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Title: THE ESSENTIAL FATTY ACID REQUIREMENTS OF RAINBOW TROUT (SALMO GAIRDNERI)

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The essential fatty acid (EFA) requirements of rainbow trout were investigated. Three sets of experimental diets, varying only in lipid content, were fed. The first set was a preliminary experiment in which 10 percent salmon oil (Diet 1), no lipid (Diet 2), or 10 percent safflower oil was included in the diet. Set 2 contained the following lipids:

Diet 2, fat free
Diet 4, 5% oleic acid
Diet 5, 4% oleic acid, 1% linoleic acid
Diet 6, 4% oleic acid, 1% linolenic acid

Set 3 diets all were isocaloric and contained 2 percent lipid. Diets 7-11 contained 0.0, 0.1, 0.5, 1.0 and 2.0 percent ethyl linolenate, being made up to 2 percent lipid with ethyl laurate. Diets 12-16 were

made up with increasing proportions of 18:2ω6/18:3ω3,[1] with the total of these two acids being one percent of the diet.

The best growth rates and feed efficiencies were obtained when the diets contained fatty acids of the linolenic or ω3 series. The optimal level of linolenate was found to be one percent of the dry weight or about 2 percent of the dietary calories. Linoleate in the diet was less effective than linolenate in promoting good growth and feed efficiency. Unlike linolenate, linoleate was unable to prevent all of the EFA deficiency symptoms.

The symptoms of EFA deficiency in rainbow trout included the following: Poor growth, low feed conversion, deterioration of fins, fatty livers, a fainting syndrome which was accentuated by handling or excitation of the fish, excrescense of the heart caused by hyperplasia of sections of the blood vessels, increased rates of swelling of isolated mitochondria in sucrose solutions, elevated respiration rates of liver homogenates, increased tissue water content and decreased hemoglobin levels.

Fatty acid composition and metabolism was altered by EFA deficiency. Absence of ω6 or ω3 fatty acids in the diet resulted in an increase in the deposition of ω9 acids in the fish lipids. The ratio of 20:3ω9/22:6ω3 in fish lipids is suggested as a possible index of EFA

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[1] The ω number refers to the position of the first double bond counting from the methyl end of a fatty acid, eg. 18:1ω9 means 18 carbon fatty acid with one double bond in the 9-10 position counting from the methyl end.

deficiency. If this ratio is above 0.4 the fish are not receiving sufficient dietary ω3 fatty acids. Quadratic equations were derived by regression analysis for relationships between dietary linolenate and the various fatty acids of the fish lipids. A complete set of intermediates have been identified for each of the following interconversions:

$$18{:}1\omega9 \longrightarrow 20{:}3\omega9$$
$$18{:}2\omega6 \longrightarrow 22{:}5\omega6$$
$$18{:}3\omega3 \longrightarrow 22{:}6\omega3$$

# The Essential Fatty Acid Requirements of Rainbow Trout (Salmo gairdneri)

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# THE ESSENTIAL FATTY ACID REQUIREMENTS OF RAINBOW TROUT (SALMO GAIRDNERI)

## INTRODUCTION

Fish culture to augment man's food supply has been practiced for centuries and only recently has it attracted attention as a potential contribution to the protein needs of mankind. Rearing fish has been and still is largely an art, but in the past few years a number of nutritionists and biochemists have reported experiments which portend that fish culture is fast becoming a science. Procedures which established the nutritional requirement of experimental and domestic animals are being modified to good advantage on salmon, trout and catfish. The protein, amino acid and vitamin levels necessary for these aquatic species have appeared in the literature, but little definitive work has been reported on the lipid requirements or the need for essential fatty acids. Recent developments in analytical procedures, particularly gas liquid chromatography, have revealed the complex nature of fish lipids and the striking difference in composition that exists between aquatic and warm blooded animals. Fish have large amounts of the highly unsaturated fatty acids of the $\omega 3$ configuration containing 20 and 22 carbons with limited amounts of the essential fatty acids; linoleic linolenic and arachidonic. Before fish culture may be called a science, it seems important that the role of lipids in the diet and metabolism of fish be understood.

This study had several objectives. The first was to investigate the nutritional aspects of lipids in the diet of rainbow trout and the particular fatty acids important and essential for good growth and feed efficiency.

The second objective was to establish the optimal levels of essential fatty acid required in the diet and describe the physiological and pathological symptoms of essential fatty acid deficiency.

The final objective was to correlate the fatty acid composition of the diet with that found in the tissue and re-establish relationships between the essential and non-essential fatty acids.

# LITERATURE SURVEY

The fact that certain fatty acids are required in the diet of animals was first recognized in 1929 by Burr and Burr (40). By feeding a diet with highly purified casein, sucrose, and fat soluble vitamins from non-saponifiables of cod liver oil they were able to produce deficiency symptoms in rats which were cured or prevented by adding a few drops of lard per day to the diet. The first symptom observed was an abnormal, scaly condition of the skin on the tail which occurred between the 70th and 90th day. The tip of the tail became inflamed and swollen. The hair became filled with dandruff and began to fall out, especially around the face, back and throat. The hind feet became swollen. The growth rate was decreased when compared with that of animals receiving fat in their diet. In severe cases there was blood in the urine and autopsy revealed degeneration of the kidney. Male rats did not mate and female rats often had irregular ovulation.

Further work by Burr and Burr (41) and Burr, Burr and Miller (42) showed that the essential nutrient of the lipid was in the fatty acid portion and not in the non-saponifiables. They found that neither saturated fatty acids (18:0, 16:0, 14:0, 12:0 or lower acids) nor monoenoic acids (18:1ω9) were effective. They found that both linoleic and linolenic acids were effective in curing the essential fatty acid (EFA) deficiency symptoms. They postulated that warm blooded

animals in general cannot synthesize appreciable quantities of linoleic acid and that this acid (and possibly others) is a dietary essential.

In the 40 years since the discovery of EFA a very large number of papers have been published on the subject. A number of very good review articles, such as those by Aaes-Jorgensen (1), Alfin-Slater and Aftergood (25) and Holman (77) as well as a conference edited by Sinclair (136) give an excellent review of the subject.

## Symptoms of EFA Deficiency

In addition to the symptoms described previously, EFA deficiency has also been found to affect other vital organs. Panos and Finerty (121) noted increased size of adrenals, liver, heart, and kidneys and a decrease in the size of the thyroid. Greenberg and Erchoff (60) found a reduction in prostate and seminal vesicle weights in rats on a EFA deficient diet. Menge (111) reported increased weights of spleen, pituitary, pineal, thyroid and adrenals of EFA deficient hens. Deficiency results in degeneration of spermatogenic tissue (5). Part of the explanation for the increase in liver size might be due to the accumulation of lipids, especially cholesterol and its esters (23, 24, 47, 136).

Related to the increased water uptake by deficient animals, Basnayake and Sinclair (29) have found increased permeability of the skin. EFA are required for the formation of cell membrane. Lack

of EFA results in increased permeability and loss of moisture to the atmosphere. Similarly, Kramar and Levine (96) found increased permeability and fragility of capillaries of animals on EFA deficient diets. Requirements of EFA for maintenance of proper cell membrane structure is also evidenced by the increased fragility of erythrocytes (104) and increased swelling of isolated mitochondria (43, 69, 85) in sucrose and salt solutions.

In 1938, Nunn and Smedley-MacLean (120) reported increased trienoic and decreased dienoic and tetraenoic fatty acids in the livers of EFA deficient rats. Increase in the content of 20:3ω9 derived from oleic acid and decrease in the content of 18:2ω6 and 20:4ω6 have come to be recognized as one of the outstanding characteristics of EFA-deficiency (1, 25, 57, 77, 110). Holman and his co-workers (75, 112) have suggested the ratio of 20:3ω9/20:4ω6 in the tissue lipids be taken as an index of the EFA deficiency. If the ratio is less than 0.4, the animal is receiving the minimum required linolenic acid in its diet.

There are other symptoms of EFA deficiency which have been reported and are listed in the review articles (1, 25, 77). These include a respiratory disease syndrome in chickens (83), reduced serum lysozyme in rats (122), changes in the activity of several enzyme systems (70, 98, 145, 146), depressed total serum protein (27), anemia (56), decreased feed efficiency (101), enlarged mitochondria with marked disorganization of crystae and swelling (140), and

decreased visual accuity in subdued light (65).

## Factors Affecting EFA Deficiency

### Humidity

There are several factors which will affect the severity of EFA deficiency. As noted above EFA deficiency results in increased permeability of the skin and increased water consumption not accounted for in the urine. In reviewing these findings it is not surprising that Burr (39) and Brown and Burr (38) found that the severity of the dermal symptoms of deficiency was lessened by increased humidity. Holman and Ener (78) also reported seasonal variations in dermatitis related to the changes in relative humidity. Holman (77), in his review article, recommends that in EFA feeding studies the relative humidity should be kept constant at 50 percent so that results at various laboratories can be compared.

### Age

Most of the EFA deficiency studies have been done with weanling rats or very young animals of the species involved. It has been found that with age it becomes increasingly difficult to produce EFA deficiencies (1, 77). Guggenheim and Jurgens (62) were able to produce the most severe signs of EFA deficiency when the mother was fed

a fat free diet during the gestation period. Aaes-Jørgensen (1) suggests that the explanation might be that EFA are necessary for active growth (formation of new tissue). Adult animals do not develop new tissue in the sense of true growth but rather use EFA primarily in the synthesis of replacement components.

Sex

Burr and Burr (40) found that EFA deficiency inhibited growth to a greater extent in male rats than in females. Greenberg et al. (59) found that the optimum dose of 18:2ω6 for male rats exceeded 50 mg daily, while that for the female was between 10 and 20 mg per day. Workers at the University of Minnesota (128) have found that the minimum requirement for the male rat is 1.3 percent of calories in the diet as linoleate and only 0.5 percent of calories for the female. Alfin-Slater et al. (24) found that EFA deficiency caused an increased cholesterol content in the liver and adrenal glands and a reduced cholesterol content in the plasma of male rats but not in females. It has been suggested (2) that the sex difference is due to the smaller size of the females. An adequate explanation for the sex differences is yet to be found.

Dietary Non-Essential Lipids

The addition of cholesterol to an EFA deficient diet has been shown to accelerate the appearance of the deficiency symptoms (8, 9, 67, 80, 118, 119). There is generally a high proportion of unsaturated fatty acids esterfied to cholesterol in animal fats (1). When a diet is deficient in EFA cholesterol accumulates in the liver and adrenal gland (24) and is found to be esterfied to fatty acids with a lower degree of unsaturation than normal. It is possible that cholesterol accentuates EFA deficiency by tying up some of the reserve unsaturated fatty acids.

Feeding saturated fatty acids, such as are present in hydrogenated coconut oil also accentuates EFA deficiency (2, 3, 4, 6, 7, 26, 27, 49, 54, 116). Saturated fatty acids added to the EFA deficient diet cause decreased growth (2, 3) increased fluid consumption (4), an increase in the 20:3ω9/20:4ω6 ratio (116) as well as increased dermal symptoms (54). Adding oleic acid or ethyl oleate to the diet also enhances EFA deficiency symptoms (49). The effect of oleate is much more pronounced then its trans isomer, elaidate. Dhopeshwaker and Mead (49) suggest that this is due to competitive inhibition of oleate for the enzymes which desaturate and elongate linoleic acid to form arachidonate. Mohrhauer and Holman (76, 79, 113) have found in rats that fatty acids of the linolenic family exhibit an ever greater inhibition of these enzymes than oleate. Century and Horwitt (44) found a similar

inhibition in the chick. Although fatty acids of the linolenic series are unable to cure the dermal symptoms of EFA deficiency, and inhibit conversion of 18:2ω6 to 20:4ω6, they are able to promote growth (1).

There are other factors which affect EFA deficiency that will not be dealt with in detail here. In his review, Aaes-Jorgensen (1) mentions coprophage might supply the animals with some EFA. Deficiency of some vitamins accentuates EFA deficiency. Certain hormones affect the severity of deficiency symptoms and this might be a partial explanation for the sex differences observed. Hypercholesterolemia and diabetes increase susceptibility to EFA deficiency. These subjects are covered in the review articles mentioned previously (1, 2, 77).

## Functions of EFA

In order to explain the very rigid requirement for fatty acids with all cis double bonds, methylene interrupted and with the terminal double bond six carbons from the methyl end, it is important to discover the biological function of these essential fatty acids. A great deal of work must still be done before we will have the complete picture of EFA functions. Several clues to their metabolic role are known, however, and this section will discuss the function of EFA in tissue and membrane production, transportation of other lipids such as cholesterol. Activation of several specific enzymes and regulation of various vital processes by prostaglandins, will also be mentioned.

Formation of Tissues and Membranes

As previously noted the absence of EFA from the diet results in increased permeability of the skin (29), skin lesions (5), increased permeability and fragility of capillaries (97), increased fragility of erythrocytes (105), and increased swelling of isolated mitochondria (43, 69, 86). Thus, it appears that cell membranes require EFA to form a normal permeability barrier around the cells and subcellular particles. Most of the modern theories of cell membranes are based on the original proposal of Danielli and Davison (46). These theories recognize that lipids play an integral part in membrane function.

As noted by Van Deener (147), the major lipid components of cellular and subcellular membranes are cholesterol and its esters and phospholipids. The fact that cholesterol esters and phospholipids, which are present in the membranes of healthy animals, have a high proportion of EFA suggests that one of their functions might be structural (1). Recent work by Moore and co-workers (116) demonstrated that artificial membranes made from phospholipids from EFA deficient rats were more permeable to $Na^+$ ions than those made from phospholipids of control rats. Thus, it appears that EFA are necessary in membranes for the maintenance of the normal permeability. A good biological test for EFA deficiency would be to measure the increase in membrane permeability. The increased rate of swelling of isolated

mitochondria from EFA deficient animals is probably related to the increased permeability of the surrounding membranes.

Transportation of Other Lipids

As noted in an earlier section (24) EFA deficiency results in an accumulation of cholesterol primarily esterified to saturated fatty acids. The suggestion has been made (40) that polyunsaturated fatty acids (PUFA) are important in the transportation of cholesterol out of the liver and that feeding cholesterol stresses EFA deficient animals because the PUFA must be released from the tissues for transportation. Many workers have shown that feeding diets high in PUFA will lower cholesterol levels in patients or animals with above normal blood fat and cholesterol (22, 37, 66, 67, 68).

Mead and Fillerup (107) fed labeled linoleate and found that within half an hour 50 percent was esterified to blood phospholipids. The amount of linoleate in the initial triglyceride slowly dropped and the amount esterified to cholesterol increased. Mead and Howton (108) state that the major portion of the fatty acids are transported as protein-lipid complexes stabilized by phospholipids. All evidence so far accumulated indicates that PUFA have a vital role to play in the normal transportation of lipids in the blood.

Enzyme Activity

Swanson and Artom (142) and Holman and Widmer (81) found that fractions containing a high level of enzyme activity from rat livers also had a relatively high portion of lipid associated with them. It is possible that these lipids have some function in enzyme activity. Tulpule and Patwardhan (145) reported that EFA deficiency resulted in reduction of succinic, glutamic and butyric dehydrogenase in rat livers. There was a correlation between liver lipid iodine value and glutamic and butyric dehydrogenase. They found that the actinomycin A sensitive factor and cytochrome oxidase had increased activity in the deficient rat liver (146). The ratio of phosphate esterified to oxygen consumed was reduced for the oxidation of both reduced NADH and reduced cytochrome C in the deficient rats. Kunkel and Williams (98) also observed the increased cytochrome oxidase activity as well as increased choline oxidase activity in deficient rats. Contrary to the findings of Tulpule and Patwardhan (145), Hayashida and Portman (70) reported that dietary PUFA raised the succinic dehydrogenase activity compared with control, but not as much as EFA deficient diets. Johnson (85) reported uncoupling of oxidative phosphorylation in mitochondria isolated from the liver of EFA deficient rats. The uncoupling appeared to be related to loss of the phosphate esterifying activity. This would agree with the theory of Tulpule and Williams (146) that

the main site of action of EFA is the phosphate esterification system, coupled with the oxidation of reduced cytochrome C.

As further evidence for the role of lipids in enzyme activity, several enzyme systems have been purified which require particular types of lipid for activity. Sekuzu, Jurtshuk and Green (134) purified a β-hydroxybutyric dehydrogenase from beef heart mitochondria which showed an absolute requirement for lecithin. All other lipid fractions tested including other phospholipids were inactive. A definite incubation period with the lecithin was required to convert the enzyme to the fully active form. Lecithins from different sources were all effective as activators of the enzyme but the degree of activity of the lecithins increased with degree of unsaturation of the esterified fatty acids (89). One molecule of the apoenzyme requires 200 molecules of lecithin for maximum activity (134). Cerletti, Strom and Giordano (45) isolated a purified succinic dehydrogenase which was activated by adding mitochondrial lipids or commercial lecithin. Recently Yoneyama et al. (151) purified a protoheme ferrolyase which required lipid fractions for activation The acidic phospholipids, phosphatidylethanolamine, cardiolipin, phosphatidic acid and phosphatidylinositol, were strong activators and the intensity of activation was in the order of the acidity. It is clear that lipids play an important role in the function of enzymes of the oxidative phosphorylation as well as other systems. Much more research is still necessary, however, before we can

understand how these lipids function.

Prostaglandins

The discovery that prostaglandins were biosynthesized from the essentially fatty acids arachidonic and dihomo-8-linolenic acids (30, 50, 51) has suggested another explanation for the effects of EFA deficiency. Prostaglandins were isolated in 1935 by Euler (53), and Bergstrom et al. (31) were first to elucidate the structure of some of the prostaglandins. In 1965 DePury and Collins (47) suggested that the fatty livers in EFA deficient animals was related to the lack of prostaglandins which could cause the lipase in adipose tissue to become more active than normal, leading to an increased release of free fatty acids. Pawar and Tidwell (123) found that prostaglandin $E_1$ did indeed inhibit the release of free fatty acids from adipose tissue of rats on a fat free diet. This past year Horton (84) published an excellent review of the present evidence and theories on functions of prostaglandins. Only a few of the theoretical functions of prostaglandins related to the symptoms of EFA deficiency will be discussed here. Prostaglandin $E_1$ inhibits the action of vasopressin and prevents it from causing increased permeability of membranes to water. As already mentioned prostaglandin $E_1$ helps to prevent fatty liver formation by inhibiting adipose tissue lipase activity. Prostaglandins $E_1$ and $A_1$ stimulate epidermal proliferation and keratinization in the

chick embryo. Their lack in EFA deficiency might explain the dermatitis and depigmentation that develop. Prostaglandins stimulate the smooth muscles of the male and female reproductive systems and might be related to impaired reproduction in EFA deficient animals. The discovery of prostaglandins as a biosynthesized product from essential fatty acids has opened the door for a great deal of possible future research related to the function of EFA.

## Essential Fatty Acids and Fish Nutrition

Relatively little work has been done on the nutritional lipid requirements of fish. Most of the research in the field of fish lipids has been involved with analysis of the lipid components of fish from their natural environment. A complete review of the papers on analysis of fish oils is beyond the scope of this survey. Several good reviews have been written. Ackman (14) in his review noted that fatty acids from marine sources had all methylene interrupted cis double bonds and that with the exception of the 16 carbon chain length, the ultimate double bond was in the three, six or nine position, counting from the methyl end. He also noted that the predominant poly-unsaturated fatty acids characteristic of marine oils were of the linolenic family. Fatty acids of the linoleic group, considered to be essential fatty acids in higher animals were present only in small quantities. In comparing the composition of fresh water fish oils with

those of marine fish, Ackman (15) noted that the fresh water fish had a higher proportion of 16 and 18 carbon fatty acids and lower amounts of 20 and 22 carbon fatty acids than marine fish. There was a higher amount of trienoic fatty acids and the ratio of ω3 to ω6 fatty acids was lower in the fresh water fish. However, the major portion of the PUFA of the fresh-water fish were still of the linolenic series, primarily 20:5ω3 and 22:6ω3. Many other excellent reviews of fish oil analysis are available, such as those by Gruger (61) and Bailey, Carter and Swain (28).

The subject of lipid metabolism in fish was reviewed in 1951 by Lovern (102) and later in 1967 by Mead and Kayama (109). Lipid absorption, deposition and metabolism in fish appears to be very similar to that of the rat, except that the water temperature will affect the rate of these reactions, as the fish is a cold blooded animal. Fish are able to synthesize saturated fatty acids as well as unsaturated fatty acids of the oleic and palmatoleic acid families de novo. However, they are unable to synthesize fatty acids of the linoleic or linolenic acid series. Like other animals fish are able to desaturate and elongate linoleic and linolenic acids to long chain PUFA.

The fatty acid composition of the fish lipids is influenced both by the dietary lipid and the water temperature. Kelly et al. (91) showed that on a fat free diet the long chain PUFA, thought to be characteristic of fish lipids decreased markedly. When fish on a fat

free diet were fed ten percent cottonseed oil for five weeks little change was noted in the body fatty acid composition (92), but feeding ten percent menhaden oil changed the fatty acid composition of the fish to resemble the composition of the diet. The subject of effect of dietary lipids on fatty acid deposition will be dealt with in greater detail shortly.

Knipprath and Mead (94, 95, 96) and Reiser et al. (130) reported that lowering the water temperature results in a greater deposition of PUFA in fish. The fact that fish generally live in water at temperatures much below that of warm blooded animals and must adapt their whole physiological make-up to function at or only slightly above the water temperature probably helps to account for the deposition of the large quantities of long chain PUFA characteristic of marine life.

Fish like rats and other warm blooded animals are able to desaturate and elongate oleic, linoleic and linolenic acids to form longer chained PUFA (90, 106, 109). In the rat the major end products are 20:3ω9, 20:4ω6 or 20:5ω3, respectively. In the fish they are 20:3ω9, 22:5ω6, and 20:5ω3 plus 22:6ω3. In common with other animals, fish cannot synthesize linoleic or linolenic acids (93). Nicolaides and Woodall (119) reported work on the EFA requirement of chinook salmon fry. They reported depigmentation as a symptom of the deficiency. The depigmentation was not cured by the addition of triolein or linolenic acid to the diet, but was prevented by trilinolein.

The fat free diet as well as triolein resulted in decreased growth. Both trilinolein and linolenic acid resulted in a positive growth response. Based on a subjective evaluation of pigmentation these workers conclude that linoleate is essential for chinook salmon and that linolenate is not. The fact that 0.1 percent linolenic acid fed as the only fat source gave higher average gains and lower mortality than one percent trilinolein was not discussed.

Lee et al. (100) reported poor growth and high mortalities in fingerling rainbow trout fed purified diets containing ten percent corn oil which is an excellent source of linoleic acid and serves well in preventing EFA deficiency in warm blooded animals. The ten percent corn oil diet was modified by substituting one percent salmon oil, five percent salmon oil, ten percent soybean oil or one percent linolenic acid. The average four week gain per fish over a 12 week feeding period was 4.2 gm for the ten percent corn oil diet and 7.9, 13.9, 9.2 and 8.4 gm respectively for the substitutions mentioned above. In each case raising the content of ω 3 fatty acids increased the growth response. The 12 week mortalities was 25 percent for the 10 percent corn oil diet and less than six percent for all other diets. Thus, the work of both Nicolaides and Woodall (119) and of Lee et al. (100) suggest that fatty acids of the linolenic family have more EFA value in fish than the linoleic acid series.

This conclusion was also reached by Higashi and his

co-workers (73). These workers tested ethyl linoleate, ethyl linolenate and ethyl esters of highly unsaturated fatty acids prepared from squid oil for their essential fatty acid value in the diet of rainbow trout. Although linoleate improved growth over that obtained with a fat free diet, linolenate was more effective and the PUFA ethyl esters gave the best growth. A high incidence of tail-rot was noted in the fat free fish which was suggested as a possible symptom of EFA deficiency. When 200 μgm/gm body weight per day of each of the ethyl esters was fed the ratios of food per weight gain were 2.11, 1.78 and 1.60 respectively for ethyl linoleate, ethyl linolenate and ethyl esters of PUFA. Thus fatty acids of the ω 3 family give better growth and more efficient feed conversion than those of the ω6 family. Just as arachidonic acid is more effective than linoleic acid in other animals, the PUFA of squid oil were more effective than linolenic acid in fish.

Work by several authors who were not considering EFA requirements of fish also supports the view that ω3 fatty acids are of greater nutritional value than ω6 to fish. Phillips and his co-workers (125, 126, 127) found that when fish oils such as cod liver oil were added to the diet there was a consistent increase in the rate of growth for each increase in concentration up to 15 percent of cod liver oil in the diet. Adding corn oil to trout diets did not alter the growth rate. Several other authors (56, 63, 64, 72, 89, 143, 144) also report better results when fish were fed diets containing oils with ω3 fatty acids, such as

are in corn oil.

Fish oils are high in fatty acids of the ω3 series. Their diet in the natural environment contains oils high in the ω3 fatty acids. It is of interest to note that when Sinnhuber (139) analyzed ten commercially prepared fish feeds he found high amounts of ω6 fatty acids and low levels of ω3 acids. Considering the ever increasing use of commercial diets in hatcheries and fish farms in this country, as well as many others, it seems very important to establish the nutritional requirements for fatty acids by fish. With this in mind the purpose of this thesis is to establish the essential fatty acid requirements of rainbow trout. The effect of various fatty acids on growth, feed efficiency, mortality and tail deformity will be confirmed. The effect on rate of swelling of isolated mitochondria, respiration of liver homogenates and fatty acid composition of the fish will also be checked as further indications of presence or absence of EFA deficiency.

# EXPERIMENTAL PROCEDURE

## Experimental Animals

Rainbow trout (Salmo gairdneri) was the experimental animal used in this study. Eggs were obtained in January from brood stock held at the Food Toxicology and Nutrition Laboratory of the Department of Food Science and Technology. The fry began feeding about one month after hatching. The fish were maintained in 75-liter fiberglass tanks in water supplied by wells. The water temperature was constant at 11.5 C.

All stock fish were fed a moist diet (Diet 1) containing 65 percent water and 35 percent dry mix. The composition of the dry mix was as follows:

| | |
|---|---|
| Casein (NBC vitamin-free) | 49.5% |
| Gelatin | 8.7% |
| Dextrin[1] | 15.6% |
| Mineral Mix[2] | 4.0% |
| Carboxy methyl cellulose[3] | 1.3% |
| Alpha-cellulose (Alphacel-NBC) | 7.7% |
| Vitamin E (supplies 660 IU/Kgm) | 0.2% |
| 70% Choline chloride | 1.0% |
| Vitamin mix no. 3 | 2.0% |
| Salmon oil | 10.0% |

[1] American Maize Products Co., 1818 W. Lake Ave. North, Seattle, Washington.

[2] Bernhart-Tomerelli Salt Mix; Modified by adding NaF and $CoCl_2$ at 0.002 and 0.02%, respectively.

[3] Hercules Powder Co., 120 Montgomery St., San Francisco, California.

The composition of the vitamin mix and the mineral mix is found in Appendix I. In all of the experimental diets only the composition and concentration of the lipid were changed. Three sets of diets were fed.

SET 1

In this preliminary experiment, trout (350) which had been maintained on a diet containing 10 percent salmon oil (Diet 1) for 4 months and then a fat free diet (Diet 2) for 8 months, were divided into 3 groups. Diet 1, containing 10 percent salmon oil was fed to 100 fish. In another group of 100 fish, 10 percent safflower oil (Diet 3) replaced the salmon oil. The remaining 150 fish were maintained on the fat free diet (Diet 2). The fish were fed ad libitum except before weighing and sampling, when food was withheld for one day.

SET 2

Semi-purified fatty acids were used in this experiment. Oleic acid was crystallized in acetone at -20 C to improve the purity of the technical grade acid obtained from the Nutritional Biochemical Corporation. Linoleic and linolenic[4] acids (99+ % pure) were obtained from The Hormel Institute.

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[4]The 99+% linolenic acid from Hormel was found by GLC analysis on a 300' by 0.01" capillary column coated with BDS, to contain two impurities thought to be cis, trans isomers of linolenic acid.

The fish had been held on Diet 1 (stock diet with salmon oil) 2 months after hatching and 3 months on Diet 2 (fat free). Eight hundred of these fish were evenly distributed among 8 tanks with duplicate lots receiving the following diets with lipids indicated:

Diet 2: Fat free

Diet 4: 5% oleic acid (18:1)

Diet 5: 4% oleic acid (18:1) and 1% linoleic acid (18:2)

Diet 6: 4% oleic acid (18:1) and 1% linolenic acid (18:3)

SET 3

Only highly purified lipids from The Hormel Institute were used in this experiment. The fish were fed a definite amount based on their body weight. All fish were weighed every 2 weeks. The diet ingredients (casein, dextrin, gelatin and cellulose) were extracted 3 times with hot isopropanol to remove trace lipids and then dried to remove the isopropanol.

After hatching, sufficient fry for the experiment were fed a fat free diet (Diet 2) for one month. Twenty sets of 20 random numbers were used to distribute 2,000 fish, 5 at a time, among 20 tanks. Duplicate lots were fed diets with 2 percent ethyl esters of the following acids:

| Diet | Laurate (12:0) | Linoleate (18:2) | Linolenate (18:3) |
|---|---|---|---|
| | | Percent | |
| 7 | 2.0 | 0.0 | 0.0 |
| 8 | 1.9 | 0.0 | 0.1 |
| 9 | 1.5 | 0.0 | 0.5 |
| 10 | 1.0 | 0.0 | 1.0 |
| 11 | 0.0 | 0.0 | 2.0 |
| 12 | 1.0 | 0.1 | 0.9 |
| 13 | 1.0 | 0.3 | 0.7 |
| 14 | 1.0 | 0.5 | 0.5 |
| 15 | 1.0 | 0.7 | 0.3 |
| 16 | 1.0 | 1.0 | 0.0 |

## Physiological Tests

A detailed outline of the procedures is given in Appendix II. The following is a brief discussion of the experiments conducted:

### Mitochondrial Swelling

The fish livers were homogenized and the mitochondria isolated by fractional centrifugation in 0.44 M sucrose following the procedure of Dounce et al. (52). Swelling was then observed as a decrease in absorbance at 520 mμ in 0.25 M sucrose, 20 mM Tris-HCl buffer at pH 7.4. The absorbancies of SET 3 mitochondria were adjusted to 100 μgm protein/ml based on the Lowry protein determination (103).

### Respiration

Fish liver samples were homogenized in a modified Kreb's saline media (82). Samples of this homogenate were then added to

Kreb's saline with disodium succinate in Warburg flasks. These were attached to a Gilson Respirometer. The oxygen absorption was noted every 5 minutes over a 30 minute time interval and plotted on graph paper. With SET 1 fish, the oxygen absorption values were based on the weight of the liver. The protein concentration was determined for SET 3 liver homogenates and absorption values based on protein content.

## Blood Analysis

Hematocrit determinations were performed on 10 fish from each diet of SET 3 after 20 weeks to obtain the packed red blood cell volume. The hemoglobin level was determined in the blood from 10 fish on each diet by the acid hematin analysis (71, 99). These two values were used as a measure of the degree of anemia.

## Flesh Water Content

Ten fish from each diet in SET 3, which had been used for blood analysis, were skinned and filleted. Weighed samples were dried in an oven overnight at 110 C and the water content determined.

## Lipid Analysis

The detailed description of all the procedures involved in lipid analysis is given in Appendix III. Only a brief outline is given here.

All samples were extracted with chloroform-methanol by the procedure of Bligh and Dyer (34). Lipids were separated into neutral lipid and phospholipid on a silicic acid column, eluting with chloroform and methanol, respectively. Methyl esters of fatty acids were prepared by transesterification with 7 percent boron trifluoride in methanol.

All methyl ester samples were analyzed by gas liquid chromatography on packed columns in an Aerograph HyFi model 500 B or on capillary columns in a Perkin-Elmer model 226. Both instruments were equipped with hydrogen flame detectors. Peak areas were measured with a disc chart integrator. Each fatty acid methyl ester was tentatively identified on the basis of relative retention times. Area percents were corrected to weight percents by conversion factors based on the "active" carbon theory of Ackman (13).

# RESULTS AND DISCUSSION

## Growth

In all cases fish fed diets free of unsaturated fatty acids gained the least weight. Diets containing linoleic acid improved growth. In all cases, superior growth was obtained with diets containing ω3 polyunsaturated fatty acids (PUFA).

Set 1:

In this preliminary experiment the data shown in Table 1 were obtained. The fish were one year of age when placed on the diets and the experimental rations were fed for 22 weeks. A comparison may be made between Diet 1 and 3, since only these two diets were isocaloric. It would appear from these data that salmon oil, which is high in ω3 fatty acids, resulted in better growth than safflower oil, which contained 76 percent ω6 fatty acids as 18:2. No significance is attached to the mortality figures in this experiment.

Table 1. Growth Data

| | Diet 1 | Diet 2 | Diet 3 |
|---|---|---|---|
| Type of Lipid | Salmon | Fat Free | Safflower |
| Init. No. Fish | 100 | 150 | 100 |
| Init. Av. Wt. (gm) | 32 | 39 | 35 |
| Final Av. Wt. (gm) | 103 | 65 | 91 |
| Av. Wt. Gain (gm) | 71 | 26 | 57 |
| Mortality | 4 | 7 | 2 |

Set 2:

The total population of each tank was weighed every 3 weeks during the experimental feeding period. The growth curves are presented in Figure 1. Diet 2 (fat free) cannot be directly compared with Diets 4, 5 and 6, as it has a lower caloric content. Diets 4, 5 and 6 are isocaloric and a comparison shows adding 1 percent ω6 fatty acid increased growth. The diet containing 1 percent 18:3ω3 was about twice as effective in improving growth rate as that with 1 percent ω6.

Set 3:

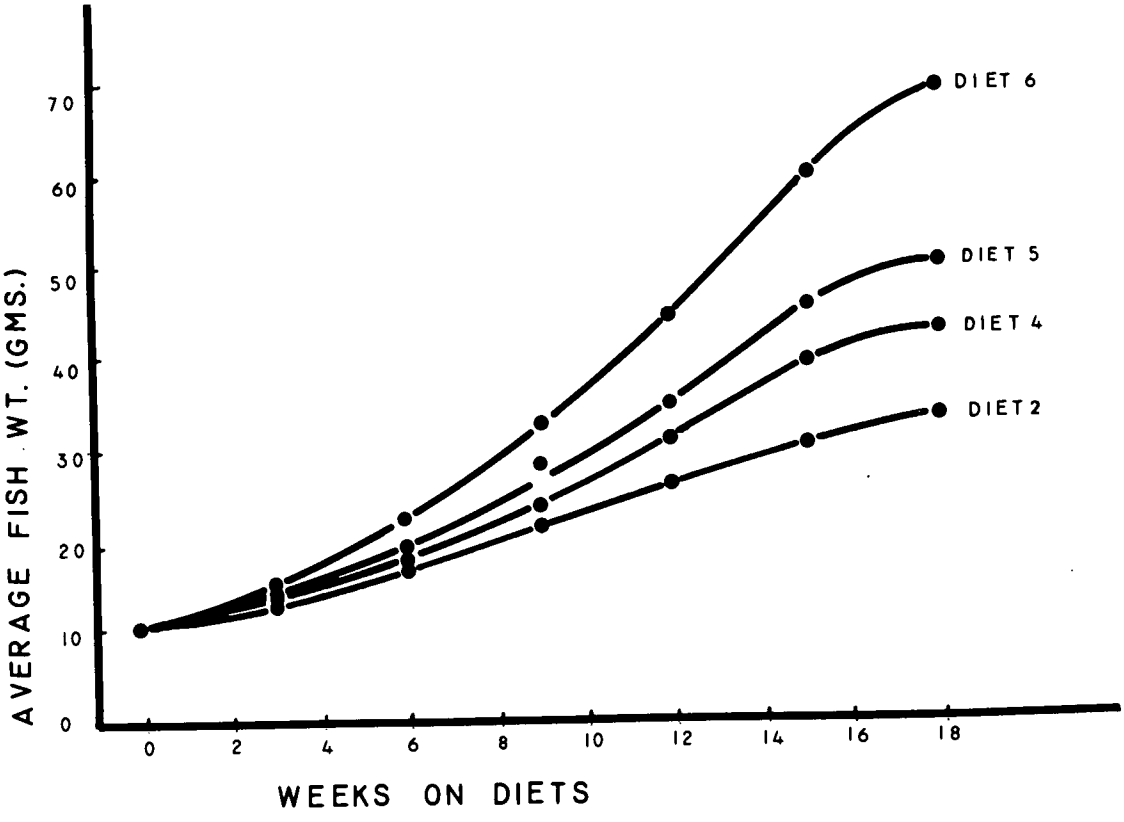
Linoleic and linolenic acids were shown to have similar effects on the growth of fish fed Set 3 diets (Figures 2, 3 and 4). These diets were all isocaloric, each having 2 percent lipid added.

Different ratios of 18:2/18:3 were fed (all adding up to 1 percent PUFA in the diet) to see if any combination promoted better growth than either fatty acid alone. The growth curves in Figure 3 indicate that no combination of ω6 with ω3 is better than 1 percent ω3 alone.

Using growth as the criterion, it seems apparent that linolenic acid has much more EFA value than linoleic acid. The growth enhancing ability of linolenate found in these experiments is similar to that reported by Nicolaides and Woodal (119), Lee et al. (100) and Higashi et al. (73). Higashi et al. (73) found that the ethyl esters of the PUFA fraction of squid oil was even more effective than ethyl

Figure 1. Set 2 growth curves.
Diet 2 - Fat Free
Diet 4 - 5% 18:1
Diet 5 - 4% 18:1, 1% 18:2
Diet 6 - 4% 18:1, 1% 18:3

Figure 2. Growth curves for Set 3 fish.
Diet 7 - 2% 12:0
Diet 8 - 1.9% 12:0, 0.1% 18:3
Diet 9 - 1.5% 12:0, 0.5% 18:3
Diet 10 - 1.0% 12:0, 1.0% 18:3
Diet 11 - 0.0% 12:0, 2.0% 18:3


AVERAGE FISH WT. (GMS.)
0
10
20
30
40
50
60
70
0
2
4
6
8
10
12
14
16
18
WEEKS ON DIETS
DIET 6
DIET 5
DIET 4
DIET 2

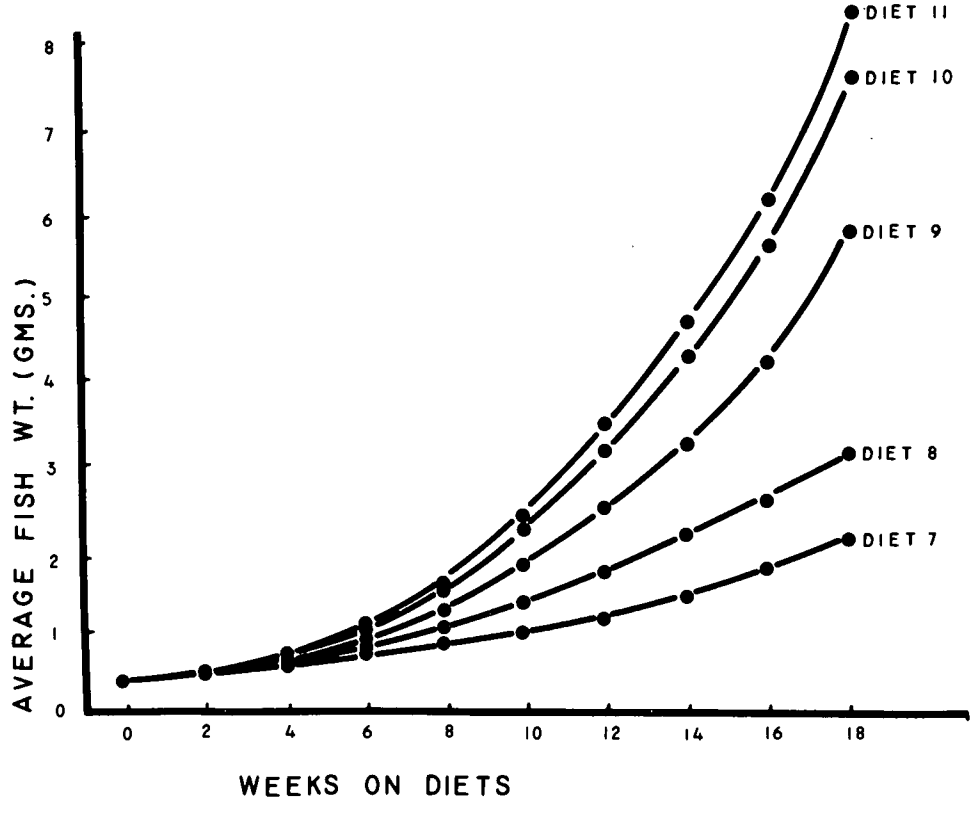
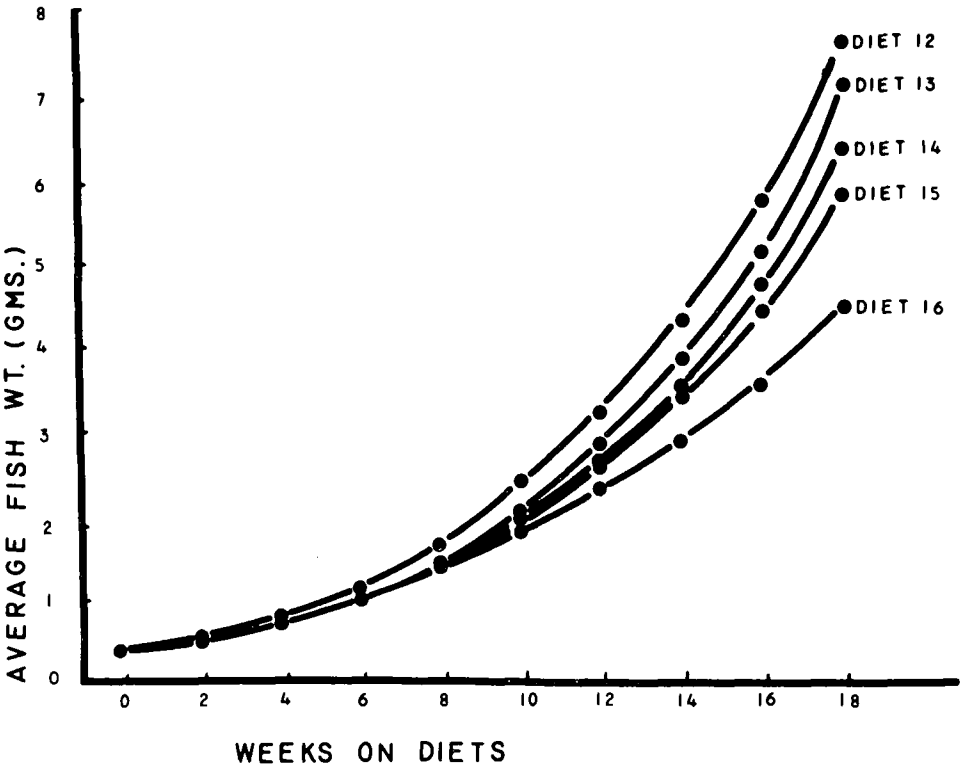
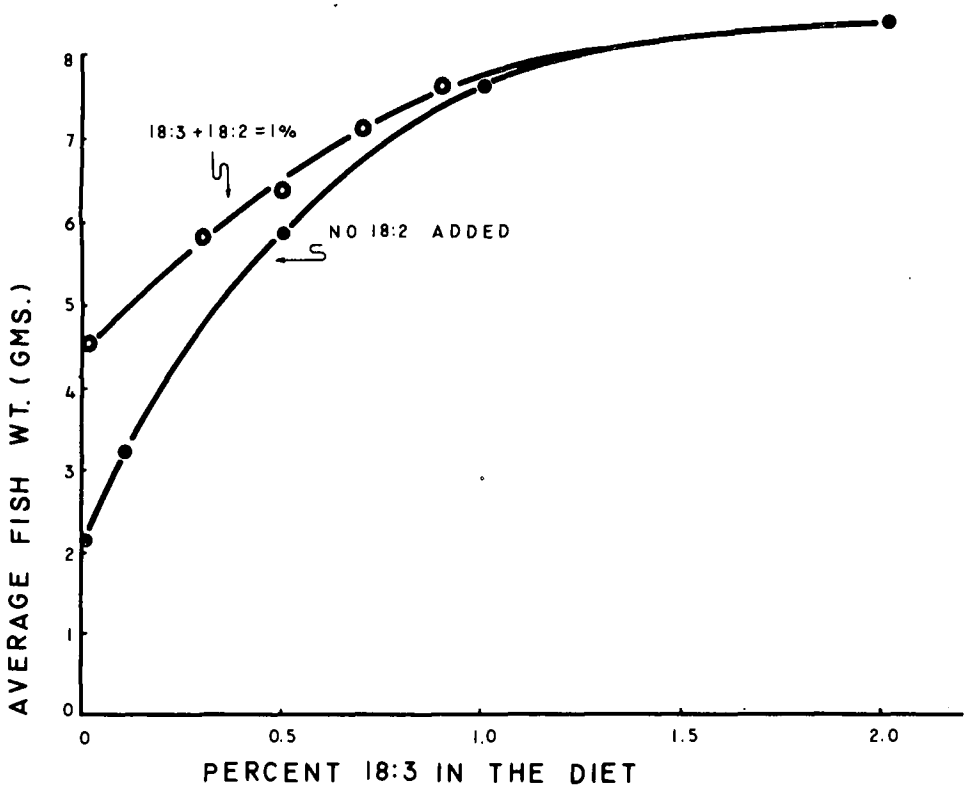

AVERAGE FISH WT. (GMS.)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
14
16
18
WEEKS ON DIETS
DIET 11
DIET 10
DIET 9
DIET 8
DIET 7

Figure 3. Growth curves for Set 3 fish.
Diet 12 - 1% 12:0, 0.9% 18:3, 0.1% 18:2
Diet 13 - 1% 12:0, 0.7% 18:3, 0.3% 18:2
Diet 14 - 1% 12:0, 0.5% 18:3, 0.5% 18:2
Diet 15 - 1% 12:0, 0.3% 18:3, 0.7% 18:2
Diet 16 - 1% 12:0, 0.0% 18:3, 1.0% 18:2

Figure 4. Relationship between 18:3$\omega$3 content in the diet and average fish weight after 18 weeks on the experimental diets of Set 3.


DIET 12
DIET 13
DIET 14
DIET 15
DIET 16
AVERAGE FISH WT. (GMS.)
WEEKS ON DIETS
0
2
4
6
8
10
12
14
16
18


18:3 + 18:2 = 1%
NO 18:2 ADDED
AVERAGE FISH WT. (GMS.)
PERCENT 18:3 IN THE DIET
0
0.5
1.0
1.5
2.0

linolenate. It would be expected that eicosapentaenoic or decosahexaenoic acids of the ω3 series would have more EFA value and result in better growth. Supplies of these acids in a purified state were not available at the time of these experiments.

## Feed Conversion

Feed conversion, which may be considered as the units of gain per unit of dry food consumed, is a measure of feed efficiency or quality of the diet. In every case the low fat diets had the poorest feed conversion. Adding oleic or linoleic acids improved the conversion. However, in all cases an equivalent amount of linolenic or other ω 3 fatty acid was much more effective in improving feed conversion.

Set 2:

The feed conversion results obtained after 30 weeks are shown in Table 2. In this experiment the best feed conversion was obtained with the diet containing 1 percent 18:3ω3. The diet containing 5 percent 18:1 was better than that with 1 percent 18:2 plus 4 percent 18:1. This might be due to the small amount of 18:3ω3 in the oleic acid used in this experiment (see Appendix I, Table 3). The added 18:2 of Diet 5 seems to have inhibited the small amount of 18:3 in the oleic acid.

Table 2. Set 2 feed conversion after 30 weeks.

| Diet | 18:1 | 18:2 | 18:3 | Gain (gm) | Feed (gm) | Feed Conversion |
|---|---|---|---|---|---|---|
| | | percent | | | | |
| 2 | 0 | 0 | 0 | 6,482 | 10,954 | 0.592 |
| 4 | 5 | 0 | 0 | 11,351 | 14,022 | 0.810 |
| 5 | 4 | 1 | 0 | 12,546 | 16,335 | 0.768 |
| 6 | 4 | 0 | 1 | 19,874 | 22,511 | 0.883 |

Set 3:

The total feed conversion after 18 weeks on the diets is shown in Figure 5. The optimal feed conversion is obtained with 1 percent 18:3ω3 in the diet.

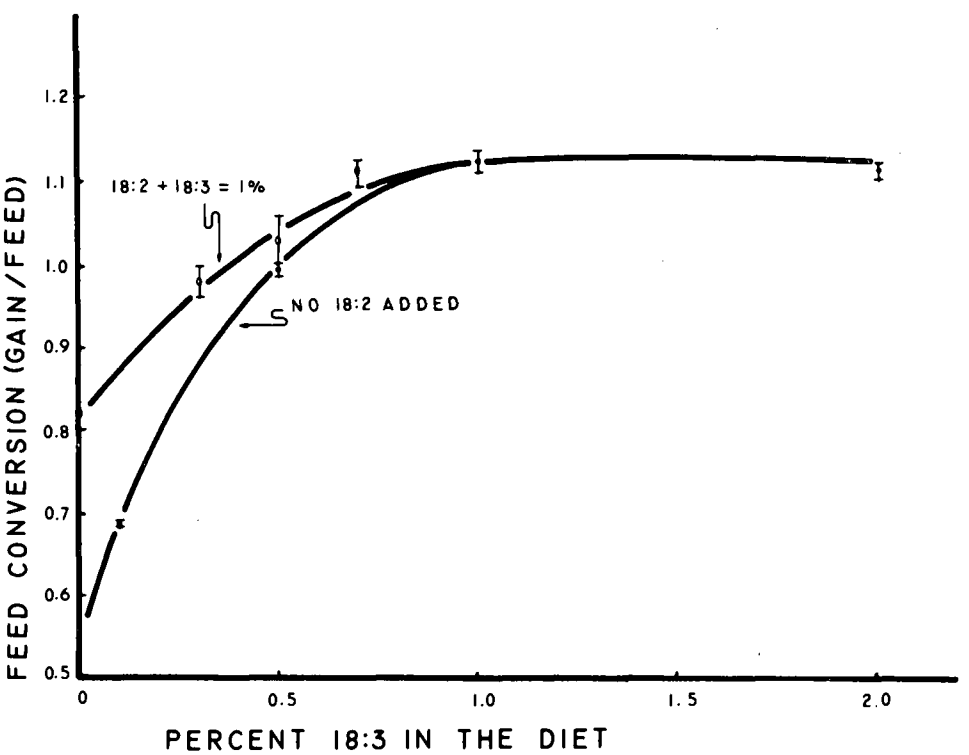



Figure 5. Relationship between feed conversion and percent 18:3ω3 in the diet.

## Physical Symptoms of Deficiency

There are a number of physical symptoms which appear in fish on EFA deficient diets. These include a shock or fainting syndrome upon handling or excitation of the fish, erosion of fins (especially the tail fin), pale, swollen, fatty livers, and deformed hearts in a few cases. The fainting syndrome and heart troubles seem to be accentuated by feeding ω6 fatty acids. The fin erosion and fatty livers are somewhat reduced by feeding ω6 fatty acids. All deficiency symptoms are prevented by including ω3 fatty acids in the diet.

In all tanks where the diet contained no added fat or where ethyl laurate or oleic acid was added, fin erosion was noted after 2 to 4 months. This deterioration was very similar to that reported by Higashi et al. (73). The condition became worse with time. In some cases the erosion became so severe that the entire tail fin was lost. The Diet 2 fish on the right in Figure 6 is a good example of this. No fin erosion was noted in fish receiving diets supplemented with linoleate or linolenate. Diet 4 (5% 18:1), Figure 6, shows some erosion, but not as much as Diet 2 (fat free). Fish, like other animals, are able to synthesize oleic but not linoleic or linolenic acid. The reduction of tail erosion by Diet 4 might be due to the traces of linoleic and linolenic acids present in the oleic acid used in the preparation of this diet.

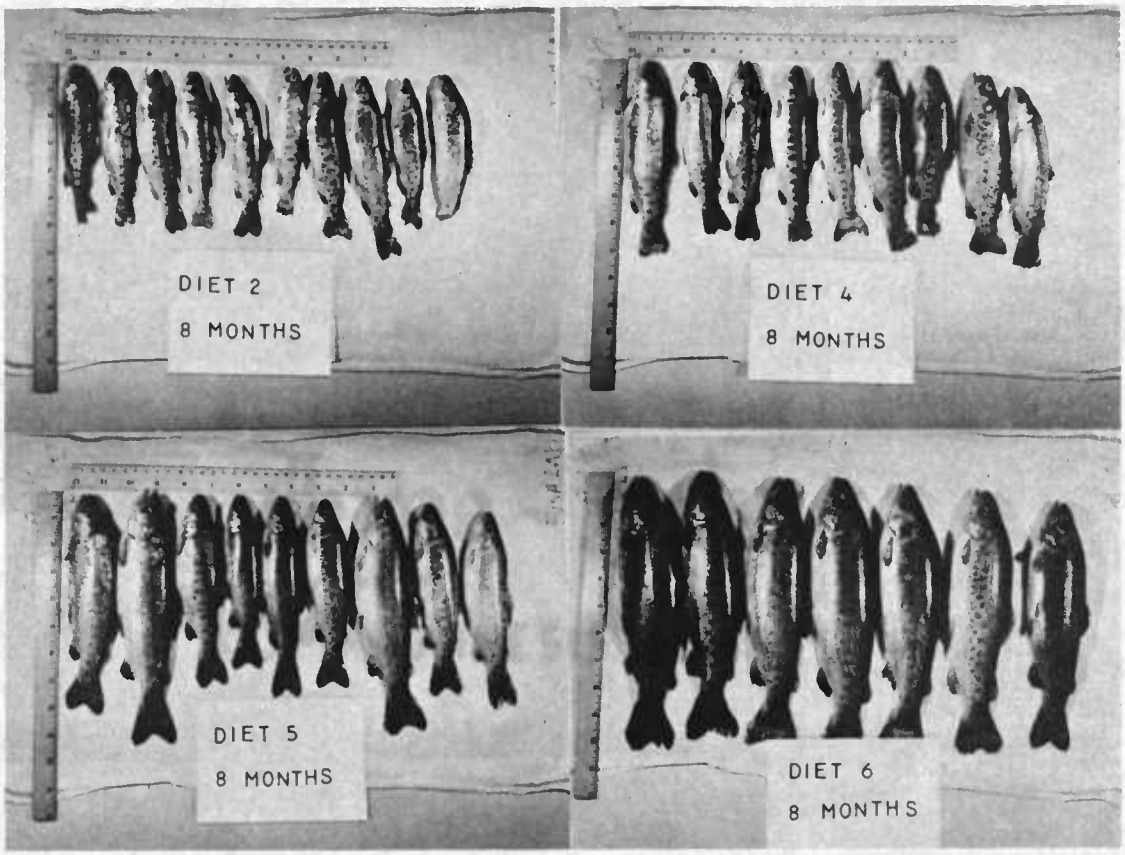



Figure 6. Fish from Set 2 at the age of 1 year, after 8 months on the diets.

Diet 2 - No Fat Added
Diet 4 - 5% 18:1
Diet 5 - 4% 18:1, 1% 18:2
Diet 6 - 4% 18:1, 1% 18:3

Several similarly affected fish from Diet 7 (2% 12:0) were microscopically examined and found to have a bacterial infection in the affected areas of the fins. The microorganisms were isolated by inoculation into Cytophaga medium at 18 C for two weeks. The organism was a Gram negative, slender rod, forming yellow to pink colored colonies. The microorganism was tentatively identified as a Cytophaga species. It appears that EFA deficiency results in increased susceptibility to bacterial infection, since similar infections were not noted among fish receiving linoleate, linolenate or salmon oil in their diet.

The fainting or shock symptoms began to appear 3 weeks to 2 months after a fat free diet or, one which was deficient in ω3 fatty acids, was fed. Careful observation of fish of Set 3 showed that when linoleate was added without linolenate the fainting symptoms were more severe and appeared even before they did with fish on diets containing 2 percent ethyl laurate. When these fish were handled many would faint and float belly-up or sink to the bottom of the tub. They remained in this state for 30 seconds to 6 minutes, depending upon how long they had been on the deficient diet. Often these fish would revive momentarily when the tub was tapped and then resume the belly-up position. This symptom was most obvious at weighing time. All of the fish were netted and moved so the tanks could be thoroughly cleaned at these times. In Set 3 fainting first occurred among fish of

Diets 16 (1% 18:2) and 15 (0.7% 18:2, 0.3% 18:3). At the 4 week weighing there were 12/200 and 6/200 fish, respectively, which fainted. At the 6 week weighing fainting was also observed in Diets 7 (2% 12:0) and 8 (1.9% 12:0, 0.1% 18:3). Figure 7 is a photograph of fish on Diet 16 at the time of the 6 week weighing. Several fish had recovered and there were only about 6 fish remaining belly-up. After several months on these diets, over 50 percent of the fish on Diets 7 and 16 would faint during weighing.

The fainting syndrome noted here is very similar to the transportation shock reported by Black (32) and Black and Barrett (33). They noted that this condition was a "common experience of fish culturists." The diets used in the experiments of these workers was not listed. Knowing that linoleic acid accentuates this fainting condition and, as reported by Sinnhuber (138), that many of the commercial fish feeds are high in linoleic and low in fatty acids of the linolenic series, one might speculate that this commonly occurring experience is related to dietary lipids and the lack of ω3 fatty acids.

The livers of fish on a low fat diet or one containing only saturated or oleic acids became swollen and very pale. Figure 8 shows two fish from Set 2. The fish on the right (Diet 2) had a pale and swollen liver. The fish on the left (Diet 1) had a normal dark liver. After only one month the average percent of the body weight represented by the liver in Diet 2 (fat free) of Set 2 was 2.03 percent,

Figure 7. Six week weighing of fish from Diet 16 of Set 3, showing several fish that have fainted.

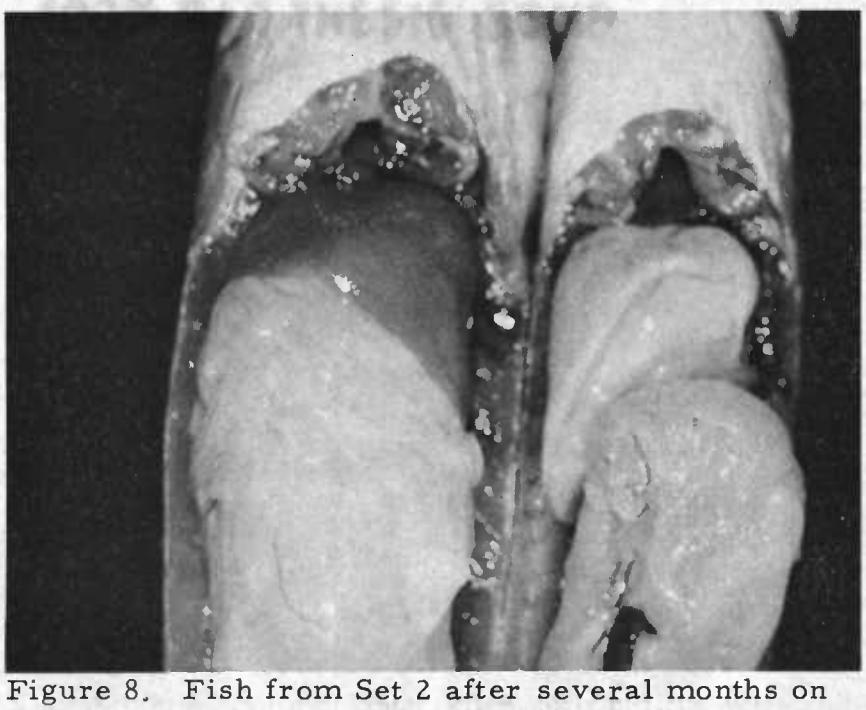


Figure 8. Fish from Set 2 after several months on their respective diets. The fish on the left received 10% salmon oil; the one on the right received a fat free diet.

while Diets 1 (10% salmon oil) and 3 (10% safflower oil) were 1.51 percent and 1.47 percent, respectively. The percent of the body weight represented by the liver decreased in all fish with age. After 30 weeks the average percent body weights were 1.83, 1.11 and 0.95 for Diets 2, 1 and 3, respectively.

The heart condition first was noted in a dead fish on Diet 16 (1% 18:2ω6, 1% 12:0) of Set 3. The heart was deformed and had a considerable degree of excrescence. Figure 9A reveals that the excrescence is due to greatly enlarged sections of blood vessels. Higher magnification (Figure 10A) shows the walls of the blood vessels to be hyperplastic. Figures 9B and 10B are sections of normal hearts from fish fed Diet 1 (salmon oil). The incidence of this cardiac condition was not very high in any of the diets fed. The number of abnormal hearts found in the 30 fish examined from each diet were 5 and 2 for Diets 16 (ω6) and 7 (2% laurate), respectively, and one each for Diets 15 (.7% ω6, .3% ω3) and 14 (.5% ω6, .5% ω3).

## Mitochondrial Swelling

### Effect of Temperature

Mitochondria were isolated by fractional centrifugation in 0.44 M sucrose from a 385 gm rainbow trout on the control diet (Diet 1). The swelling was observed in 0.25 M sucrose 20 mM tris-HCl buffer at

A

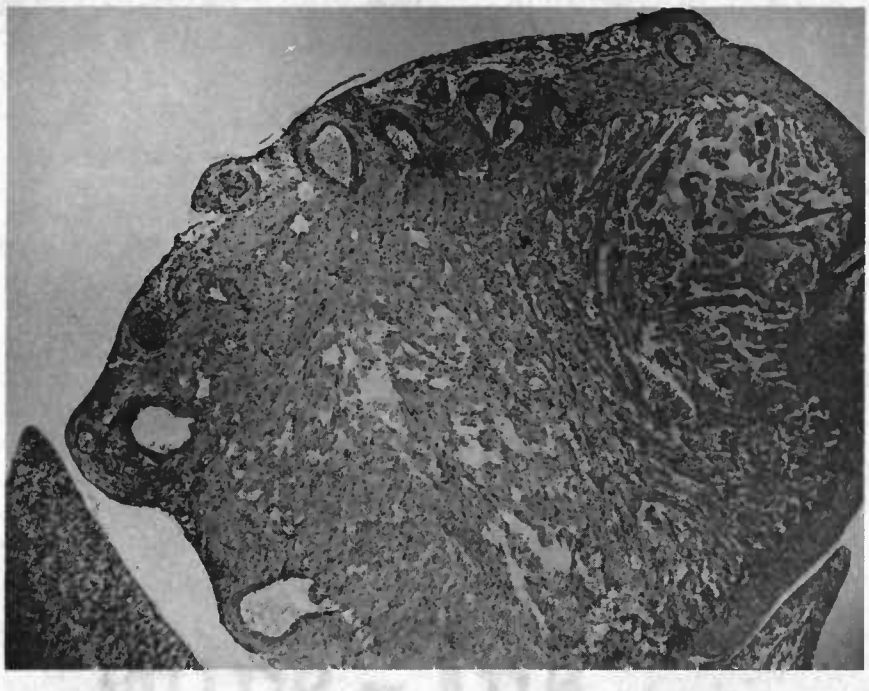


Figure 9A. The abnormal heart from a fish on Diet 16 after 18 weeks.

B

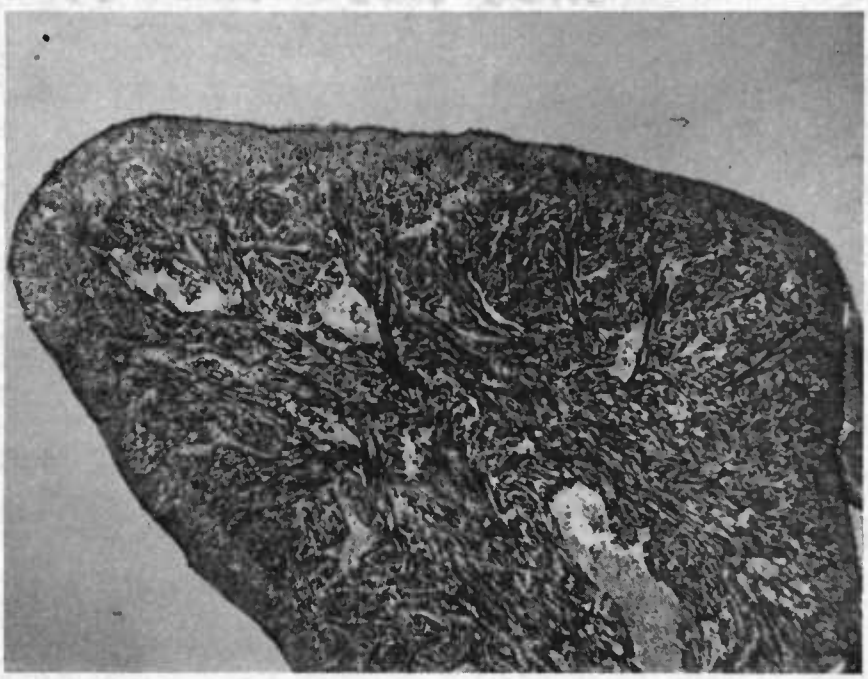


Figure 9B. Normal heart from stock fish receiving Diet 1 (salmon oil).

A

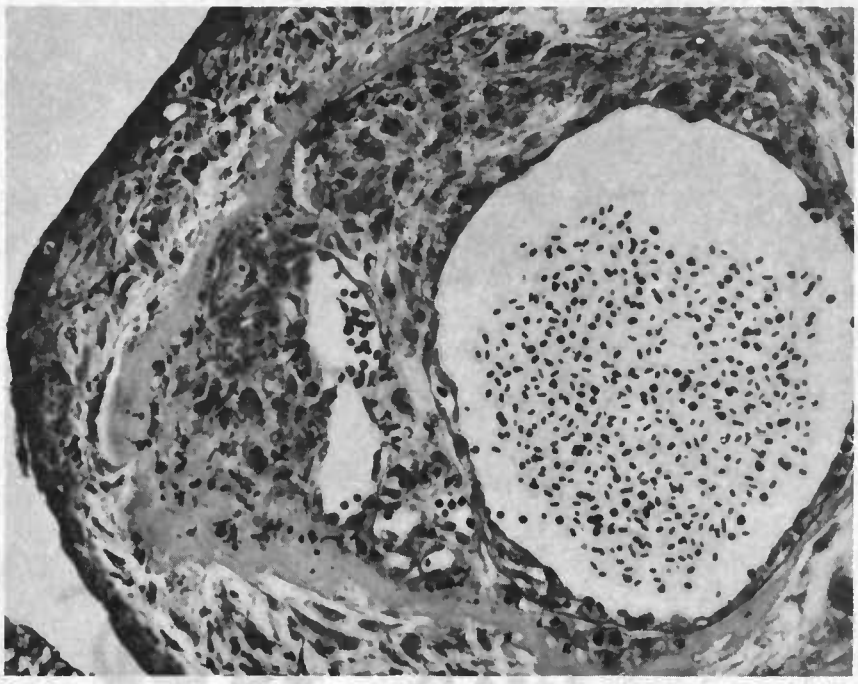


Figure 10A. High magnification of enlarged blood vessel of Diet 16 fish heart.

B

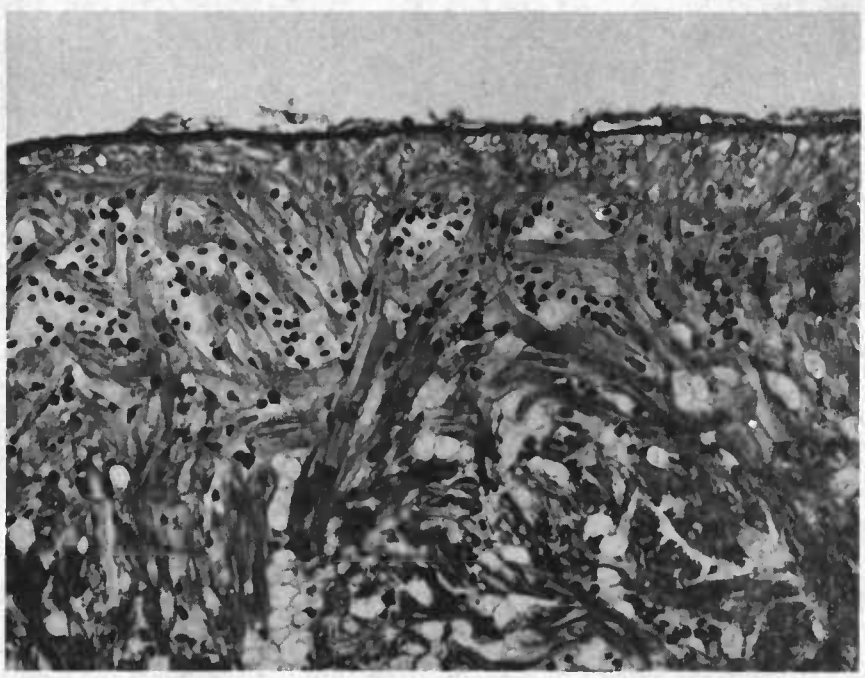


Figure 10B. High magnification of section of heart from normal fish on salmon oil diet.

pH 7.4 at 9, 15, 28 and 37 C. The swelling curves, Figure 11, show that the greatest swelling occurred at 28 C. At 37 C there was an initial fast rate of swelling, but the rate decreased faster than lower temperature swelling. Swelling at 30 C (not shown in Figure 11) was almost identical to that at 28 C.

Effect of Sucrose Concentration

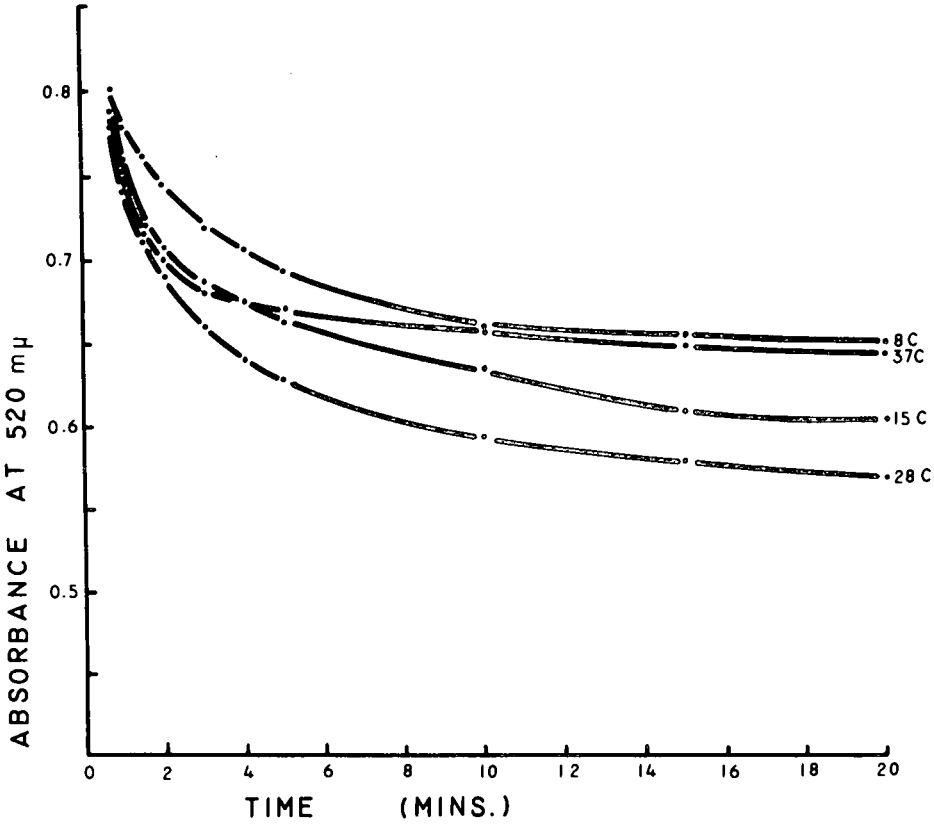
A 401 gm trout on the same diet was killed to obtain liver mitochondria used to determine the effect of sucrose concentration on swelling. Figure 12 shows the curves obtained for 0.1, 0.2, 0.3 and 0.4 M sucrose in 20 mM tris-HCl buffer at pH 7.4. Decreasing sucrose concentration increased the rate of swelling.

Effect of a Fat Deficient Diet

A comparison of mitochondria prepared from liver and brain of control fish receiving 10 percent salmon oil and fish which had been on a fat free diet (Diet 2) for six months revealed a considerable increase in swelling rates of mitochondria from the animals on a fat free diet (Figures 13 and 14). These results were reproducible. Little difference was noted between the mitochondria from the brain or liver of fish on the same diet.

Figure 11. Effect of temperature on mitochondrial swelling.

Figure 12. Effect of sucrose concentration on mitochondrial swelling.


ABSORBANCE AT 520 mμ
0.8
0.7
0.6
0.5
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS.)
8C
37C
15 C
28 C

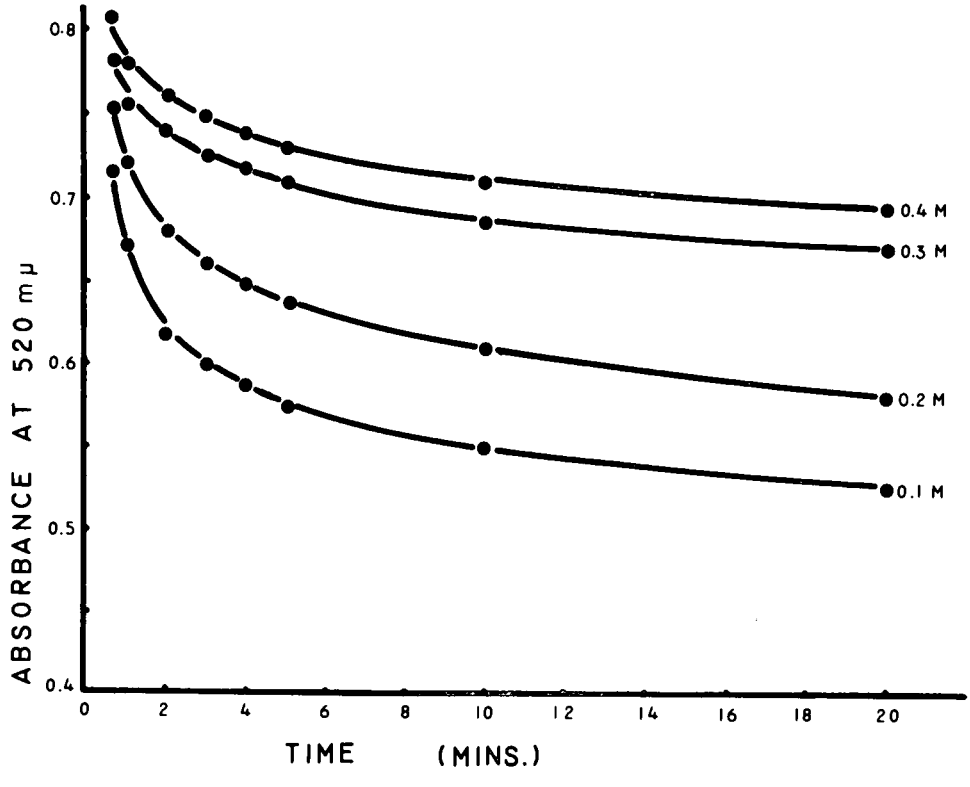
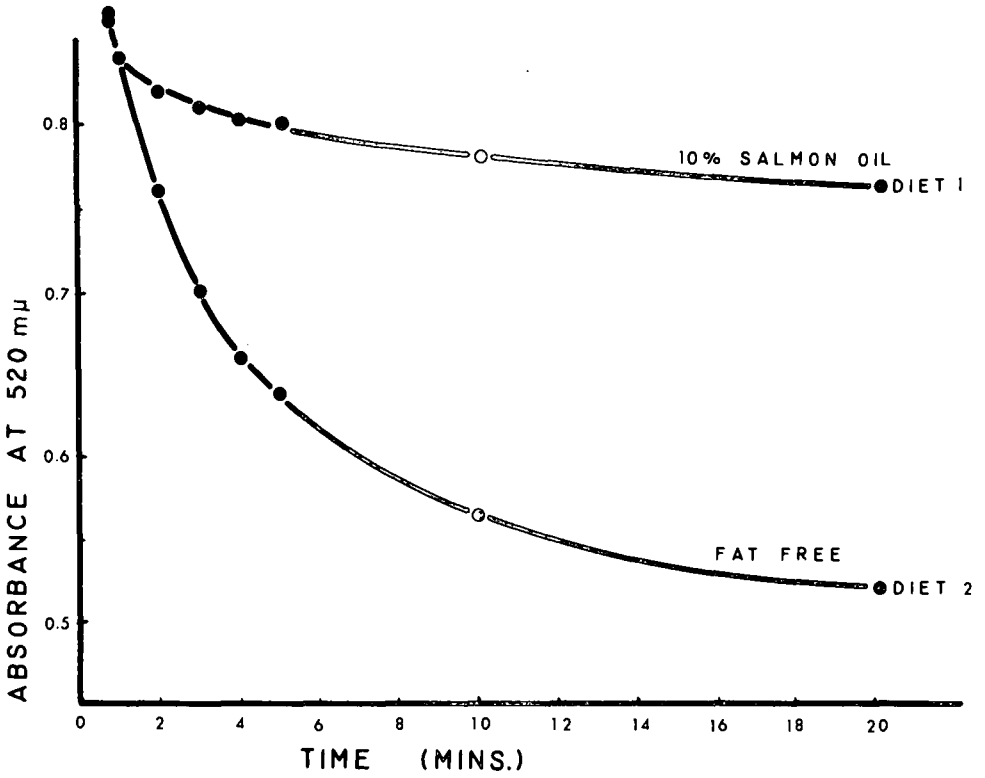
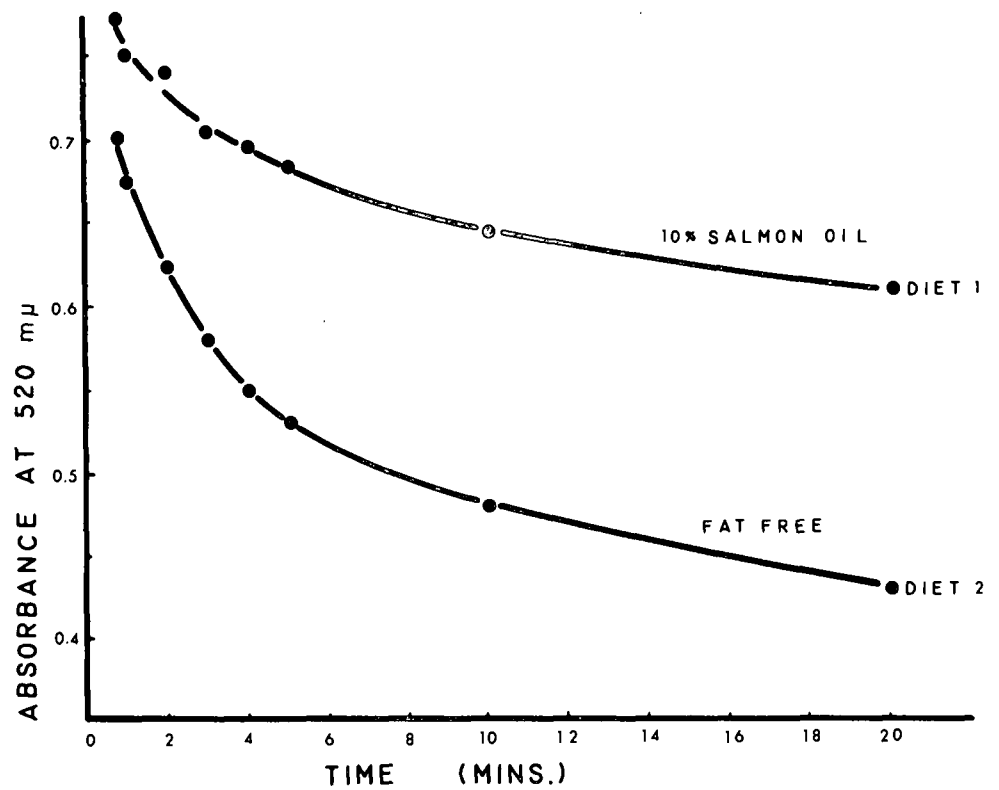

ABSORBANCE AT 520 mμ
0.8
0.7
0.6
0.5
0.4
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS.)
0.4 M
0.3 M
0.2 M
0.1 M

Figure 13. Swelling of liver mitochondria from fish receiving salmon oil (Diet 1) and a fat free diet (Diet 2).

Figure 14. Swelling of brain mitochondria from fish receiving salmon oil (Diet 1) and a fat free diet (Diet 2).


ABSORBANCE AT 520 mμ
0.8
0.7
0.6
0.5
10% SALMON OIL
DIET 1
FAT FREE
DIET 2
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS.)


ABSORBANCE AT 520 mμ
0.7
0.6
0.5
0.4
10% SALMON OIL
DIET 1
FAT FREE
DIET 2
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS.)

Set 1

Figure 15 indicates that even after only two days on ten percent salmon oil diet (Diet 1) fish in Set 1 showed considerable reduction in the mitochondrial swelling. The ten percent safflower oil diet (Diet 3) had not changed when compared with the fish which were continued on the fat free diet (Diet 2). Figure 16 demonstrates that the picture was the same after 40 days on the diets. Ten percent safflower oil had not reduced the rate of swelling at 30 C compared with the fat free diet and in fact appeared to swell slightly more. At 4 C the swelling picture is different (Figure 17). Here the salmon oil and safflower oil fed fish both show reduced mitochondrial swelling compared to the fat free fish. The swelling of all the mitochondria is reduced at 4 C compared with that at 30 C.

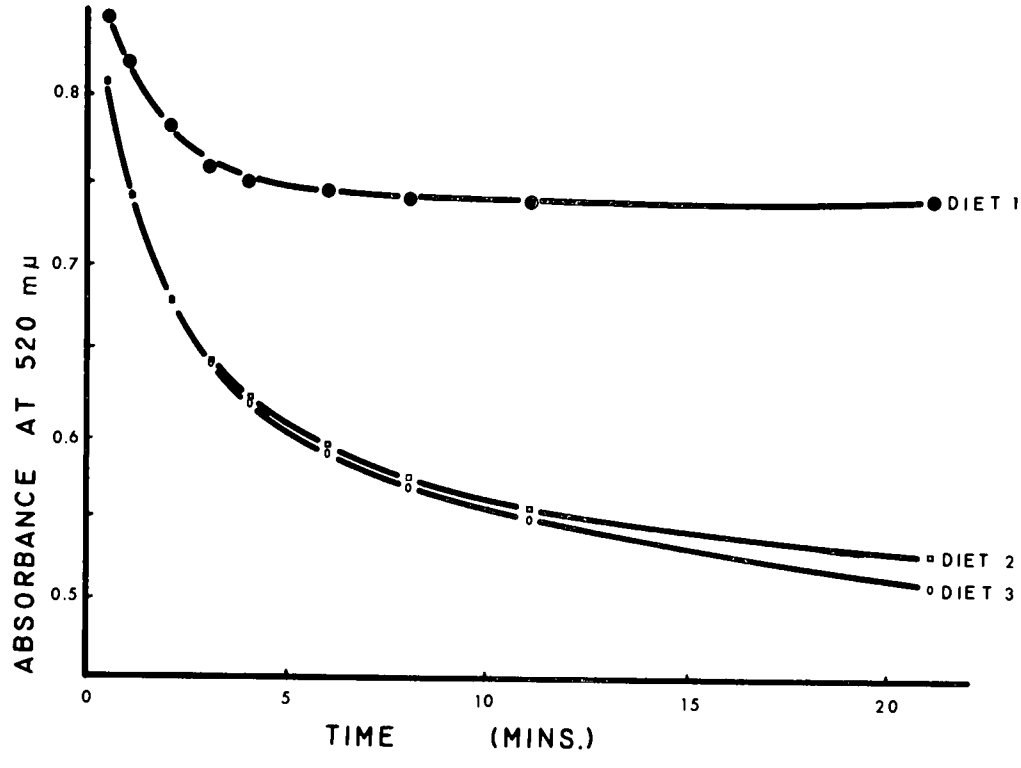
Set 2

With Set 2 fish the effect of changing the dietary lipid is very rapidly observed in the mitochondrial swelling. In Figure 18 the effects of five percent oleic acid, four percent oleic plus one percent linoleic acids and four percent oleic plus one percent linolenic acids are compared with the fat free diet after two days on the new diets. Oleic alone enhanced the swelling. The 18:3ω3 was again the most effective in reducing mitochondrial swelling.

The mitochondrial swelling picture remains very similar for the

Figure 15. Swelling of liver mitochondria at 30 C after two days on diets. Diet 1 contained ten percent salmon oil, Diet 2 was fat free and Diet 3 contained ten percent safflower oil.

Figure 16. Swelling of liver mitochondria at 30 C after 40 days on diets.


ABSORBANCE AT 520 mμ
0.8
0.7
0.6
0.5
0
5
10
15
20
TIME (MINS.)
DIET 1
DIET 2
DIET 3

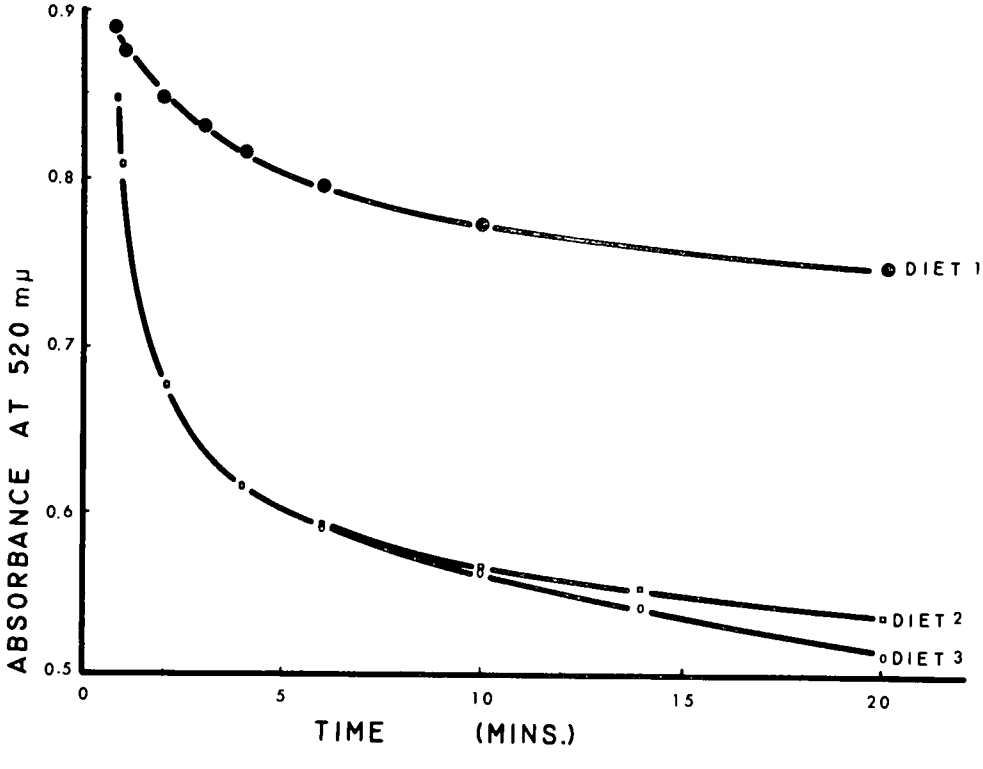
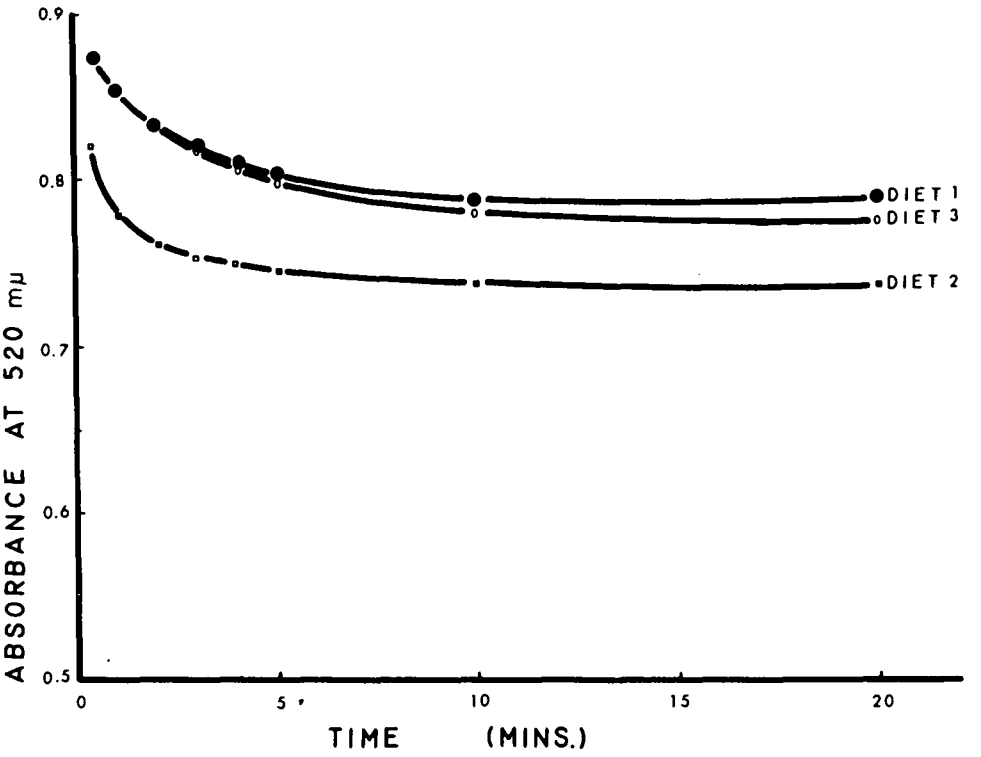
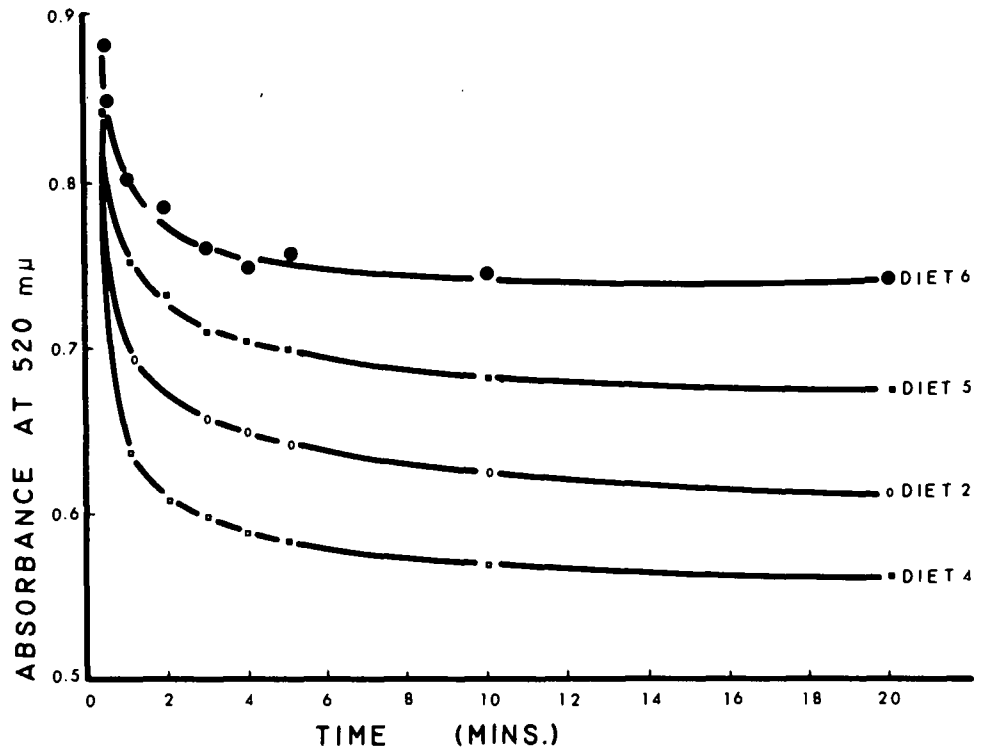

ABSORBANCE AT 520 mμ
0.9
0.8
0.7
0.6
0.5
0
5
10
15
20
TIME (MINS.)
DIET 1
DIET 2
DIET 3

Figure 17. Swelling of liver mitochondria at 4 C after 40 days on diets.

Figure 18. Swelling of liver mitochondria at 30 C two days after commencing experimental diets.


0.9
0.8
0.7
0.6
0.5
ABSORBANCE AT 520 mμ
0
5
10
15
20
TIME (MINS.)
DIET 1
DIET 3
DIET 2


0.9
0.8
0.7
0.6
0.5
ABSORBANCE AT 520 mμ
0
2
4
6
8
10
12
14
16
18
20
TIME (MINS.)
DIET 6
DIET 5
DIET 2
DIET 4

remainder of the experiment with Set 2 fish.

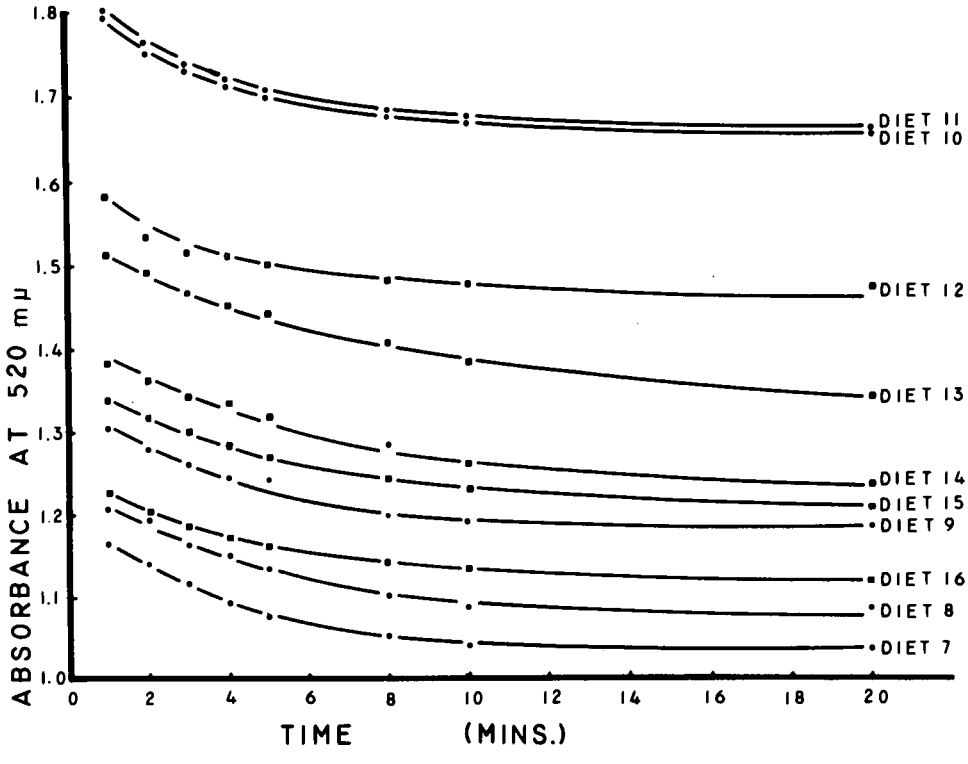
Set 3

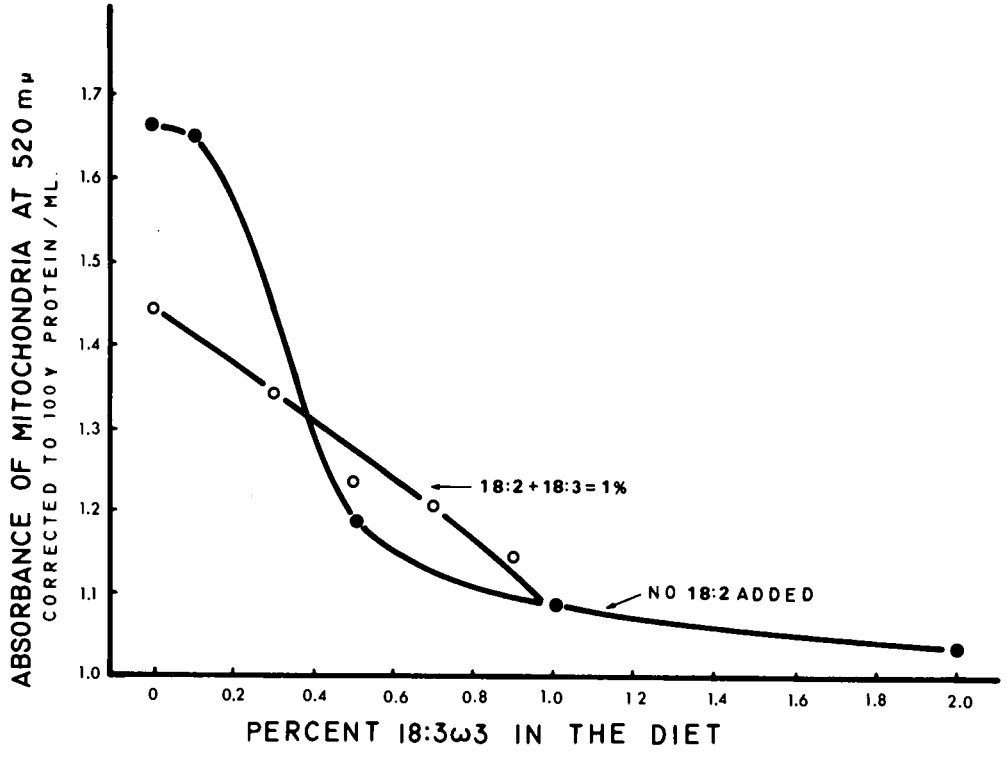
The fish in Set 3 gave unexpected swelling curves compared with those just reported. Even after 13 weeks there appeared to be little difference in the rate of mitochondrial swelling of all the diets (Figure 19). Unlike the fish in Set 2 and 1 these fish had not received salmon oil diets after hatching. It is known that age of the experimental animal plays an important role in the development of EFA deficiency. Because these fish were all fed a fat free diet at a very early age and then changed to diets containing only two percent lipids, the deficiency symptoms would be expected to be more severe in these fish. This does not appear to be the case with mitochondria. It is possible that all the mitochondria of fish in the experiment were much more fragile than those of previous studies and were ruptured during the homogenization and centrifugation procedures.

If the absorbance of the mitochondrial suspension (corrected to read for 100 $\mu$ gm per ml), at any particular time after innoculation into the swelling medium, is plotted against the percent 18:3$\omega$3 in the diet, an interesting relationship is observed. Figure 20 demonstrates this relationship. The absorbency per mg of protein decreased as the content of 18:3$\omega$3 was increased. Feeding one percent 18:2$\omega$2 resulted in a lower absorbency than the ethyl laurate alone, but was not nearly

Figure 19. Swelling of mitochondria at 30 C. The absorbance readings are for a suspension containing 100 μ gm protein/ml.

Figure 20. Absorbance of mitochondrial suspensions, ten minutes after innoculation into the swelling media, plotted against 18:3 content in the diet.


ABSORBANCE AT 520 mμ
TIME (MINS.)
DIET 11
DIET 10
DIET 12
DIET 13
DIET 14
DIET 15
DIET 9
DIET 16
DIET 8
DIET 7


ABSORBANCE OF MITOCHONDRIA AT 520 mμ
CORRECTED TO 100 γ PROTEIN / ML.
18:2 + 18:3 = 1%
NO 18:2 ADDED
PERCENT 18:3ω3 IN THE DIET

as effective as 18:3ω3.

Richardson and Tappel (131) found that the swelling rates for mitochondria from normal fish were comparable with those of rats between 30 and 40 C. At lower temperatures the fish mitochondria were more flexible and would swell at a more rapid rate than rat mitochondria between 0 and 30 C. The results with control fish presented in this thesis agree well with their findings. Richardson et al. (132) also noted that fish heart and liver mitochondria were much higher in PUFA of the linolenic family and lower in those of the linoleic series than rats and other land animals.

Mitochondria from brains and livers of fish fed a fat free diet showed a much increased rate of swelling. This is in agreement with the results for other animals (43, 69, 86, 87, 148). EFA deficiency causes increased mitochondrial swelling in fish also. There remains a great deal of research to be done related to EFA deficiency and mitochondrial function in fish. Although linoleic acid does reduce the rate of swelling as it has in other animals, linolenic acid is much more effective in this regard. In fish, linolenic acid seems to play the role that linoleic acid does in rats. Whether the EFA function only in the formation of membranes and affect permeability or aid enzyme functions such as oxidative phosphorylation has yet to be established.

## Respiration

Set 1: The plots of oxygen uptake by 0.5 ml of 1/5 (liver wt/vol) Set 1 fish liver homogenate in 2.5 ml 3mM disodium succinate in Krebs saline at 30 C are shown in Figure 21. Each curve is the average of four runs. Although there is overlapping of individual fish on different diets, the averages show a general trend. The lowest oxygen uptake was noted for stock fish which had always been on a diet containing ten percent salmon oil. The next lowest oxygen uptake was noted with fish on ten percent salmon oil for ten months after four months on fat free diets. Diet 2 fish were on a fat free diet for 14 months. The highest oxygen uptake was noted for fish on ten percent safflower oil (Diet 3) for ten months after four months on a fat free diet.

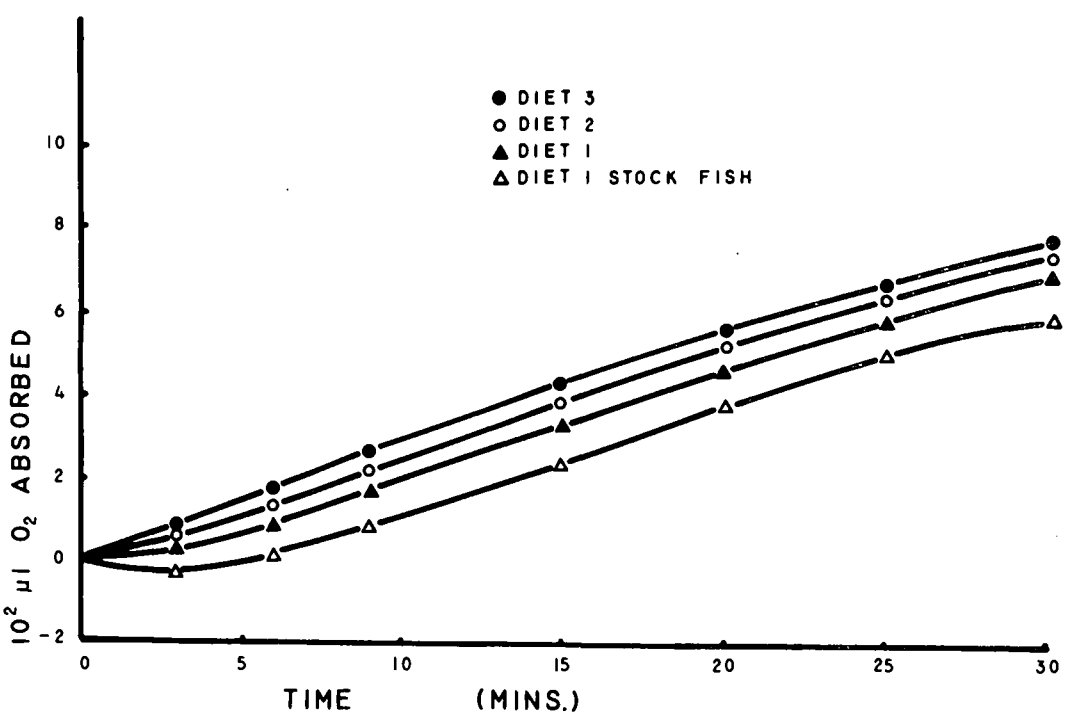


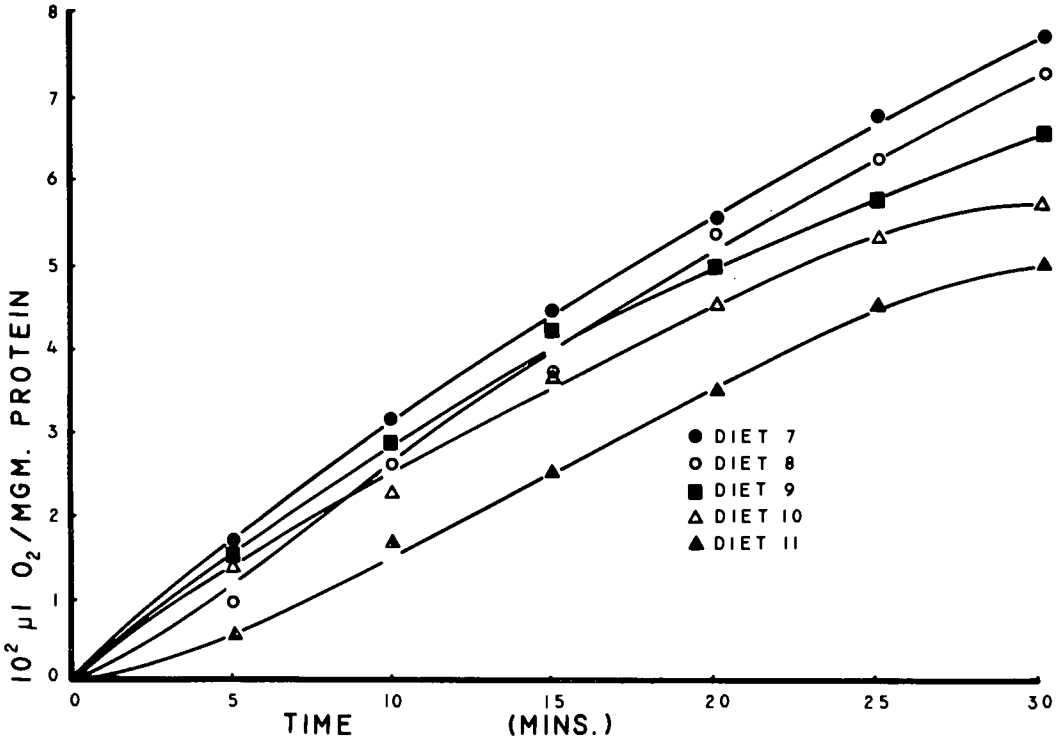

Figure 21. Set 1 liver homogenate respiration in Krebs saline with 20 mM disodium succinate.

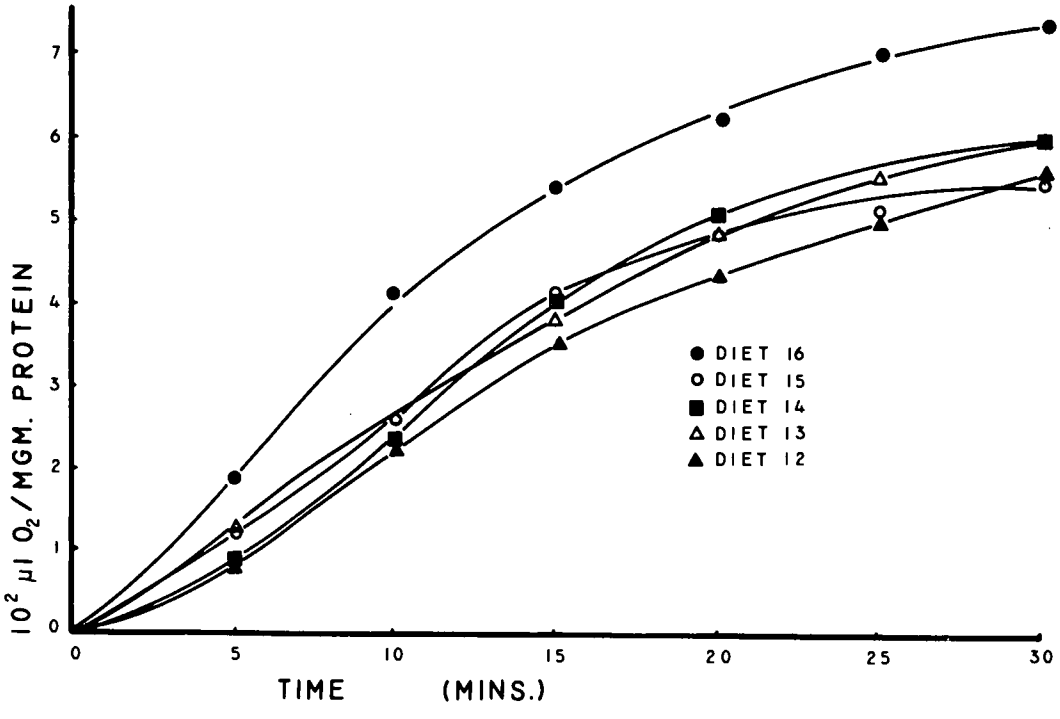
Set 3. The livers from fish on diets in Set 3 were homogenized and allowed to respire at 30 C in 3mM succinate Krebs saline. The oxygen uptake was calculated on the basis of μl per mg of protein. The respiration rate is highest for tissues from fish deficient in 18:3ω3 (Figures 22 and 23, Diets 7 and 16). The respiration rate decreases with increasing 18:3ω3 content in the diet. With one exception, Diet 15 Figure 23, after 15 minutes had absorbed more oxygen than any diet with higher 18:3ω3. However, after 30 minutes it had absorbed less oxygen than any other diet in Figure 23. Each curve is the average of four runs from each diet (two runs from each of two duplicate tanks on each diet).

EFA deficiency in the fish resulted in increased respiration of liver homogenates. These data are similar to the findings of Smithson (139) with rat liver homogenates. However, in the rats, adding a source of linoleate to the diet caused a lowering of respiration rates. In the fish, linoleate resulted in even higher respiration rates than the fat free diets. In the case of the fish, feeding linolenate or fish oil brought about a lower liver homogenate respiration rate. Hayashida and Portman (70) reported EFA deficiency in rats caused an increase in succinic dehydrogenase activity in the liver mitochondria. Since the respiration media used in this study contained 3mM succinate, it is possible that the increased respiration rate reflects a similar increase in the activity of this enzyme system in the fish. In the work

Figure 22. Set 3 liver homogenate respiration. Diets 7-11 containing 18:3 and 12:0 but no 18:2.

Figure 23. Set 3 liver homogenate respiration. Diets 12-16 containing one percent 12:0 and mixed 18:2 + 18:3 = 1%.


$10^2$ µl $O_2$/MGM. PROTEIN
TIME (MINS.)
DIET 7
DIET 8
DIET 9
DIET 10
DIET 11


$10^2$ µl $O_2$/MGM. PROTEIN
TIME (MINS.)
DIET 16
DIET 15
DIET 14
DIET 13
DIET 12

with rats, fatty acids of the linoleic family gave lower activity than those of the linolenic family. Both of these groups had lower activity than rats on a fat free diet. Again it appears as though linolenic acid plays a similar role in fish, to that of linoleic acid in rats and several other animals. Linoleate in some ways appears to stress fish even more than a fat free diet.

## Flesh Water Content

Ten fish from each diet in Set 3 were analyzed for water after 18 weeks on the diets. The average values with the 95 percent confidence limits as calculated by the sums of the squares of error are given in Table 3. Lowering the 18:3ω3 content in the diet increases the percent water in the flesh. Presence of 18:2ω6 results in a lower moisture content than ethyl laurate alone. Analysis of variance of the water data indicates that Diets 7, 8, 9 and 16 are all significantly higher, at the 95 percent confidence level, than Diets 10 through 13.

Considering present theories on deficiency and permeability, it does not seem unreasonable to postulate that the higher levels of moisture in the flesh result from increased cell membrane permeability due to EFA deficiency. If this is the case, linoleate does not prevent EFA deficiency. For rainbow trout it appears as though fatty acids of the linolenate family are essential fatty acids.

Table 3. Water content of muscle tissue.

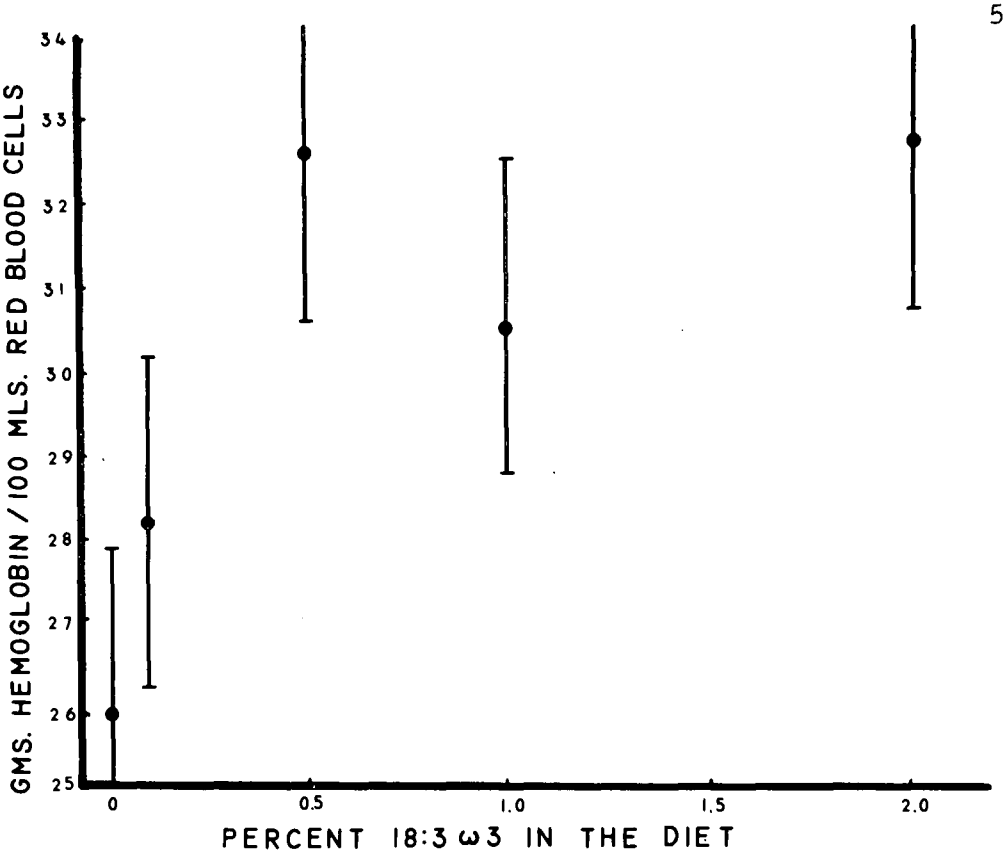
| Diet | Percent 18:3 | 18:2 | 12:0 | Moisture |
|---|---|---|---|---|
| 7 | 0 | 0 | 0 | 80.12 ± 0.45 |
| 8 | 0.1 | 0 | 1.9 | 79.46 ± 0.48 |
| 9 | 0.5 | 0 | 1.5 | 79.34 ± 0.22 |
| 10 | 1.0 | 0 | 1.00 | 78.55 ± 0.07 |
| 11 | 2.0 | 0 | 0 | 78.34 ± 0.27 |
| 12 | 0.9 | 0.1 | 1.0 | 78.64 ± 0.28 |
| 13 | 0.7 | 0.3 | 1.0 | 78.78 ± 0.14 |
| 14 | 0.5 | 0.5 | 1.0 | 79.13 ± 0.63 |
| 15 | 0.3 | 0.7 | 1.0 | 79.17 ± 0.03 |
| 16 | 0.0 | 1.0 | 1.0 | 79.23 ± 0.14 |

## Blood Hemoglobin

Hematocrit (or packed red blood cell volume) and hemoglobin content were determined in the blood of ten fish from each diet in Set 3 at the same time as the flesh moisture was being determined. Table 4 gives average hematocrit and hemoglobin analysis of each diet with the sum of the squares of error 95 percent confidence limits. Analysis of variance performed on the calculated hemoglobin content in the red blood cells gives a least significant difference of 4.04 at the 95 percent confidence limit. The results (Table 4, Figures 24 and 25) show a definite lowering of the hemoglobin content of the red blood cells when 18:3ω3 is excluded from the diet. It can also be seen that more analyses must be run before a mathematical relationship between 18:3ω3 and hemoglobin content of red blood cells can be derived.

Figure 24. Hemoglobin content of red blood cells versus dietary linolenate content. Diets 7-11.

Figure 25. Hemoglobin content of red blood cells versus dietary linolenate content. Diets 12-16.


GMS. HEMOGLOBIN / 100 MLS. RED BLOOD CELLS
34
33
32
31
30
29
28
27
26
25
0
0.5
1.0
1.5
2.0
PERCENT 18:3 ω3 IN THE DIET

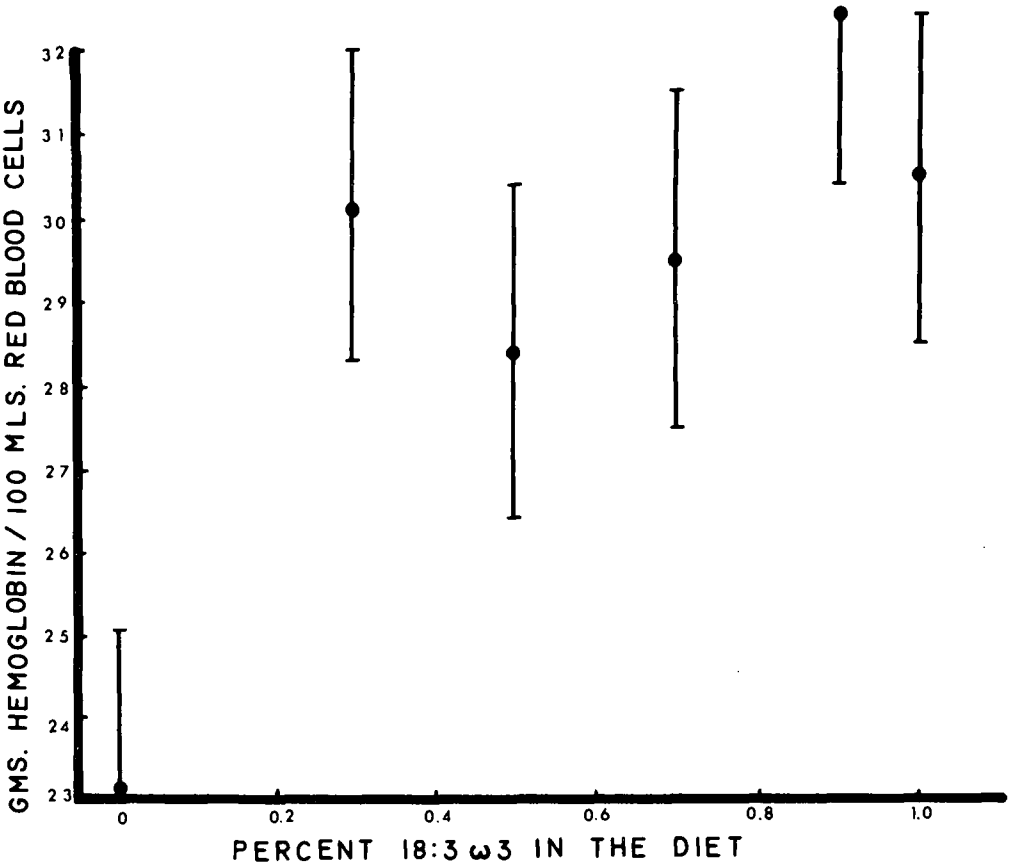

GMS. HEMOGLOBIN / 100 MLS. RED BLOOD CELLS
32
31
30
29
28
27
26
25
24
23
0
0.2
0.4
0.6
0.8
1.0
PERCENT 18:3 ω3 IN THE DIET

Table 4. Hematocrit and hemoglobin levels.

| Diet | Hematocrit | Hemoglobin gm/100 ml Blood | Hemoglobin gm/100 ml Red Blood Cells |
|---|---|---|---|
| 7 | 38.8 ± 4.5 | 10.03 ± 1.11 | 25.9 |
| 8 | 42.5 ± 4.1 | 11.98 ± 0.58 | 28.2 |
| 9 | 39.2 ± 2.8 | 12.79 ± 1.08 | 32.6 |
| 10 | 40.5 ± 3.5 | 12.34 ± 0.98 | 30.5 |
| 11 | 37.8 ± 4.2 | 12.36 ± 0.86 | 32.7 |
| 12 | 38.9 ± 2.2 | 12.59 ±0.92 | 32.4 |
| 13 | 41.8 ± 4.3 | 12.35 ± 0.67 | 29.5 |
| 14 | 42.2 ± 4.1 | 11.97 ± 0.41 | 28.4 |
| 15 | 41.1 ± 5.4 | 12.36 ± 0.93 | 30.1 |
| 16 | 48.5 ± 5.6 | 11.22 ± 1.12 | 23.1 |

Snieszko et al. (140) found that the normal hematocrit values for rainbow trout were between 45 and 53. Most of the values reported in this thesis are below that. However, all of these fish were unusually stressed by being fed a fat free diet for the first month that they were eating. Fowler and Wood (56) have already reported lowered hematocrit values in fish on EFA deficient diets. Their values were as low as 22.7, after 22 weeks on a diet high in saturated fat. It is possible that we would have found greater differences if we had analyzed our fish after they had been on the diets for a longer period of time.

Although linoleate gave high hematocrit values, the content of hemoglobin per volume of red blood cells was lower than that of fish receiving linolenate, indicating a tendency toward anemia. This could well be related to decreased activity of certain enzymes in the heme synthesis system. Yoneyama et al. (151) reported that

phospholipids were required for activity of certain enzymes involved in heme synthesis.

## Lipids of Experimental Fish

### Total Lipids

The total body lipid content of our stock rainbow trout fed the ten percent salmon oil diet (Diet 1), as determined by the procedure of Bligh and Dyer, ranged between four and six percent and averaged around five percent of the wet weight of the fish. Knipprath and Mead (94) reported values of 7.1 and 5.9 percent for the muscle of rainbow trout on a diet of slaughter house scraps and ocean fish. Removal of added lipids from the diet for six months gave a lower lipid content of the total fish. Values range from 3.4 to 5.5 percent. This is visibly evident by the greatly reduced adipose tissue.

Set 1. After one month on the diets of Set 1 the lipid content of six evicerated fish from each diet was found to be:

| | | |
|---|---|---|
| 10% Salmon Oil | Diet 1 | 4.7% |
| Fat Free | Diet 2 | 3.4% |
| 10% Safflower Oil | Diet 3 | 6.0% |

Set 2. The fish used in Set 2 were younger at the beginning of the experiment than those used in Set 1. The average initial weight being 11.4 gm as opposed to 35 gm for Set 1. These fish started out with an average lipid content of about two percent. In Table 5 the lipid

content of evicerated fish from Set 2 after three, six and twelve weeks on the various diets are given. Three fish from each tank were extracted for each analysis. There were duplicate tanks on each diet. In all cases the lipid content of the fish increases with age even in the low fat diet (Diet 2). The addition of five percent 18:1, four percent 18:1 plus one percent 18:2ω6 or four percent 19:3ω3, Diets 4, 5 and 6 respectively, results in increased lipid deposition in the body compared with the fat free diet (Diet 2).

Table 5. Total lipid content of evicerated fish.

| Diet | Percent Lipid | | |
|---|---|---|---|
| | Three weeks average | Six weeks average | Twelve weeks average |
| 2 fat free | 1.32 | 2.20 | 4.59 |
| 4 ω9 | 1.74 | 3.17 | 8.56 |
| 5 ω6 | 1.72 | 6.49 | 8.42 |
| 6 ω3 | 3.18 | 6.21 | 7.14 |

Adding 18:3ω3 results in a faster initial build up of lipid than any of the other diets in Set 2. However, both 18:1 alone and 18:1 plus 18:2 results in a higher level of body lipid after 12 weeks.

Set 3. Increased body lipid in Sets 1 and 2 might have been due to increased caloric intake. After 12 weeks on the diets of Set 3 there did not appear to be any significant differences in percent total body lipid (Table 6). These diets were all iso-caloric, each containing

two percent lipid. There were differences in the proportion of phospholipid. Increasing 18:3ω3 from Diet 7 to Diet 11 resulted in increased phospholipid. The phospholipid decreased from Diet 12 to 16 with increasing 18:2ω6 and decreasing 18:3ω3.

Table 6. Lipid content of total body of Set 3 fish after 12 weeks on diets. Percent:

| Diet | Body Lipid | Average | Phospholipid | Average | Neutral Lipid | Average |
|---|---|---|---|---|---|---|
| 7 | 2.39<br>4.39 | 3.39 | 30.7<br>23.5 | 27.1 | 69.3<br>76.5 | 72.9 |
| 8 | 4.03<br>3.81 | 3.92 | 20.8<br>22.4 | 21.6 | 79.2<br>77.6 | 78.4 |
| 9 | 2.91<br>4.09 | 3.50 | 32.3<br>21.0 | 26.7 | 67.7<br>79.0 | 73.3 |
| 10 | 2.74<br>3.51 | 3.12 | 40.1<br>30.6 | 35.4 | 59.9<br>69.4 | 64.6 |
| 11 | 3.86<br>3.25 | 3.06 | 38.4<br>30.0 | 34.2 | 61.6<br>70.0 | 65.8 |
| 12 | 2.68<br>2.88 | 2.78 | 38.7<br>30.2 | 34.5 | 61.3<br>69.8 | 65.5 |
| 13 | 3.30<br>2.98 | 3.14 | 34.9<br>27.4 | 31.2 | 65.1<br>72.6 | 68.8 |
| 14 | 3.18<br>3.06 | 3.12 | 29.4<br>35.2 | 32.3 | 70.6<br>64.8 | 67.7 |
| 15 | 3.13<br>2.75 | 2.94 | 30.6<br>31.7 | 31.2 | 69.4<br>68.3 | 68.8 |
| 16 | 2.69<br>3.20 | 2.95 | 28.2<br>26.6 | 27.4 | 71.8<br>73.4 | 72.6 |

Liver Lipids

Set 1. The dietary lipids also affected the lipid content of the liver. Adding ten percent safflower oil to the fish in Set 1 (Diet 3) raised the liver lipid and lowered the percent phospholipid (Table 7). The salmon oil diet had about the same percent total lipid but with a greater proportion of phospholipid.

Table 7. Set 1 lipid content of livers.

| Diet | Percent Total Lipid | Phospholipid | Neutral Lipid |
|---|---|---|---|
| 1 Salmon oil | 2.52 | 75.4 | 24.6 |
| 2 Fat Free | 2.44 | 56.4 | 43.6 |
| 3 Safflower oil | 3.70 | 48.5 | 51.5 |

Set 2. The picture with the younger fish used in Set 2 was somewhat different, Table 8. The low fat fish had very fatty livers and adding either 18:2ω6 or 18:3ω3 lowered this lipid level considerably after six weeks. The linolenic acid resulted in the lower liver lipid content.

Table 8. Liver lipid content of fish from Set 2 fish.

| Diet | Six weeks average | Twelve weeks average |
|---|---|---|
| 2 | 10.09 | 9.28 |
| 4 | 9.58 | 7.95 |
| 5 | 2.73 | 6.04 |
| 6 | 1.82 | 4.53 |

In order to evaluate the effect of the dietary lipids on other vital organs samples of brain, heart, kidney and liver were taken from ten fish on each diet of Set 2 fish, after 24 weeks. Although the six and twelve week sample showed the livers of fish on a fat free diet to be very high in lipid, the 24 week samples (Table 9) had much lower lipid levels. The liver lipid levels of Diets 4, 5 and 6 had not varied greatly between 12 and 24 weeks. After 24 weeks on the diets there appeared to be differences in lipid content which were not directly related to calories alone. Table 9 shows that one percent 18:3ω3 plus four percent 18:1ω9 (Diet 6) resulted in the highest level of kidney lipid. Brain lipid was higher in all fish fed five percent lipid than in the fish fed the low fat diet. No significant difference was noted in the total lipid content of hearts from fish on each diet. Adding a non-essential fatty acid such as oleic acid should increase the triglyceride and thus neutral lipid. This was the case in the liver, kidney, and heart but not in brain. Since the essential fatty acids are concentrated in the phospholipids, one might expect to find higher phospholipid in fish receiving ω3 fatty acids. This was found to be the case in total body lipids of fish from Set 3 Table 6. In all the vital organs examined (Table 9) except the liver, ω6 fatty acids resulted in higher phospholipid content and ω3 fatty acids lowered it.

Table 9. Lipid content of liver, kidney, brain and heart.

| Organ | Diet | % Lipid | Phospholipid | Neutral Lipid |
|---|---|---|---|---|
| Liver | 2 | 5.46 | 77.6 | 22.4 |
| | 4 | 8.42 | 38.0 | 62.0 |
| | 5 | 6.74 | 54.5 | 45.5 |
| | 6 | 4.30 | 68.9 | 31.1 |
| Kidney | 2 | 3.23 | 49.7 | 50.3 |
| | 4 | 4.40 | 32.3 | 67.7 |
| | 5 | 3.69 | 43.3 | 56.7 |
| | 6 | 6.88 | 34.6 | 65.4 |
| Brain | 2 | 5.08 | 66.3 | 33.7 |
| | 4 | 7.40 | 66.9 | 33.1 |
| | 5 | 9.58 | 71.3 | 28.7 |
| | 6 | 8.75 | 58.5 | 41.5 |
| Heart | 2 | 2.10 | 60.0 | 40.0 |
| | 4 | 3.46 | 53.7 | 46.3 |
| | 5 | 2.69 | 64.8 | 35.2 |
| | 6 | 3.54 | 54.1 | 45.9 |

Blood Cholesterol Levels of Set 1

Blood samples were taken by hypodermic syringe from the hearts of ten fish on each of the diets in Set 1 after 24 weeks. After centrifugation to remove the red blood cells the serum cholesterol levels were determined.[5] Table 10 shows the average total and free cholesterol with the 95 percent confidence limits calculated by the method of sums of squares of error. The lowest cholesterol levels

[5] Appreciation is expressed to George Putman for performing these analysis.

were found in fish receiving the fat free diet and the highest in those fed the salmon oil diet. Oils or lipids high in ω3 fatty acids have generally been found to be the most effective in lowering blood cholesterol in other animals. It is surprising that the salmon oil diet resulted in the highest cholesterol levels. This might be the result of the cholesterol in the salmon oil. Safflower oil does not contain cholesterol.

Table 10. Average cholesterol levels in blood plasma.

| Diet | Total Cholesterol mg/100 ml | % Free Cholesterol |
|---|---|---|
| 1 Salmon oil | 234 ± 46 | 44.2 ± 3.3 |
| 2 Fat free | 151 ± 43 | 42.4 ± 2.4 |
| 3 Safflower oil | 171 ± 52 | 43.4 ± 9.6 |

Fatty Acid Analysis

Set 2. The GLC analysis of phospholipid and neutral lipids from liver, heart, brain and kidney of fish from Set 2 are given in Tables 11 to 18. There is a much greater incorporation of 20 and 22 carbon chain unsaturated fatty acids into the phospholipids of all these organs than into the neutral lipids. Fish fed very low fat (Diet 2) or diet high in oleic acid (Diet 4) incorporate high levels of 20:3ω9 into the phospholipids of all organs studied except the brain. Adding either ω3 or ω6 to the diet is sufficient to give a 4-16 fold reduction in the

Table 11. Liver phospholipid fatty acid composition.

| Fatty Acid[a] | Ret. Rt.[b] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|---|
| 14:0 | 0.309 | 1.6 | 1.8 | 1.1 | 1.2 |
| 16:0 | 0.550 | 12.7 | 12.9 | 13.4 | 13.2 |
| 16:1ω7[c] | 0.660 | 11.1 | 9.9 | 4.3 | 5.9 |
| 18:0 | 1.000 | 5.0 | 4.6 | 5.6 | 5.2 |
| 18:1ω9[c] | 1.170 | 30.3 | 33.7 | 19.3 | 21.1 |
| 18:2ω9 | 1.370 | 1.1 | ---- | ---- | ---- |
| 18:2ω6 | 1.440 | 2.1 | 2.1 | 2.3 | 1.3 |
| Unk | 1.640 | 0.8 | ---- | ---- | ---- |
| 18:3ω6 | 1.770 | ---- | ---- | 0.2 | ---- |
| 18:4ω3 | 2.300 | 0.9 | 0.6 | ---- | ---- |
| 18:3ω3<br>20:1ω11[c] | 2.020 | 5.0 | 5.6 | 3.0 | 3.8 |
| 20:2ω9 | 2.400 | 3.2 | 3.9 | 2.0 | 1.2 |
| 20:3ω9 | 2.740 | 16.6 | 16.8 | 3.1 | 2.3 |
| 20:3ω6 | 2.990 | ---- | ---- | 2.2 | ---- |
| 20:4ω6 | 3.380 | ---- | ---- | 12.6 | 0.7 |
| 22:1ω11 | 3.420 | 2.0 | 2.7 | ---- | 0.6 |
| 20:5ω3 | 4.500 | ---- | ---- | ---- | 2.1 |
| 22:4ω9 | 5.550 | 1.3 | ---- | ---- | ---- |
| 22:4ω6 | 5.850 | ---- | ---- | 1.0 | ---- |
| 22:4ω3 | 5.97 | ---- | ---- | ---- | 1.2 |
| 22:5ω6 | 6.700 | 1.6 | 2.0 | 24.3 | ---- |
| 22:5ω3 | 7.140 | ---- | ---- | ---- | 1.2 |
| 22:6ω3 | 9.200 | 4.7 | 3.4 | 5.6 | 39.0 |
| ω9 | | 52.5 | 54.5 | 24.4 | 24.6 |
| ω6 | | 3.7 | 4.1 | 42.6 | 2.0 |
| ω3 | | 5.6 | 4.0 | 5.6 | 43.5 |
| 20:3ω9/22:5ω6 | | 10.4 | 8.4 | 0.13 | ---- |
| 20:3ω9/22:6ω3 | | 3.5 | 4.9 | 0.55 | 0.05 |
| Dietary lipid | | Fat free | 5%18:1 | 4%18:1<br>1%18:2 | 4%18:1<br>1%18:3 |

[a] Fatty Acids less than 0.1% are not reported

[b] Retention times relative to 18:0 on 12' x 1/8" stainless steel column packed with 15% DEGS on Chromosorb GAW, DMCS treated 100/120 mesh, at 190°C, 20 ml/min $N_2$ carrier Gas.

[c] Other isomers may be present.

Table 12. Liver neutral lipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 3.2 | 3.1 | 2.1 | 1.7 |
| 15:0 | 0.4 | 0.6 | 0.1 | ---- |
| 16:0 | 5.6 | 9.8 | 10.9 | 11.4 |
| 16:1ω7[b] | 17.8 | 16.3 | 10.6 | 9.0 |
| 16:2ω7 | ---- | 0.7 | 0.2 | 0.4 |
| 18:0 | 3.6 | 3.7 | 3.1 | 2.9 |
| 18:1ω9[b] | 61.3 | 58.2 | 55.8 | 52.0 |
| 18:2ω6 | 1.0 | 1.7 | 3.2 | 2.9 |
| 18:3ω6 | ---- | ---- | 0.2 | ---- |
| 18:3ω3<br>20:1 | 4.7 | 4.1 | 5.8 | 7.6 |
| 18:4ω3 | ---- | ---- | ---- | 3.8 |
| 20:2ω9 | 1.6 | 1.2 | 3.6 | ---- |
| 20:3ω9 | 0.4 | ---- | 0.4 | 0.5 |
| 20:3ω6 | ---- | ---- | 0.3 | ---- |
| 20:4ω6 | 0.4 | 0.6 | 2.7 | ---- |
| 22:5ω6 | ---- | ---- | 0.9 | ---- |
| 22:6ω3 | ---- | ---- | ---- | 9.8 |
| ω9 | 63.3 | 59.4 | 59.8 | 52.5 |
| ω6 | 1.4 | 2.3 | 7.3 | 0.9 |
| ω3 | ---- | ---- | ---- | 13.6 |
| 20:3ω9/22:5ω6 | ---- | ---- | 0.44 | ---- |
| 20:3ω9/22:6ω3 | ---- | ---- | ---- | 0.05 |
| Dietary lipid | Fat Free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 0.1% are not reported.

[b]Other isomers may be present.

Table 13. Heart phospholipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 3.1 | 3.2 | 3.6 | 2.5 |
| 15:0 | 0.4 | ---- | ---- | ---- |
| 16:0 | 17.2 | 12.4 | 15.2 | 19.8 |
| 16:1ω7[b] | 9.5 | 5.9 | 4.8 | 4.4 |
| 16:2ω7 | ---- | 0.4 | ---- | 0.2 |
| 18:0 | 3.6 | 4.1 | 6.0 | 5.4 |
| 18:1ω9[b] | 25.8 | 32.1 | 20.0 | 23.8 |
| 18:2ω6 | 1.9 | 2.7 | 5.4 | 1.4 |
| A | 0.6 | ---- | ---- | ---- |
| 18:3ω3<br>20:1ω11[b] | 1.7 | 3.0 | ---- | 3.3 |
| 18:4ω3 | ---- | ---- | ---- | 2.3 |
| 20:2ω9 | 2.4 | 4.6 | 1.3 | ---- |
| 20:3ω9 | 16.9 | 18.1 | 2.2 | 2.0 |
| 20:3ω6 | ---- | 1.8 | 2.0 | ---- |
| 20:4ω6 | 1.9 | 4.8 | 10.9 | 0.7 |
| 20:4ω3 | ---- | ---- | ---- | 0.4 |
| 20:5ω3 | 1.0 | ---- | ---- | 4.3 |
| 22:4ω9 | 0.8 | 0.9 | ---- | ---- |
| 22:4ω6 | 0.4 | ---- | 3.0 | ---- |
| 22:5ω6 | 2.8 | 2.5 | 22.0 | 0.4 |
| 22:5ω3 | | ---- | | 0.5 |
| 22:6ω3 | 10.0 | 3.5 | 3.6 | 28.6 |
| ω9 | 45.9 | 55.7 | 23.5 | 25.8 |
| ω6 | 7.0 | 11.8 | 43.3 | 2.5 |
| ω3 | 11.0 | 3.5 | 3.6 | 36.1 |
| 20:3ω9/22:5ω6 | 6.04 | 7.24 | 0.10 | ---- |
| 20:3ω9/22:6ω3 | 1.69 | 5.17 | 0.61 | 0.07 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty Acids less than 0.1% are not reported.

[b]Other isomers may be present.

Table 14. Heart neutral lipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 2.6 | 3.7 | 3.2 | 2.4 |
| 14:1 | 0.3 | 0.4 | 0.5 | 0.5 |
| 15:0 | 0.3 | 0.3 | ---- | ---- |
| 16:0 | 19.9 | 14.1 | 15.4 | 13.7 |
| 16:1ω7[b] | 13.3 | 11.8 | 12.1 | 11.6 |
| 16:2ω7 | 0.1 | 0.4 | 0.5 | 0.6 |
| 18:0 | 5.1 | 2.7 | 4.2 | 3.1 |
| 18:1ω9[b] | 50.4 | 57.6 | 53.2 | 56.4 |
| 18:2ω6 | 1.4 | 0.8 | 5.3 | 0.9 |
| 18:3ω6 | 0.1 | ---- | 0.6 | ---- |
| 18:3ω3<br>20:1ω11[b] | 3.1 | 3.0 | 2.4 | 4.3 |
| 18:4ω3 | ---- | 0.7 | ---- | 3.4 |
| 20:2ω9 | 1.5 | 1.8 | 1.3 | ---- |
| 20:3ω9 | 1.8 | 1.9 | 0.2 | ---- |
| 20:4ω6 | 0.1 | 0.8 | 1.1 | ---- |
| 22:5ω6 | ---- | ---- | 0.9 | ---- |
| 22:6ω3 | ---- | ---- | ---- | 3.1 |
| ω9 | 53.7 | 61.3 | 55.8 | 56.4 |
| ω6 | 1.6 | 1.6 | 7.9 | 0.9 |
| ω3 | ---- | ---- | ---- | 6.5 |
| 20:3ω9/22:5ω6 | ---- | ---- | 0.22 | 0.0 |
| 20:3ω9/22:6ω3 | ---- | ---- | ---- | 0.0 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 0.1% are not reported.

[b]Other isomers may be present.

Table 15. Brain phospholipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 1.5 | 0.8 | 0.5 | 0.7 |
| 16:0 | 20.1 | 19.8 | 19.2 | 20.9 |
| 16:1ω7[b] | 8.2 | 7.7 | 5.6 | 5.3 |
| 16:2ω7 | 0.4 | 0.5 | 0.4 | ---- |
| 18:0 | 5.9 | 7.3 | 7.5 | 8.2 |
| 18:1ω9[b] | 24.8 | 31.6 | 26.4 | 26.3 |
| 18:2ω6 | 0.7 | 1.2 | 0.9 | 0.7 |
| 18:2ω3<br>20:1ω11[b] | 4.9 | 4.6 | 3.1 | 3.1 |
| 20:2ω9 | 0.9 | 1.1 | 0.7 | 0.4 |
| 20:3ω9 | 5.3 | ---- | ---- | ---- |
| 22:1ω13 | 2.9 | 4.6 | 2.1 | 1.3 |
| 20:4ω6 | ---- | 3.3 | 6.3 | ---- |
| 20:5ω3 | 1.6 | ---- | ---- | 4.2 |
| 22:4ω6 | ---- | 1.5 | 0.8 | ---- |
| 22:4ω3 | 4.3 | ---- | ---- | 5.4 |
| 22:5ω6 | 1.7 | 3.6 | 14.8 | ---- |
| 22:5ω3 | 1.0 | ---- | ---- | 1.7 |
| 22:6ω3 | 15.8 | 12.4 | 11.7 | 21.8 |
| ω9 | 31.0 | 32.7 | 27.1 | 26.7 |
| ω6 | 2.4 | 9.6 | 22.8 | 0.7 |
| ω3 | 22.7 | 12.4 | 11.7 | 33.1 |
| 20:3ω9/22:5ω6 | 5.30 | 0.0 | 0.0 | 0.0 |
| 20:3ω9/22:6ω3 | 0.34 | 0.0 | 0.0 | 0.0 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 0.1% are not reported.

[b]Other isomers may be present.

Table 16. Brain neutral lipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 5.0 | 3.9 | 2.7 | 2.7 |
| 14:1ω5 | 0.6 | 0.6 | 0.5 | 0.7 |
| 16:0 | 8.4 | 14.1 | 15.0 | 14.5 |
| 16:1ω7[b] | 8.4 | 12.3 | 11.8 | 10.4 |
| 16:2ω7 | ---- | ---- | ---- | 0.7 |
| 18:0 | 5.4 | 3.3 | 4.0 | 4.6 |
| 18:1ω9[b] | 48.2 | 54.7 | 50.4 | 54.3 |
| 18:2ω6 | 1.1 | 1.5 | 4.5 | 2.8 |
| 18:3ω6 | ---- | ---- | 0.8 | ---- |
| 18:3ω3<br>20:1ω11[b] | 4.1 | 2.7 | 1.7 | 4.6 |
| 20:2ω9 | 2.2 | 0.6 | 0.7 | ---- |
| 20:3ω9 | 1.9 | ---- | ---- | ---- |
| 22:1ω13 | 1.5 | 0.8 | 1.0 | 3.0 |
| 20:5ω3 | ---- | ---- | ---- | 0.5 |
| 22:6ω3 | 1.5 | ---- | ---- | 1.2 |
| Unknown[c] | 11.7 | 5.5 | 6.9 | ---- |
| ω9 | 52.3 | 55.3 | 51.1 | 54.3 |
| ω6 | 1.1 | 1.5 | 5.3 | 2.8 |
| ω3 | 1.5 | ---- | ---- | 1.7 |
| 20:3ω9/22:5ω6 | ---- | ---- | ---- | ---- |
| 20:3ω9/22:6ω3 | 1.26 | ---- | ---- | 0.0 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 1.0% are not recorded.

[b]Other isomers may be present.

[c]Unidentified fatty acid, methyl ester has relative retention time of 10.52.

Table 17. Kidney phospholipid fatty acid composition.

| Fatty Acid[a] | Percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 14:0 | 1.7 | 1.8 | 1.5 | 1.3 |
| 15:0 | 0.1 | 0.1 | 0.2 | 0.2 |
| 15:1 | 0.1 | 0.1 | 0.2 | ---- |
| 16:0 | 15.1 | 17.5 | 19.4 | 22.8 |
| 16:1ω7[b] | 9.3 | 9.8 | 6.3 | 4.9 |
| 16:2ω7 | 0.2 | 0.1 | 0.3 | 0.1 |
| 18:0 | 4.3 | 4.2 | 5.3 | 4.8 |
| 18:1ω9[b] | 27.7 | 31.8 | 25.3 | 23.4 |
| 18:2ω6 | 1.1 | 1.4 | 4.2 | 1.2 |
| 18:3ω6 | 0.6 | ---- | 0.4 | ---- |
| 18:3ω3<br>20:1ω11[b] | 2.7 | 3.6 | 1.2 | 2.5 |
| 20:2ω9 | 2.9 | 3.4 | 1.2 | 2.1 |
| 20:3ω9 | 16.2 | 15.9 | 1.8 | 1.7 |
| 20:3ω6 | ---- | ---- | 3.3 | ---- |
| 20:3ω3 | ---- | ---- | ---- | 1.4 |
| 20:4ω6 | 3.8 | 5.2 | 12.7 | ---- |
| 20:4ω3 | ---- | ---- | ---- | 2.5 |
| 20:5ω3 | 1.2 | ---- | ---- | 4.8 |
| 22:4ω9 | 2.1 | 1.2 | ---- | ---- |
| 22:4ω6 | ---- | ---- | 0.6 | ---- |
| 22:5ω6 | 2.5 | 2.0 | 12.0 | 0.8 |
| 22:5ω3 | ---- | ---- | ---- | 1.8 |
| 22:6ω3 | 8.4 | 1.9 | 4.1 | 23.7 |
| ω9 | 48.9 | 52.3 | 28.3 | 27.2 |
| ω6 | 4.2 | 8.6 | 33.2 | 2.0 |
| ω3 | 8.4 | 1.9 | 4.1 | 34.2 |
| 20:3ω9/22:5ω6 | 6.48 | 7.85 | 0.15 | 2.13 |
| 20:3ω9/22:6ω3 | 1.93 | 8.37 | 0.44 | 0.07 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 0.1% are not recorded.

[b]Other isomers may be present.

Table 18. Kidney neutral lipid fatty acid composition.

| Fatty Acid[a] | percent in Diet 2 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|
| 12:0 | 0.3 | 0.1 | 0.2 | 0.1 |
| 14:0 | 3.7 | 3.6 | 4.0 | 2.5 |
| 14:1 | 0.6 | 1.0 | 0.6 | 0.4 |
| 16:0 | 17.7 | 14.3 | 17.0 | 15.9 |
| 16:1ω7[b] | 16.1 | 12.9 | 14.6 | 10.6 |
| 16:2ω7 | ---- | 0.1 | 0.5 | 0.3 |
| 18:0 | 3.4 | 2.5 | 3.6 | 3.4 |
| 18:1ω9[b] | 49.7 | 56.2 | 48.9 | 54.1 |
| 18:2ω6 | 0.6 | 2.4 | 4.1 | 2.7 |
| 18:3ω6 | 0.1 | ---- | 1.1 | ---- |
| 18:3ω3<br>20:1ω11[b] | 4.7 | 3.7 | 2.7 | 5.4 |
| 20:2ω9 | 0.8 | 0.6 | ---- | ---- |
| 22:1ω13 | 0.3 | 0.4 | 0.5 | ---- |
| 22:5ω6 | ---- | ---- | 0.9 | ---- |
| 22:6ω3 | 0.6 | ---- | ---- | 0.9 |
| ω9 | 51.9 | 58.9 | 50.3 | 57.8 |
| ω6 | 0.7 | 2.4 | 6.1 | 2.7 |
| ω3 | 0.6 | ---- | ---- | 0.9 |
| 20:3ω9/22:5ω6 | ---- | ---- | 0.0 | ---- |
| 20:3ω9/22:6ω3 | 1.33 | ---- | ---- | 0.0 |
| Dietary lipid | Fat free | 5% 18:1 | 4% 18:1<br>1% 18:2 | 4% 18:1<br>1% 18:3 |

[a]Fatty acids less than 0.1% are not reported.

[b]Other isomers may be present.

percent of 20:3ω9 in the phospholipids of the various organs studied, including the brain. It is very interesting to note that the brain has only very low levels of ω9 fatty acids in all cases (Table 15 and 16). Addition of ω6 or ω3 fatty acids to the diet completely prevents the formation or deposition of 20:3ω9 into brain lipids. Even Diet 4, which is very high in 18:1ω9 and has only traces of ω6 and ω3 fatty acids, prevented 20:3ω9 deposition in the brain. This is especially surprising because the 20:3ω9 levels of all other organs from fish on Diet 4 were either equal to that of the fat free fish or higher, as in the case of heart phospholipid. The increase in 20:3ω9 caused by the EFA deficient diets noted in this thesis further substantiates the findings of other workers with rats (35, 114, 133), chicks (74), rabbits (21) and fish (36). For most animals studied, in which fatty acids of the linoleic family satisfies the EFA requirement, Holman and his co-workers (72, 114) have suggested that the ratio of 20:3ω9/20:4ω6 in the animal's lipids be taken as an index of EFA deficiency. A ratio cannot be applied to fish lipids. Most fish have very low concentrations of fatty acids of the linoleic family and by this ratio would be judged to be EFA deficient. Alfin-Slater and Aftergood (25) in their review include fatty acids of the linolenate family in the classification of "Essential Fatty Acids." They propose the ratio of 20:3ω9/20:5ω3 as an estimate of the adequacy of linolenate intake in the diet. A ratio of 0.4 or less indicates that sufficient fatty acids of the ω3 family are

metabolized to depress the synthesis of 20:3ω9 from 18:1ω9. However, in fish the predominant polyunsaturated fatty acid is docosahexaenoic acid (22:6ω3) when acids of the linolenic family are fed or docosapentaenoic acid (22:5ω6) when acids of the linoleic family are fed. Thus in fish it might be appropriate to look at the ratio of trienoic to pentaenoic (20:3ω9/22:5ω6), or trienoic to hexaenoic (20:3ω9/22:6ω3). The polyunsaturated fatty acids are deposited in the phospholipids to a much greater extent than in the neutral lipids. The 20:3ω9/22:5ω5 or 20:3ω9/22:6ω3 ratios will thus be of greater interest for phospholipids. In Tables 11, 13, 15 and 17 either of these last two ratios would predict that Diets 2 and 4 were deficient in both the ω6 and ω3 fatty acids. The ω6 fatty acids do not prevent all of the deficiency symptoms thus a low 20:3ω9/22:5ω6 ratio may indicate depression of 18:1ω9 conversion to a 20 carbon polyunsaturate but it does not indicate absence of EFA deficiency. The linolenic acids series appear to prevent all of the deficiency symptoms and the 20:3ω9/22:6ω3 ratio is the best of the above indexes of deficiency for rainbow trout.

An interesting observation in Tables 11 to 18 is that the sum of all the ω9 fatty acids in the phospholipids is reduced to almost half the value of fat free diet fish by ω6 or ω3 in the diet. In the neutral lipids there is no significant difference in the total ω9 fatty acids between diets. This further substantiates the theory that EFA are selectively

incorporated into the phospholipids.

Set 3. Table 19 gives the GLC analysis of the phospholipid fatty acid methyl esters from the lipid extracted from five whole fish from each tub of each diet in Set 3. Analysis on the BDS capillary column allows resolution of the monoenoic isomers which are unresolved on ordinary packed columns (18). It can be seen that increasing 18:3ω3 from 0-2% (Diets 7-11) reduced the 18:1ω9 formation and incorporation into phospholipids from about 38 percent to 28 percent while the 18:1ω7 was not altered. In Diets 12-16 where the sum of 18:2ω6 plus 18:3ω3 was equal to one percent, changing the 18:3ω3 from 0.9-0.0 resulted in no change in the 18:1ω9 content. It appears that 18:2ω6 and 18:3ω3 are about equivalent in their effectiveness for depressing 18:1ω9 incorporation into the phospholipids. However, it can be seen that linolenate was more effective than linoleate in reducing the 20:3ω9 incorporation or formation.

The content of polyunsaturates in the phospholipids of the fish in Set 3 was surprisingly lower than that of the phospholipids of fish in Set 2. This difference very likely results from the fact that the Set 3 fish, after hatching, were fed a fat free diet for one month. Set 2 fish were fed a 10 percent salmon oil diet for four months after hatching and then fed a low fat diet for an additional three months. Set 3 fish were much younger at the time of sampling and were on the experimental diet for a shorter time than Set 2 fish. In the Set 2 fish

Table 19. Set 3 phospholipid fatty acids.

| F.A. | R.T.[b] | Percent in Diet[a] 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12:0 | 0.12 | 1.2 | 1.8 | 1.9 | 1.5 | ---- | 1.2 | 1.2 | 1.0 | 1.2 | 1.2 |
| UnK. | 0.23 | ---- | ---- | 0.4 | 0.6 | 0.8 | 0.9 | 0.7 | 0.7 | 0.4 | 0.4 |
| 14:0 | 0.25 | 4.4 | 4.8 | 4.3 | 3.4 | 2.5 | 3.3 | 3.2 | 3.3 | 2.8 | 2.8 |
| 14:1ω5 | 0.29 | ---- | ---- | 0.5 | 0.5 | 0.6 | 0.8 | 0.5 | 0.7 | 0.4 | 0.4 |
| UnK. | 0.41 | 0.2 | 0.5 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 | 0.4 | 1.0 | 0.6 |
| 16:0 | 0.50 | 23.3 | 25.9 | 28.5 | 29.3 | 31.9 | 30.8 | 29.7 | 27.9 | 24.1 | 23.3 |
| 16:1ω9 | 0.55 | 4.4 | 5.1 | 3.3 | 2.9 | 1.7 | 2.8 | 3.2 | 3.2 | 3.5 | 3.5 |
| 16:1ω7 | 0.56 | 8.3 | 8.8 | 8.9 | 7.7 | 5.5 | 8.4 | 8.6 | 8.4 | 8.0 | 7.5 |
| 16:1ω5 | 0.60 | ---- | ---- | ---- | 0.1 | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.4 |
| 16:2ω7 | 0.62 | ---- | ---- | 0.2 | 0.3 | 0.4 | 0.5 | 0.4 | 0.5 | 0.4 | 0.5 |
| UnK. | 0.95 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.2 | 0.4 | 0.3 |
| UnK. | 0.97 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.1 | 0.3 | ---- |
| 18:0 | 1.00 | 4.3 | 4.2 | 4.4 | 5.0 | 6.0 | 4.7 | 4.5 | 4.8 | 5.8 | 5.3 |
| 18:1ω9 | 1.07 | 38.9 | 34.7 | 30.9 | 29.5 | 28.4 | 28.0 | 28.7 | 27.2 | 27.7 | 27.0 |
| 18:1ω7 | 1.13 | 1.9 | 1.8 | 1.8 | 1.3 | 1.9 | 1.5 | 1.9 | 1.7 | 2.1 | 1.9 |
| 18:1ω5 | 1.17 | 1.0 | 1.0 | ---- | ---- | ---- | ---- | ---- | 0.1 | 0.1 | 0.1 |
| 18:2ω11 | 1.20 | 0.4 | 0.4 | 0.6 | 0.4 | 0.3 | 0.4 | 0.5 | 0.5 | 0.6 | 0.5 |
| 19:2ω9 | 1.23 | 0.4 | 0.4 | 0.4 | 0.3 | 0.4 | 0.2 | 0.3 | 0.4 | 0.4 | 0.3 |
| 18:2ω6 | 1.26 | 0.6 | 0.6 | 0.6 | 0.5 | 0.5 | 1.4 | 1.8 | 4.5 | 6.5 | 8.1 |
| 18:3ω6 | 1.40 | ---- | ---- | ---- | ---- | ---- | 0.1 | ---- | 0.4 | 0.5 | 0.6 |
| 18:3ω3 | 1.57 | ---- | ---- | 1.2 | 3.2 | 5.5 | 2.5 | 2.2 | 1.4 | 0.7 | ---- |
| 18:4ω3 | 1.77 | ---- | ---- | 0.3 | 0.8 | 1.7 | 0.6 | 0.7 | 0.3 | 0.2 | ---- |
| 20:1ω11 | 2.10 | 1.4 | 1.3 | 1.1 | 1.0 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 | 1.2 |
| 20:1ω9 | 2.16 | 0.3 | 0.1 | 0.1 | 0.2 | 0.2 | 0.3 | 0.2 | 0.1 | 0.2 | 0.3 |
| UnK | 2.26 | 5.0 | 1.5 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- |
| 20:2ω9 | 2.30 | 1.0 | 1.0 | 1.3 | 0.9 | 0.5 | 0.9 | 1.0 | 1.0 | 0.9 | 0.7 |
| 20:3ω9 | 2.47 | 1.9 | 2.9 | 1.6 | 0.9 | 0.5 | 1.1 | 1.2 | 1.7 | 1.8 | 2.0 |
| 20:2ω6 | 2.54 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.1 | 0.2 | 0.8 |
| 20:3ω6 | 2.66 | ---- | 0.3 | 0.2 | 0.1 | 0.2 | 0.6 | 0.6 | 1.2 | 1.5 | 1.7 |
| 20:4ω6 | 2.89 | 0.4 | 0.7 | 0.2 | 0.2 | 0.1 | 0.5 | 0.5 | 1.2 | 1.9 | 2.4 |
| 20:2ω3 | 3.04 | ---- | ---- | ---- | 0.4 | 0.5 | 0.2 | 0.3 | ---- | ---- | ---- |
| 20:3ω3 | 3.20 | ---- | ---- | 0.6 | 0.5 | 0.5 | 0.4 | 0.4 | 0.2 | 0.1 | ---- |
| 20:4ω3 | 3.34 | ---- | ---- | 0.5 | 0.6 | 0.9 | 0.4 | 0.5 | 0.1 | 0.1 | ---- |
| 20:5ω3 | 3.60 | ---- | 0.3 | 1.0 | 1.0 | 1.9 | 0.9 | 0.7 | 0.6 | 0.3 | ---- |
| 22:2ω6 | 4.75 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.1 | 0.2 |
| 22:3ω6 | 5.12 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.2 |
| 22:4ω6 | 5.50 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.3 |
| 22:5ω6 | 5.98 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0.4 | 1.0 | 3.8 |
| 22:5ω3 | 6.95 | ---- | ---- | 0.2 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 | ---- | ---- |
| 22:6ω3 | 7.50 | ---- | 1.8 | 4.2 | 5.4 | 4.7 | 4.1 | 4.5 | 3.9 | 3.8 | 1.1 |
| 24:1 | 8.18 | 0.5 | 0.4 | 0.5 | 1.1 | 0.4 | 0.8 | 0.6 | 0.5 | 0.4 | 0.6 |
| 20:3ω9/22:6ω3 | | | 1.61 | 0.61 | 0.02 | 0.01 | 0.27 | 0.27 | 0.44 | 0.56 | 1.82 |

[a] Average of analysis of duplicate tanks.

[b] Retention time relative to 18:0.

the lipids are from vital organs. In Set 3 the total body lipid was analyzed. These differences and the differences in the lipid content of the experimental diets rule out any comparison between lipid composition of Set 2 and Set 3 fish.

If the ratio of 20:3ω9/22:6ω3 in the phospholipids is used as a criterion for evaluating EFA deficiency, one would predict that all fish receiving 0.5 percent or less 18:3ω3 in the diet are receiving less than the optional level of ω3 fatty acids. This judgment is being based on a ratio of 0.4 or greater indicating a deficiency.

Table 20 gives the fatty acid composition of neutral lipids of the same fish from Set 3. As expected, the polyunsaturated fatty acid content of the neutral lipids was lower than that of the phospholipids. Increasing 18:3ω3 in the diet decreased 18:1ω9 in both the phospholipid and neutral lipid. At the same time an increase in the palmitic acid content was noted. A similar increase in palmitic acid was noted in some of the tissue lipids of Set 2 fish when ω6 or ω3 was added to the diet (Tables 12, 13, 17 and 18).

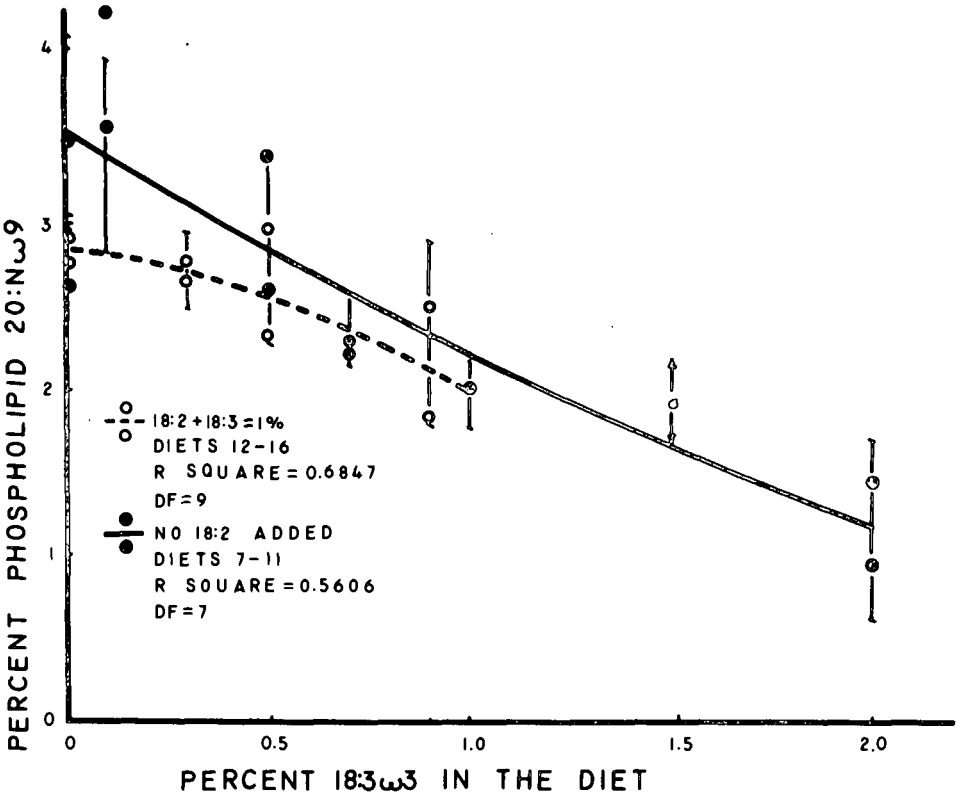
In an attempt to determine the significance of the correlation between dietary lipid composition and the concentration of particular types of fatty acids in the animal lipids a number of quadratic regression analyses were performed. Figures 26 and 27 show the curves which correlate total 20 carbon fatty acids of the oleate (ω9) family with the percent 18:3ω3 in the diet, for phospholipids and neutral

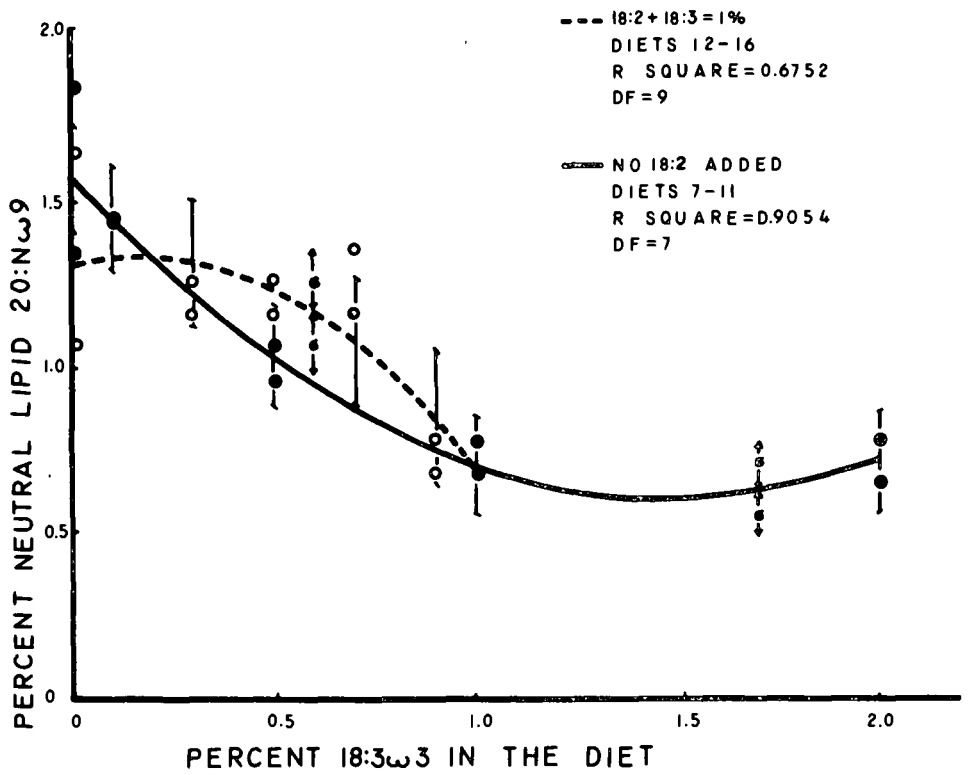
Table 20. Set 3 neutral lipid fatty acids.

| | | Percent in diet | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| F. A. | R. T. | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 12:0 | 0. 12 | 7. 4 | 15. 5 | 11. 0 | 9. 2 | 0. 3 | 8. 5 | 9. 0 | 8. 2 | 7. 8 | 9. 1 |
| 14:0 | 0. 21 | 7. 0 | 7. 2 | 5. 1 | 4. 9 | 3. 3 | 4. 5 | 5. 2 | 4. 5 | 4. 6 | 4. 4 |
| 14:1 | 0. 29 | 0. 5 | 0. 2 | 0. 3 | 0. 4 | 0. 2 | 0. 3 | 0. 2 | 0. 1 | 0. 2 | 0. 4 |
| 16:0 | 0. 50 | 15. 7 | 16. 5 | 19. 3 | 20. 3 | 20. 7 | 20. 6 | 21. 3 | 21. 1 | 19. 9 | 18. 0 |
| 16:1 ω 9 | 0. 54 | 1. 6 | 2. 0 | 1. 5 | 2. 1 | 1. 2 | 1. 7 | 1. 3 | 1. 8 | 2. 1 | 1. 2 |
| 16:1 ω 7 | 0. 56 | 14. 9 | 13. 8 | 15. 0 | 14. 1 | 12. 7 | 13. 0 | 13. 8 | 13. 5 | 12. 2 | 11. 7 |
| 16:2 ω 7 | 0. 63 | 0. 1 | 0. 3 | 0. 2 | 0. 3 | 0. 1 | 0. 3 | 0. 3 | 0. 2 | 0. 2 | 0. 1 |
| 18:0 | 1. 00 | 1. 4 | 2. 9 | 1. 7 | 4. 0 | 5. 4 | 3. 9 | 3. 7 | 4. 1 | 4. 2 | 4. 6 |
| 18:1 ω 9 | 1. 06 | 44. 3 | 34. 4 | 38. 3 | 34. 1 | 37. 0 | 34. 4 | 33. 5 | 34. 6 | 34. 3 | 36. 0 |
| 18:1 ω 7 | 1. 12 | 2. 7 | 3. 4 | 1. 9 | 1. 7 | 2. 3 | 1. 7 | 1. 7 | 1. 7 | 1. 7 | 1. 8 |
| 18:2 ω 11 | 1. 20 | 0. 2 | 0. 2 | 0. 3 | 0. 3 | 0. 1 | 0. 4 | 0. 4 | 0. 4 | 0. 3 | 0. 4 |
| 18:2 ω 9 | 1. 22 | 0. 3 | 0. 4 | 0. 5 | 0. 3 | 0. 1 | 0. 5 | 0. 5 | 0. 4 | 0. 4 | 0. 4 |
| 18:2 ω 6 | 1. 26 | 0. 3 | 0. 3 | 0. 5 | 0. 6 | 0. 5 | 1. 6 | 3. 1 | 4. 4 | 5. 9 | 7. 7 |
| 18:3 ω 6 | 1. 39 | ---- | ---- | ---- | ---- | ---- | 0. 1 | 0. 1 | 0. 2 | 0. 2 | 0. 5 |
| 18:3 ω 3 | 1. 57 | 0. 1 | 0. 1 | 1. 7 | 4. 9 | 10. 6 | 4. 3 | 2. 5 | 1. 7 | 2. 4 | ---- |
| 18:4 ω 3 | 1. 77 | ---- | ---- | 0. 2 | 0. 4 | 2. 0 | 0. 6 | 0. 3 | 0. 2 | 0. 2 | ---- |
| 20:1 ω 11 | 2. 09 | 1. 8 | 1. 3 | 1. 1 | 0. 9 | 1. 0 | 1. 1 | 1. 3 | 1. 1 | 1. 0 | 1. 3 |
| 20:1 ω 9 | 2. 15 | 0. 5 | 0. 3 | 0. 2 | 0. 1 | 0. 1 | 0. 1 | 0. 2 | 0. 1 | 0. 6 | 0. 2 |
| 20:2 ω 9 | 2. 30 | 0. 5 | 0. 6 | 0. 6 | 0. 5 | 0. 6 | 0. 7 | 0. 7 | 0. 6 | 0. 6 | 0. 6 |
| 20:3 ω 9 | 2. 43 | 0. 5 | 0. 5 | 0. 3 | 0. 1 | 0. 2 | 0. 2 | 0. 4 | 0. 4 | 0. 5 | 0. 7 |
| 20:3 ω 6 | 2. 66 | ---- | ---- | ---- | ---- | ---- | ---- | 0. 1 | 0. 2 | 0. 3 | 0. 3 |
| 20:4 ω 6 | 2. 89 | ---- | ---- | ---- | ---- | ---- | 0. 1 | 0. 2 | 0. 2 | 0. 2 | 0. 4 |
| 20:3 ω 3 | 3. 04 | ---- | ---- | ---- | ---- | 0. 3 | ---- | ---- | ---- | ---- | ---- |
| 20:4 ω 3 | 3. 34 | ---- | ---- | 0. 1 | 0. 2 | 0. 4 | ---- | ---- | ---- | ---- | ---- |
| 20:5 ω 3 | 3. 60 | ---- | ---- | 0. 1 | 0. 3 | 0. 4 | ---- | ---- | ---- | ---- | ---- |
| UnK. | 4. 33 | 0. 2 | 0. 1 | 0. 1 | ---- | ---- | ---- | ---- | ---- | ---- | ---- |
| 22:5 ω 6 | 5. 98 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0. 1 | 0. 2 |
| 22:6 ω 3 | 7. 50 | ---- | ---- | 0. 3 | 0. 3 | 0. 5 | 0. 4 | 0. 3 | 0. 2 | 0. 1 | ---- |

Figure 26. Regression analysis curves for phospholipid 20 carbon fatty acids of the oleic series versus ω3 content of the diet.

Figure 27. Regression analysis curves for neutral lipid content of 20 carbon, ω9 fatty acids versus ω3 content of the diet.


PERCENT PHOSPHOLIPID 20:Nω9
PERCENT 18:3ω3 IN THE DIET
18:2+18:3=1%
DIETS 12-16
R SQUARE=0.6847
DF=9
NO 18:2 ADDED
DIETS 7-11
R SQUARE=0.5606
DF=7
0
0.5
1.0
1.5
2.0
1
2
3
4


PERCENT NEUTRAL LIPID 20:Nω9
PERCENT 18:3ω3 IN THE DIET
18:2+18:3=1%
DIETS 12-16
R SQUARE=0.6752
DF=9
NO 18:2 ADDED
DIETS 7-11
R SQUARE=0.9054
DF=7
0
0.5
1.0
1.5
2.0

lipids respectively. Either with or without 18:2ω6 in the diets, the greatest reduction in 20 carbon acids of the ω9 family, resulted from increasing the 18:3ω3 content of the diet. The total ω9 in the phospholipids appears to be equally reduced by either 18:2ω6 or 18:3ω3 (Figure 28). The total ω9 content in the neutral lipids (Figure 29) does not appear to be greatly altered by either 18:2ω6 or 18:3ω3.

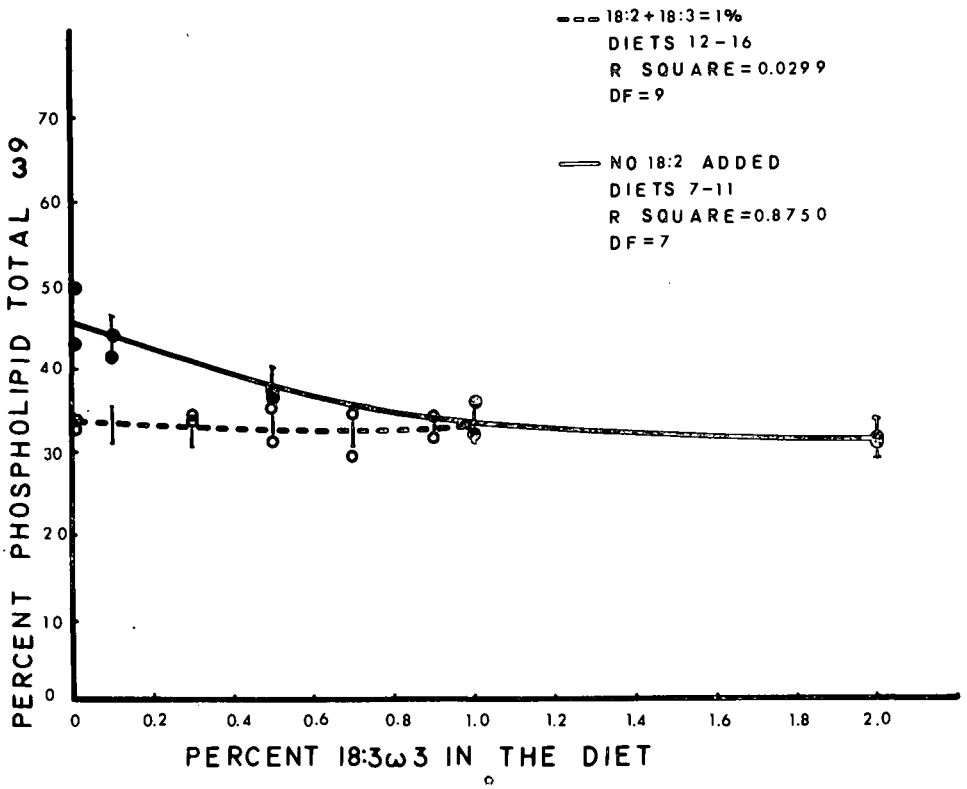
The effect of dietary ω3 on the ω3 content of the phospholipids (Figure 30) and the neutral lipids (Figure 31) was quite different. In the phospholipids, the incorporation of long chain unsaturated fatty acids derived from linolenate rose rapidly as the dietary level of 18:3ω3 was increased to one percent in the diet. Above one percent there was little increase in conversion of 18:3ω3 to longer chained, more highly unsaturated fatty acids. The diets with added 18:2ω6 resulted in lower chain elongation and greater direct incorporation of linolenic acid directly into the phospholipids.

In the neutral lipids (Figure 31) linolenic acid is for the most part, incorporated unchanged. There was almost a linear relationship between ω3 content in total neutral lipids and 18:3ω3 content in the diet. Again there was a slight decrease in chain elongation of 18:3ω3 in the fish fed mixed 18:3ω3 : 18:2ω6.

The competitive inhibition between various PUFA has been extensively investigated by Holman and his co-workers (58, 79, 115, 129). Although the inhibition of 18:3ω3 metabolism by 18:2ω6 has been

Figure 28. Regression analysis curves of phospholipid total ω9 fatty acids versus ω3 content of the diet.

Figure 29. Regression analysis curves of neutral lipid total ω9 fatty acids versus ω3 content of the diet.


18:2+18:3=1%
DIETS 12-16
R SQUARE=0.0299
DF=9
NO 18:2 ADDED
DIETS 7-11
R SQUARE=0.8750
DF=7
PERCENT PHOSPHOLIPID TOTAL ω9
0
10
20
30
40
50
60
70
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
PERCENT 18:3ω3 IN THE DIET

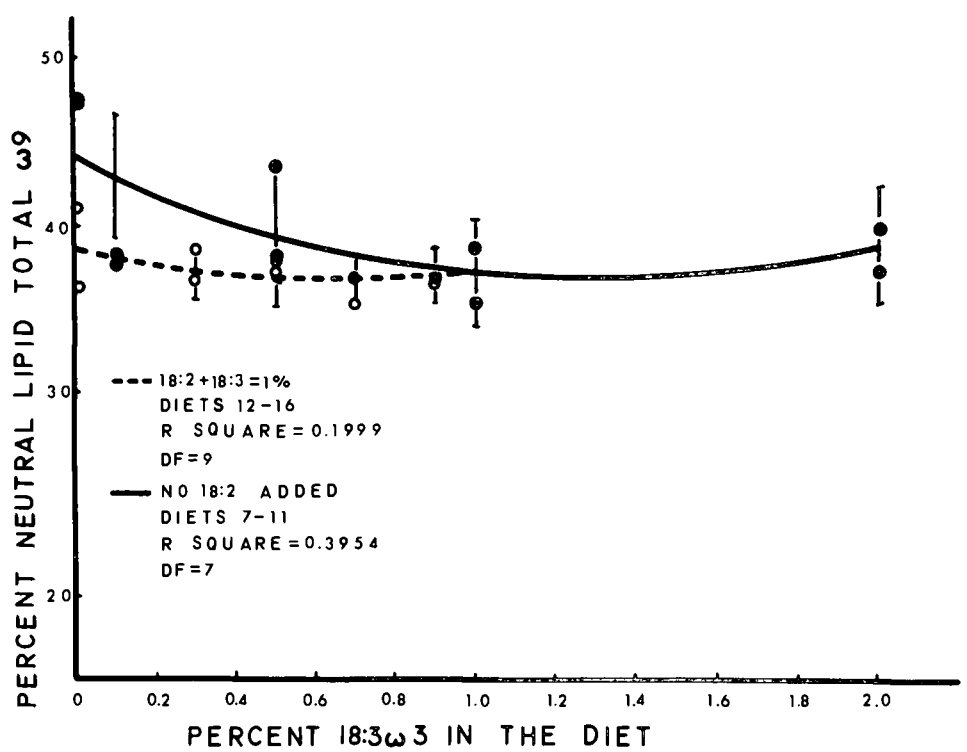
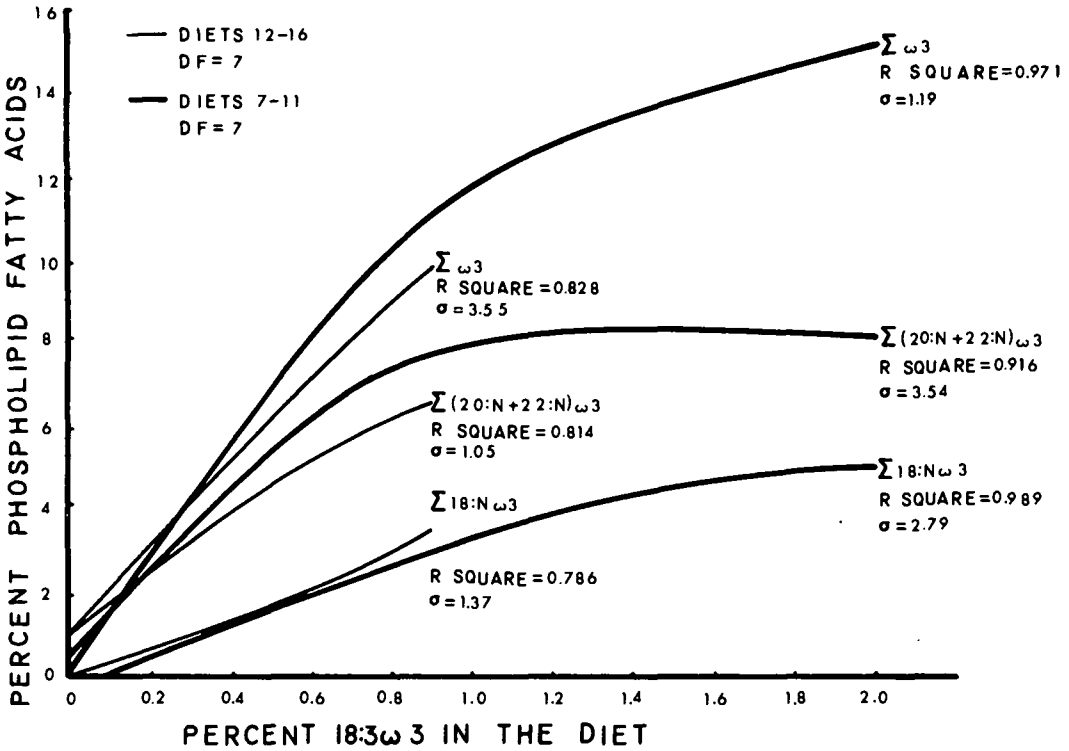
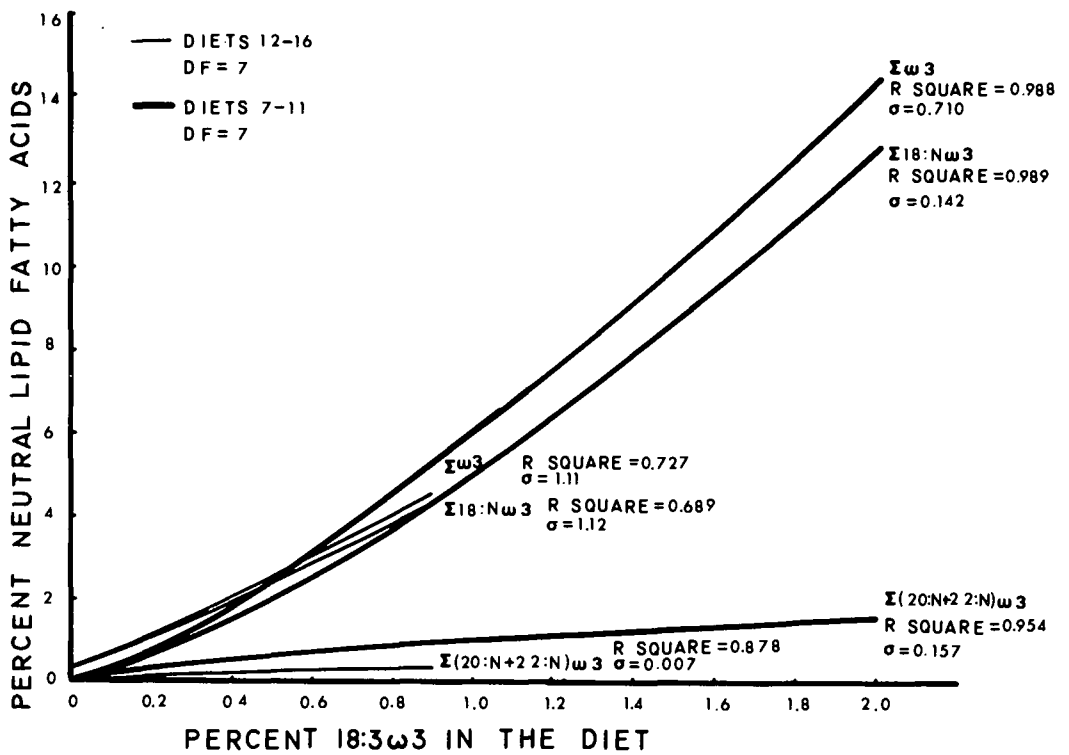

PERCENT NEUTRAL LIPID TOTAL ω9
20
30
40
50
18:2+18:3=1%
DIETS 12-16
R SQUARE=0.1999
DF=9
NO 18:2 ADDED
DIETS 7-11
R SQUARE=0.3954
DF=7
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
PERCENT 18:3ω3 IN THE DIET

Figure 30. Regression analysis curves of phospholipid $\omega 3$ fatty acids versus $\omega 3$ content of the diet.

Figure 31. Regression analysis curves of neutral lipid $\omega 3$ fatty acids versus $\omega 3$ content of the diet.


DIETS 12-16
DF = 7
DIETS 7-11
DF = 7
Σ ω3
R SQUARE=0.971
σ=1.19
Σ ω3
R SQUARE=0.828
σ=3.55
Σ(20:N+22:N)ω3
R SQUARE=0.916
σ=3.54
Σ(20:N+22:N)ω3
R SQUARE=0.814
σ=1.05
Σ18:Nω3
R SQUARE=0.989
σ=2.79
Σ18:Nω3
R SQUARE=0.786
σ=1.37
PERCENT PHOSPHOLIPID FATTY ACIDS
PERCENT 18:3ω3 IN THE DIET


DIETS 12-16
DF = 7
DIETS 7-11
DF = 7
Σω3
R SQUARE=0.988
σ=0.710
Σ18:Nω3
R SQUARE=0.989
σ=0.142
Σω3
R SQUARE=0.727
σ=1.11
Σ18:Nω3
R SQUARE=0.689
σ=1.12
Σ(20:N+22:N)ω3
R SQUARE=0.954
σ=0.157
Σ(20:N+22:N)ω3
R SQUARE=0.878
σ=0.007
PERCENT NEUTRAL LIPID FATTY ACIDS
PERCENT 18:3ω3 IN THE DIET

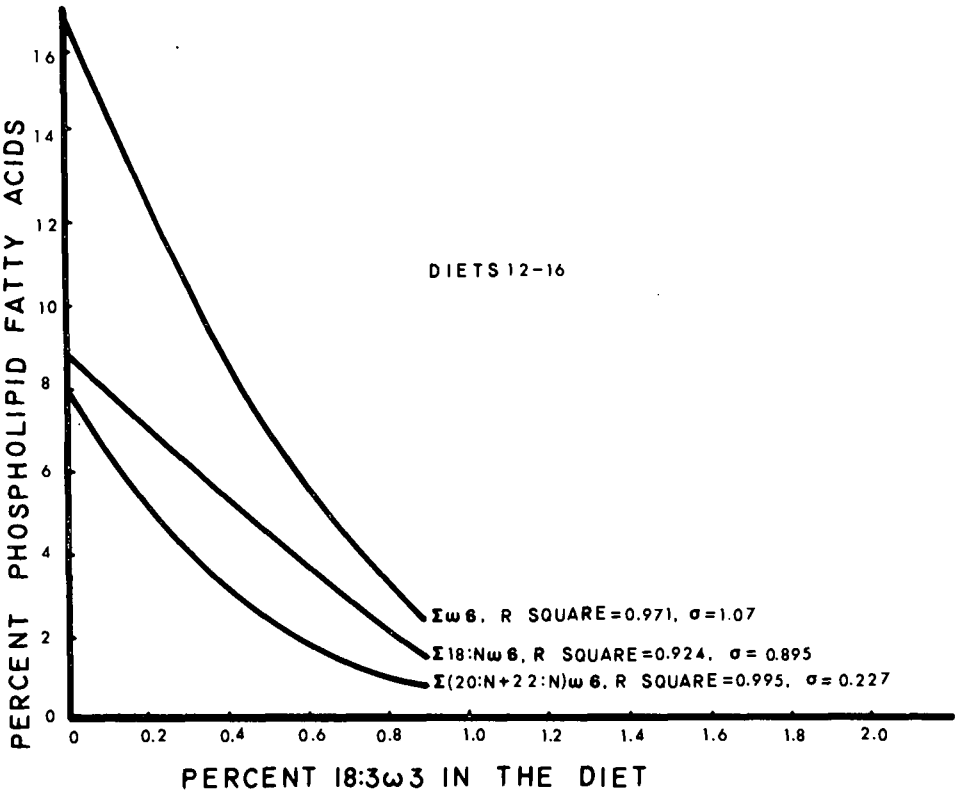
reported (129), the inhibition of 18:2ω6 by 18:3ω3 has been shown to be much more effective (29, 44, 58, 104, 113, 115). As noted by Garcia and Holman (58) the interactions were much more pronounced in the phospholipids (Figures 28, 30, 32) than in the neutral lipids (Figures 29, 31, 33). The data presented in this thesis for fish confirm the competitive interactions between fatty acids. There was a rapid non-linear reduction of 20 and 22 carbon fatty acids of the ω6 family with increasing 18:3ω3 in the diet as shown by the regression analysis curves of Figure 32. These results cannot be considered conclusive evidence as no diets were fed with equivalent amounts of 18:2ω6 without 18:3ω3. Additional evidence on the inter-relationships of 18:2 and 18:3 metabolism in fish may be gained by feeding several levels of 18:3ω3 and varying the level of added 18:2ω6.

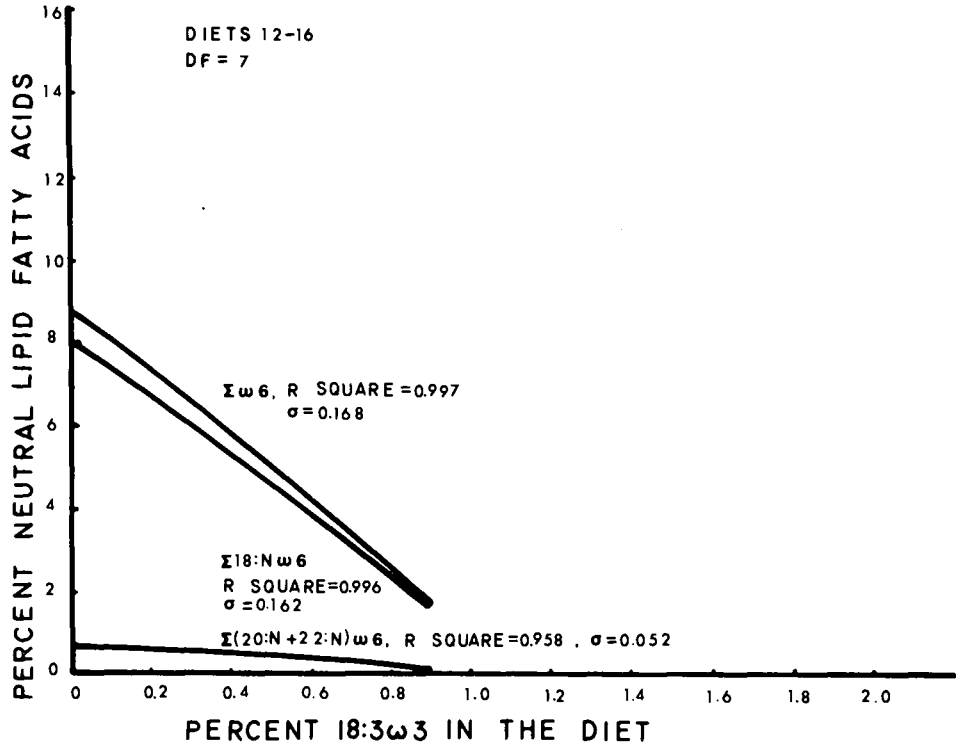
Fatty Acid Metabolism

The fatty acid analyses described in this thesis present considerable evidence for the pathways of lipid interconversions. Sufficient intermediates have been tentatively identified (e.g. see Table 19) to postulate a complete pathway for conversion of 18:1ω9 to 20:3ω9, 18:2ω6 to 22:5ω6, and 18:3ω3 to 22:6ω3. These pathways, with only fatty acids found in the fish lipids, are presented in Figure 34. This same pathway for linoleate has been postulated by Holman in 1964 (76). The patterns of desaturation and chain elongation have been outlined

Figure 32. Regression analysis curves of phospholipid $\omega 6$ fatty acids versus $\omega 3$ content of the diet.

Figure 33. Regression analysis curves of neutral lipid $\omega 6$ fatty acids versus $\omega 3$ content of the diet.


PERCENT PHOSPHOLIPID FATTY ACIDS
DIETS 12-16
Σω6, R SQUARE=0.971, σ=1.07
Σ18:Nω6, R SQUARE=0.924, σ= 0.895
Σ(20:N+22:N)ω6, R SQUARE=0.995, σ= 0.227
PERCENT 18:3ω3 IN THE DIET


PERCENT NEUTRAL LIPID FATTY ACIDS
DIETS 12-16
DF = 7
Σω6, R SQUARE =0.997
σ=0.168
Σ18:Nω6
R SQUARE=0.996
σ=0.162
Σ(20:N+22:N)ω6, R SQUARE=0.958 , σ=0.052
PERCENT 18:3ω3 IN THE DIET

extensively and discussions may be found in the review articles (1, 25, 77).

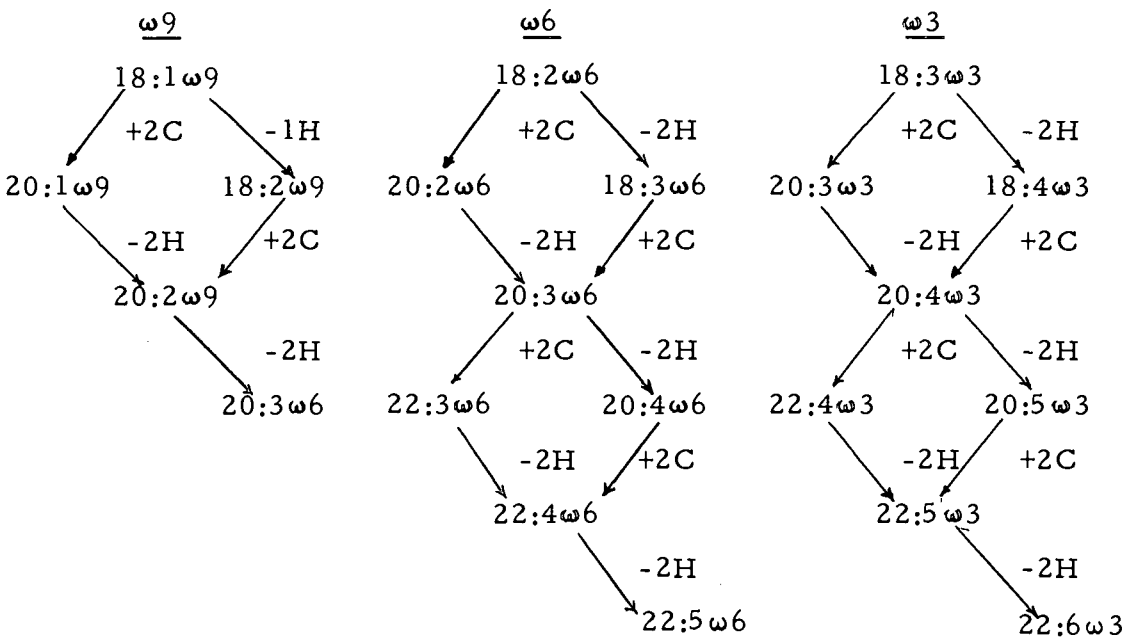



Figure 34. Pathways for fatty acid metabolism in rainbow trout.

## Deposition of Dietary ω6 and ω3 Fatty Acids

### Set 3

The percent of the total ω6 and ω3 fatty acids of the diet which were retained by the fish over the first 12 weeks of the feeding experiment are given in Table 21. These figures were based on the weight of 18:2 ω6 and 18:3ω3 in the food consumed in each tank on each diet. The actual total ω3 and ω6 fatty acids was based on the lipid analysis of five fish from each tub. A lower percent of the ω3 is retained at low dietary levels of 18:3 (Diet8 0.1% 18:3ω3) than when this fatty

acid is fed at higher levels. If part of the EFA function of ω3 fatty acids involved further metabolites this might explain the lower retention in Diet 8. Such metabolic derivatives as prostaglandins might help to explain the essentiality of particular positional isomers of PUFA. Such a proposal has been suggested by Horton for the EFA of other animals (84). A study on the retention or catabolism of labeled 18:2ω6 and 18:3ω3 would be helpful in clearing up this point. Although ω3 fatty acids have had more apparent EFA value in the rainbow trout, it appears in Table 21 that a higher percent of the dietary ω6 fatty acids were retained by the fish. This would also be more easily understood if part of the essential character of ω3 fatty acids was due to metabolic derivatives of the EFA's, such as prostaglandins.

Table 21. Percent of dietary ω3 and ω6 fatty acids deposited over a 12 week period.

| Diet | ω3 | ω6 |
|---|---|---|
| 7 | ---- | ---- |
| 8 | 17.5 | ---- |
| 9 | 25.5 | ---- |
| 10 | 29.7 | ---- |
| 11 | 27.6 | ---- |
| 12 | 23.3 | 66.5 |
| 13 | 23.7 | 36.9 |
| 14 | 23.0 | 37.3 |
| 15 | 33.8 | 35.9 |
| 16 | ---- | 31.9 |

# SUMMARY AND CONCLUSIONS

The fatty acid requirements of rainbow trout were the subject of this thesis. The types and levels of fatty acids essential in the diet of the trout were investigated. The effects of dietary lipid on growth, physical appearance and physiology were also noted. With the aid of gas liquid chromatography the effect of dietary lipids on fatty acid composition of various body tissue and organs was also examined. The conclusions are summarized below:

1. Fatty acids of the ω3 series are essential in the diet of rainbow trout. The optimal level being about one percent of the dry weight or two percent of the dietary calories.
2. Feeding fatty acids of the linoleate or ω6 series will aid in preventing some of the symptoms of EFA deficiency such as poor growth and fatty livers. Other symptoms such as the fainting syndrome and excrescent hearts, are aggravated by diets high in ω6 fatty acids.
3. A list of the EFA deficiency symptoms includes:
   (a) poor growth
   (b) fatty livers
   (c) a shock or fainting syndrome accentuated by handling or excitation of the fish,
   (d) abnormal hearts with greatly enlarged sections of the

blood vessels,

(e) increased swelling of isolated liver mitochondria in 0.25 M sucrose,

(f) increased respiration rate of liver homogenates,

(g) lowered blood hemoglobin,

(h) increased water content in muscle tissues,

(i) deposition of increased amounts of ω9 fatty acids such as 20:3ω9 in both neutral and phospholipids.

4. Alternation of dietary PUFA by fish is very similar to that by other animals. By a sequential series of desaturation and chain elongation fish convert 18:1ω9 to 20:3ω9, 18:2ω6 to 22:5ω6 and 18:3ω3 to 22:6ω3.

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# APPENDICES

# APPENDIX I

Dietary Components.

Table 1. Vitamin Premix No. 3

| Vitamin | Set 3 % in Mix | Sets 1 & 2 % in Mix |
|---|---|---|
| Thiamine (HCl) | 0.3200 | 0.3200 |
| Riboflavin | 0.7200 | 0.7200 |
| Niacinamide | 2.5600 | 2.5600 |
| Biotin | 0.0080 | 0.0080 |
| Ca-pantothenate $(D)_4$ | 1.4400 | 1.4400 |
| Pyridoxine (HCl) | 0.2400 | 0.2400 |
| Folic Acid | 0.0960 | 0.0960 |
| Menadione | 0.0800 | 0.0800 |
| $B_{12}$(Cobalamine 3,000 gm/gn) | 0.2667 | 0.0008 |
| i-inositol (meso) | 12.5000 | 12.5000 |
| Ascorbic Acid | 6.0000 | 6.0000 |
| Para-amino-benzoic Acid | 2.0000 | 2.0000 |
| Vitamine $D_2$ (500,000 USP/gm) | 0.0400 | 2.1600 |
| Butylated hydroxyanisole | 0.0750 | 0.0750 |
| Butylated hydroxytoluene | 0.0750 | 0.0750 |
| Vitamine A (250,000/IU/gm) | 1.0000 | 4.3200 |
| Celite | 72.5793 | 67.4052 |

Table 2. Modified Bernhart - Tomarelli Salt Mix

| Ingredient | Set 3 % in Mix |
|---|---|
| $CaCO_3$ | 2.100 |
| $Ca(PO_4)_2$ | 73.500 |
| Citric Acid | 0.205 |
| Curpic Citrate ($2Cu_2C_6H_5O_7 \cdot 5H_2O$) | 0.046 |
| Ferric Citrate ($FeC_6H_5O_7 \cdot 5H_2O$) | 0.558 |
| MgO | 2.500 |
| $Mn_3(C_6H_5O_7)_2$ | 0.835 |
| KI | 0.001 |
| $K_2HPO_4$ | 8.100 |
| $K_2SO_4$ | 6.800 |
| NaCl | 3.060 |
| $Na_2HPO_4 \cdot 2H_2O$ | 2.140 |
| $Zn_3(C_6H_5O_7)_2 \cdot H_2O$ | 0.133 |
| NaF | 0.002 |
| $CoCl_2$ | 0.020 |

Table 3. GLC Analysis of Diet Lipids

| F. A. | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 |
|---|---|---|---|---|---|---|
| 10:0 | - | 1.8 | - | - | - | - |
| 12:0 | tr | 3.1 | - | tr | tr | tr |
| 14:0 | 4.9 | 11.3 | 0.2 | 2.5 | 2.3 | 2.4 |
| 14:1 | 0.2 | 1.3 | - | 0.4 | 0.7 | 0.8 |
| 15:0 | 0.6 | 1.0 | - | 0.3 | 0.2 | 0.2 |
| 16:0 | 13.1 | 31.0 | 7.3 | 5.3 | 7.1 | 4.1 |
| 16:1 | 4.9 | 1.4 | - | 6.0 | 4.2 | 4.8 |
| 16:2ω6 | 0.7 | 0.8 | - | 1.0 | 0.5 | 0.5 |
| 18:0 | 3.1 | 12.2 | 2.3 | 1.4 | 0.8 | 0.8 |
| 18:1 | 20.4 | 21.2 | 13.8 | 80.8 | 64.4 | 64.0 |
| 18:2ω6 | 1.5 | 13.1 | 75.9 | 0.6 | 21.8 | 1.5 |
| 18:3ω3 | 1.2 | 1.1 | 0.5 | 0.6 | 0.6 | 20.6 |
| 20:1 | 12.4 | | | | | |
| 18:4ω3 | 1.8 | | | | | |
| 20:4ω6 | 0.8 | | | | | |
| 22:1 | 3.4 | | | | | |
| 20:4ω3 | 7.1 | | | | | |
| 20:5ω3 | 7.5 | | | | | |
| 22:4ω6 | 0.6 | | | | | |
| 22:4ω3 | 1.6 | | | | | |
| 22:5ω3 | 1.9 | | | | | |
| 22:6ω3 | 10.3 | | | | | |

# APPENDIX II

## Physical and Physiological Analysis

### A. Weighing Live Fish:

1. Tare large plastic tub with 1000-5000 g water.

2. Catch all the fish to be weighed. (Generally all the fish from one tank are weighed at the same time.)

3. Allow the water to drain from the net for a few seconds.

4. Put the fish in the tared plastic tub, weigh and calculate fish weight by difference.

5. Count fish back into original tank.

6. Determine average weight of fish in tank:

   Weight/No fish = Average weight

### B. Hematocrit (Packed Red Blood Cell Volume)

1. Kill fish by sharp blow to the back of the head.

2. Cut off tail just posterior to the anal fin.

3. Take sample by touching one end of a 100 ml glass capillary tube to the blood droplet formed by the central artery.

4. Seal the other end of the capillary in a flame.

5. Place the capillary tube in an Adams Micro-Hematocrit Centrifuge (Clay-Adams Inc., N. Y.) and spin at full speed for ten minutes.

6. Determine hematocrit by dividing the length of the packed red blood cells, by the total length of blood in the capillary tube.

C. Acid Hemoglobin Test - (71, 99)

1. Fish killed and tail removed as in section B of Appendix II.

2. Draw up ten μl in a micropipette and expell into 5.0 ml of 0.1N HCl, rinsing the pipette several times in the acid solution.

3. Allow the sample to stand for 15 minutes.

4. Record the absorbancy at 530 mμ, and determine hemoglobin reading from standard curve prepared from readings on known concentrations of NBC bovine hemoglobin.

5. Use equation of Larsen and Snieszke (99), 1961 for correcting standard acid hematin values for fish blood:

C A - Hb value = (0.534 X A - Hb value) + 2.44

C A - Hb value = corrected acid-hemoglobin value

A - Hb value = Acid-hemoglobin value from standard curve

D. Moisture Content of Flesh

1. Kill fish and remove skin.

2. Weigh a portion of the flesh in a tared foil dish.

3. Dry in 110 C oven for 24 hours.

4. Weigh dried sample.

5. Calculate percent moisture as:

$$\frac{\text{weight wet fish - weight dried flesh}}{\text{weight wet flesh}}$$

E. Mitochondrial Swelling (52, 150)

1. Mitochondrial Preparation:

   (a) Kill the fish, quickly remove the liver and wash it in 0.44 M sucrose.

   (b) Blot the liver on paper towel and weigh.

   (c) Add four times the liver weight of ice cold 0.44 M sucrose 2.3 mM citric acid in glass distilled water, and homogenize with a Potter and Elvehjem homogenizer with a teflon pestle.

   (d) Add an equal volume of ice cold 0.44 M sucrose.

   (e) Centrifuge at 675 g for 20 minutes at 3 C to remove cell debris.

   (f) Centrifuge supernatant from (e) at 13,000 g for ten minutes.

   (g) Resuspend the pellet in chilled 0.44 M sucrose and centrifuge again at 13,000 g for ten minutes. Repeat once more.

   (h) Suspend the final pellet in a volume of chilled 0.44 M sucrose equal to the original weight of the liver.

   (i) Store at 3 C and use suspension for swelling procedure.

2. Swelling Determination:

(a) Add 0.1 - 0.2 ml of mitochondrial suspension to 2.9 ml 0.25 M sucrose in 20 mM Tris-HCl Buffer, pH 7.4 at 30 C.

(b) Record mitochondrial swelling as a decrease in absorbancy at 520 mμ. The initial absorbancy should be adjusted to 0.6 - 0.9 by increasing or decreasing the volume of suspension added.

F. Respiration of Liver Homogenates

1. Fish livers removed as before and washed in ice cold Krebs saline solution (82).

Table 4. Krebs saline.

| Substance | % Concentration | Parts |
|---|---|---|
| NaCl | 0.935 | 103 |
| KCl | 1.19 | 4 |
| $KH_2PO_4$ | 2.19 | 1 |
| $MgSO_7 \cdot 7H_2O$ | 3.92 | 1 |
| $NaHCO_3$ | 1.35 | 3 |
| $PO_4$ buffer | ---- | 18 |
| $PO_4$ buffer | | |
| $Na_2HPO_4$ | 1.47 | 4 |
| $NaH_2PO_4 \cdot H_2O$ | 1.43 | 1 |

2. The liver is then weighed and homogenized with four times its weight of cold Krebs saline solution.

3. Oxygen uptake is measured with a Gilson Respirometer at 30 C.

(a) Equip the center well of a Warburg flask with a piece of folded paper and 0.1 ml 5% KOH.

(b) Add 2.5 ml 3 mM disodium succinate in Krebs saline solution to the main chamber

(c) Add 0.5 ml of liver homogenate to the saline solution and attach the flask to the respirometer.

(d) Allow all flasks to equilibrate for ten minutes then close valves and take readings of oxygen uptake every five minutes for half an hour.

# APPENDIX III

## Lipid Analysis

### Extraction of Tissue Lipids

The method of Bligh and Dyer (34) was used to extract lipids from all tissue samples.

1. In a Waring Blendor mix tissue, methanol, and chloroform (1:2:1) for two minutes.

2. Add another volume of chloroform and blend for 30 seconds.

3. Finally add one volume of water and blend for 30 seconds.

4. Filter through Whatman No. 1 filter paper with a Buchner funnel.

5. Re-extract the pulp with chloroform: methanol: water (2:2:1.8) and combine filtrates.

6. Remove water layer and dry chloroform layer over anhydrous $Na_2SO_4$.

7. Filter and remove chloroform from lipids with a rotary-flash-evaporator.

8. Weigh lipid and record percent lipid recovered. Store samples under nitrogen at -30 C.

Column Chromatographic Separation of Phospholipids and Neutral Lipids

1. Pack a 14 mm. I. D. glass column with 5 gm silicic acid (Mallinckrodt, 100 mesh, methanol washed and dried at 110 C for 16 hours) and 2.5 g celite (Johns-Manville) 545 mixed in 50 ml chloroform (USP distilled). Wash column with an additional 50 ml chloroform.

2. Add 50-200 mg of lipid dissolved in 2 ml chloroform.

3. Elute the neutral lipids with 150 ml chloroform and store under nitrogen.

4. Elute the phospholipids with 150 ml methanol (USP distilled).

5. Remove solvents by rotary-flash-evaporator and determine weight of each type lipid recovered.

6. Store each sample in small vial under a nitrogen atmosphere at -30 C.

Methyl Ester Preparation

Methyl esters of lipids were prepared for GLC by transesterification with boron-trifluoride in anhydrous methanol (117).

1. Neutral lipids: 35-150 mg lipid mixed with one ml benzene (distilled reagent grade), one ml 14% $BF_3$ in methanol (Applied Science Laboratories) and two ml anhydrous methanol in a 20 ml culture tube with a tight sealing Teflon lined screw cap.

(a) The mixture is heated at 100 C for 30 minutes. Cool. Add 10 ml saturated $Na_2SO_4$ and extract the methyl esters with diethyl ether.

(b) The ether extract is washed with water and dried over anhydrous $Na_2SO_4$.

(c) The ether is removed by a stream of $N_2$ and the esters are stored at -30 C under $N_2$ until analysis by GLC.

2. Phospholipids:

(a) Mix 25-140 mg lipid with two ml anhydrous methanol and two ml 14 percent $BF_3$ in methanol in a tube with teflon lined screw cap.

(b) Heat at 100 C for 15 minutes.

(c) Cool and add 10 ml saturated $Na_2SO_4$.

(d) Proceed as with methyl esters of neutral lipids.

Gas-Liquid Chromatography - GLC

The fatty acid composition of all lipid samples are determined by GLC analysis of their methyl esters.

1. Equipment:

(a) Aerograph Hy-Fi Model 600B with hydrogen flame detector and Barber Coleman 8000 series recorder fitted with a Barber Coleman disc chart integrator model 205.

(b) Columns used with the Hy-Fi are 12' x 1/8" stainless steel coiled columns packed with.

(i) 15% DEGS on Chromosorb G (Acid washed DMCS treated, 100/120 mesh).

(ii) 12% DEGS on Gas Chrom P 100/120 mesh.

(iii) 15% EGSS-Y (Applied Science Laboratories) on Gas Chrom P 100/120 mesh.

(iv) 15% DEGS on acid and base washed celite 100/120 mesh.

(c) Conditions used with Hy-Fi: Oven temperature 180-200 C; injection port and detector 230-260 C, carrier gas nitrogen 20 ml/min.

(d) Perkin-Elmer model 226 with hydrogen flame detector connected to Barber Coleman series 8000 recorder with disc chart integrator. 300' x 0.01" I.D. capillary column coated with BDS, Carrier gas $N_2$ at 40 psi, oven temperature 180 C, block 260 C.

2. Peak Identification:

All identifications are based on retention times relative to 18:0. Whenever possible analysis are run on two columns of different degrees of polarity. Procedures for identification include the following.

(a) Comparison with relative retnetions of known methyl esters

in standard mixtures from Applied Science Laboratories or the Hormel Institute. Known standards include 12:0, 14.0, 16:0, 16:1ω7, 18:0, 18:1ω9, 18:2ω6, 18:3ω3.

(b) Comparison with relative retention times methyl esters in oils that have been previously analyzed such as cod liver oil, salmon oil, etc.

(c) Comparison with relative retention times published in the literature.

(d) Graphical analysis based on the fact that plotting log of relative retention times versus carbon numbers gives straight lines for homologous series of methyl esters (10, 11, 12, 16, 17).

3. Quantitation:

Correlation factors for relating area percent to weight percent of fatty acid methyl esters are based on the "active" carbon theory of Ackman (13, 19, 20). The correlation factors for methyl esters in Table 5 gives very good agreement when checked with weighed quantitative standards.

Table 5. Methyl ester response factors used for correcting area percent to weight percent.

| F. A. Ester | Correction Factor |
|---|---|
| 12:0 | 1.08 |
| 14:0 | 1.04 |
| 14:1 | 1.04 |
| 15:0 | 1.03 |
| 16:0 | 1.02 |
| 16:1 | 1.01 |
| 16:2 | 1.00 |
| 16:3 | 1.00 |
| 17:0 | 1.01 |
| 17:1 | 1.00 |
| 18:0 | 1.00 |
| 18:1 | 0.99 |
| 18:2 | 0.99 |
| 18:3 | 0.98 |
| 18:4 | 0.97 |
| 20:1 | 0.98 |
| 20:2 | 0.97 |
| 20:3 | 0.97 |
| 20:4 | 0.96 |
| 20:5 | 0.95 |
| 22:1 | 0.97 |
| 22:2 | 0.96 |
| 22:3 | 0.96 |
| 22:4 | 0.95 |
| 22:5 | 0.94 |
| 22:6 | 0.94 |
| 24:1 | 0.96 |