemus goramy*
Kissing gourami	*Helostoma temminicki*
Snakeskin gourami	*Trichogaster pectoralis*
Ayu	*Plecoglossus altivelis*
Carp, common	*Cyprinus carpio*
Carp, Chinese	
Chinese (bream)	*Parabramis pekinensis*
Bighead	*Aristichythys nobilis*
Black	*Mylopharyngodon piceus*
Grass	*Ctenopharyngodon idella*
Mud	*Cirrhina molitorella*
Silver	*Hypophthalamichthys molitrix*
Carp, Indian	
Catla	*Catla catla*
Mrigal	*Cirrhinus mrigala*
Rohu	*Labeo rohita*
Catfish	
African	*Clarias gariepinus*
Brackish-water	*Chrysichthys nigrodigitalus*
Channel	*Ictalurus punctatus*
European	*Siluris glanis*
Malaysian	*Pangasius pangasius*
Walking	*Clarias batrachus*
Cod	
Atlantic	*Gadus morhua*
Colossoma	
Tambaqui	*Colossoma* spp.
Eel	
Japanese	*Anguilla japonica*
European	*Anguilla anguilla*
Flatfish	
Flounder	*Pseudopleuronectes*
Winter flounder	*Pseudopleuronectes americanus*
Halibut	*Plueronectes hippoglossus*
Common halibut	*Paralichthys olivaceus*
Sole	*Solea solea*
Turbot	*Scophthalmus maximus*
Grouper	*Epinephalus* spp.
Milkfish	*Chanos chanos*
Pacu	*Prochilodus* spp.

Table A.13 *(Continued)*

Scientific Names of Aquaculture Species[a]

Common name	Scientific name
Pickerel	
Northern pike	*Esox lucius*
Rabbit fish	*Siganus* spp.
Salmonids	
Arctic char	*Salvelinus alpinus*
Atlantic salmon	*Salmo salar*
Chinook (king) salmon	*Oncorhynchus tshawytscha*
Chun (dog) salmon	*Oncorhynchus keta*
Coho (silver) salmon	*Oncorhynchus kisutch*
Sockeye (red) salmon	*Oncorhynchus nerka*
Pink salmon	*Oncorhynchus gorbuscha*
Brook trout	*Salvelinus fontinelis*
Brown trout	*Salmo trutta*
Lake trout	*Salvelinus namaycush*
Rainbow trout	*Oncorhynchus mykiss* (*Salmo gairdneri*)
Steelhead trout	*Oncorhynchus mykiss* R. (*Salmo gairdneri* R.)
Bass	
Black large-mouth	*Micropterus salmoides*
Black small-mouth	*Micropterus dolomieui*
Striped	*Morone saxatilis*
Black sea	*Centropristis striata*
European sea	*Dicentrarchus labrax*
Giant sea perch	*Lates calcarifer*
Sea bream	
Gilthead	*Sparus auratus*
Red	*Chrysophys* (*Sparus*) *major*
Chinese	*Rhabdosargus sarba*
Snakehead	*Ophicephalus* spp.
Snapper	*Lutjanus* spp.
Tilapia	
Blue	*Oreochromis discolor*
Java (golden)	*Oreochromis aureus*
Mozambique	*Oreochromis mossambicus*
Nile	*Oreochromis niloticus*
Whitefish	*Coregonus clupeaformis*
Yellow perch	*Perca flavescens*
Yellowtail	*Seriola quinqueradiata*

[a] More than 100 other species are being investigated for fish farming in various areas of the world. Those scientific names may be found in the latest U.S. FDA list of market fish.

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