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Title: METABOLISM OF (1-^{14}C) LINOLENIC ACID
IN COHO SALMON, ONCORHYNCHUS KISUTCH

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The process of phospholipid acylation and deacylation is recognized to play an important role in the control of fatty acid composition of biological membranes. The rates of turnover of phospholipids and their constituent fatty acids could provide valuable information as to the nature of that process and the general metabolic state of the individual fatty acids. However, few detailed studies are available concerning the biological halflives of the polyunsaturated essential fatty acids in individual phospholipids of mammals, and no such data exist for poikilotherms. The purpose of this study was to describe the incorporation of (1-^{14}C) linolenic acid, an essential fatty acid for many fish, into tissues of coho salmon, and to measure halflives of the ω3 fatty acids in the

major hepatic phospholipids. Additional objectives included the description of previously unreported phospholipid composition of coho salmon liver, and acyl group analysis of liver choline and ethanolamine phosphoglycerides.

Results showed that incorporation of label into liver and heart lipids was much greater than into gill lipids. Inositol phosphoglycerides had the shortest halflife of all hepatic phospholipids. Total acyl halflife was shorter for the choline phosphoglycerides than for the ethanolamine phosphoglycerides, as were the halflives of all individual ω3 fatty acids. Eicosapentaenoic acid (20:5ω3) had the shortest halflife in both phospholipids, while docosapentaenoic acid (22:5ω3) and docosahexaenoic acid (22:6ω3) had much longer halflives. The fatty acid halflives reported here for salmon are in general agreement with those found previously in mammals, indicating that poikilotherms do not necessarily have reduced levels of lipid metabolism.

Metabolism of (1-^{14}C) Linolenic Acid in Coho Salmon, *Oncorhynchus kisutch*

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LITERATURE SURVEY

Introduction

The essential fatty acids are recognized as those fatty acids that cannot be biosynthesized by animals, but are required in the diet for proper development and the maintenance of many physiological functions. Two distinct families of polyunsaturated essential fatty acids have been identified. One, the linoleic family, is characterized by having six carbon atoms between the methyl end of the molecule and the first double bond, and is referred to as the ω-6 family. The second, the linolenic family, has three carbon atoms between the methyl group and the first double bond, and is referred to as the ω-3 family. Both linoleic acid (18:2ω-6, where 18 refers to the number of carbon atoms in the fatty acid and :2 is the number of double bonds) and linolenic acid (18:3ω-3) may be converted to other fatty acids having longer carbon chains and more double bonds, but the ω conformation is retained. Fatty acids of the two families are not interconvertible.

It has been well established that many species of fish require fatty acids of the linolenate (ω-3) family in their diets to support optimal growth and prevent the onset of essential fatty acid deficiency symptoms (1-3). Furthermore, it appears that trout and salmon do not require dietary fatty acids of the linoleate (ω-6) family. The latter acids are metabolized slowly in plaice (4) and turbot (5),

but will undergo metabolic conversion in rainbow trout and coho salmon, resulting in depression of growth and feed conversion (3, 6-8). Conversely, it has been known for many years that the ω-6 fatty acids fulfill the essential fatty acid requirement of mammals, and as yet no requirement has been demonstrated for the ω-3 acids. Nevertheless, ω-3 fatty acids can alleviate some of the symptoms of ω-6 deficiency, and are found in large quantities in certain vital tissue lipids of mammals. Efforts to deplete this ω-3 content through dietary manipulation have proved difficult (9), indicating that these fatty acids may serve some as yet undetermined function in mammalian tissues.

The polyunsaturated homologs of the ω-3 and ω-6 fatty acids of both fish and mammals are esterified primarily to phospholipids, which are in turn located almost entirely in cellular membranes (Figure 1). The pathways of phospholipid metabolism and the nature of many of the enzymes involved have been recently reviewed (10). It is evident that turnover of membrane phospholipids may involve movement of intact molecules into or out of membranes, or metabolic transfers of only a portion of the phospholipid molecule (refer to Figure 2).

In efforts to more precisely determine the physiological roles of the polyunsaturated fatty acids, various investigators have examined their rates of incorporation and depletion, and the biochemical pathways involved with the metabolism of these compounds. Such studies have dealt primarily with mammalian tissues, and little information is available on fatty acid or phospholipid turnover in fish. This emphasis on mammalian studies will be reflected in the following

review, which represents the more notable studies concerned with lipid turnover, and includes the various methods by which these processes are measured.

Studies on Lipid Turnover in Mammals

The enzyme-catalyzed replacement of fatty acids in phospholipids by acyltransferases was demonstrated in rat and guinea pig microsomes by Hill and Lands (11). These authors measured the rates of esterification

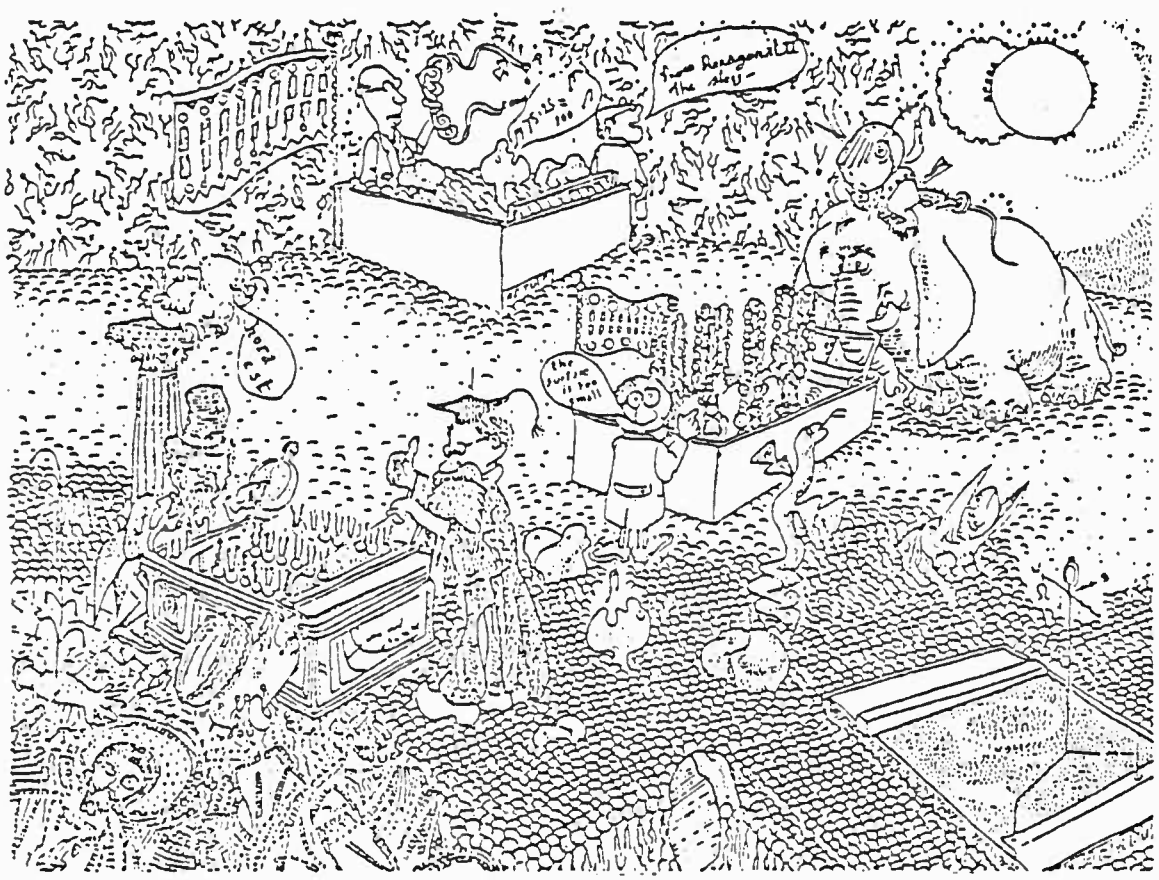

Figure 1. "The Lipid Bilayer Concept of Cell Membranes." From Zwaal, R.F.A., R.A. Demel, B. Roelofsen, and L.L.M. van Deenen, Trends in Biochemical Science 1:112 (1976).

of fatty acid-CoA esters to 1-acyl-glycerophosphate and 1-acyl-glycerophosphatidyl choline (GPC). The results showed a clear preference for 1-acyl-GPC by 20:2, 20:3, 20:4, and 20:5 acids, indicating that these long-chain unsaturated fatty acids would be more effectively incorporated into phospholipids via acyltransferase activity than by phosphatidic acid synthesis de novo (the CDP-choline pathway, Figure 2).

The incorporation of ^{3}H-arachidonate into rat brain phospholipids was studied by Baker and Thompson (12). Choline phosphoglycerides (CPG) were the most highly labelled phospholipid at 30 minutes, the time of peak incorporation, followed by monophosphoinositides and ethanolamine phosphoglycerides (EPG). The rapid appearance of ^{3}H-arachidonate in monophosphoinositide occurred in parallel with the formation of arachidonyl species of phosphatidic acid rather than in sequence, as might be expected for de novo synthesis. It was concluded that the high incorporation of arachidonate in brain was due primarily to an independent acyl exchange mechanism rather than by de novo synthesis of the various phospholipids. Furthermore, it was noted that the arachidonyl groups in brain CPG turned over more rapidly than in other phospholipids, and that this could be accounted for by acyltransferase activity. Fatty acid halflives were not calculated in this study.

Holub and Kuksis (13) found that the specific activities of the different molecular species of CPG and EPG in rat liver following injection of labelled linoleate, linolenate, and arachidonate were not all the same. The tetraenoic species formed after injection of

arachidonate indicated the arachidonate was incorporated primarily through reacylation, as found by previous authors (11,12), and that the fatty acid at the 1-position did not appreciably affect the acylation. On the other hand, the specific activity of the 16:0-18:2 species was 2-3 times higher than that of the 18:0-18:2 species following linoleate injection, perhaps reflecting equal incorporation by the acylation pathway in combination with de novo synthesis of the 16:0-18:2 species by the phosphatidic acid (CDP-choline) pathway.

The in vivo incorporation and molecular distribution of ^{14}C-linolenic and ^{14}C-arachidonic acids in rat liver lipids was investigated by de Tomas and Mercuri (14). No significant differences in the incorporation of the three labelled acids were observed. However, a low incorporation of ^{14}C-arachidonic acid into 1,2-diacylglycerol contrasted with the relatively high activity of this acid in phospholipid. This finding suggests that arachidonic acid may be incorporated primarily through acyltransferase activity as opposed to the CDP-choline pathway.

Poovaiah et al. (15) examined the effect of diet on uptake of radioactivity from $^{14}C_1$-linolenic acid into heart and liver lipids of male rats. Most radioactivity was recovered in carcass as unchanged linolenate, and very little was found in heart lipids. Maximum incorporation into liver lipids occurred at 3 hours for control rats and 4 hours for essential fatty acid-deficient rats. In liver, 20:5ω-3 was the most highly labelled fatty acid at four hours, with little activity occurring in 18:3ω-3 or 20:4ω-3. The authors concluded that 18:3ω-3 and 18:4ω-3 must have been turning over at rapid rates, and

that 20:5ω-3 had a slower rate of removal than of formation. A slow rate of turnover was also indicated for 22:6ω-3, which was still increasing in activity at 24 hours. These data also showed that dietary dosages of 22:6ω-3 greatly reduced incorporation of label into phospholipids, and that conversion of 20:5ω-3 to 22:5ω-3 was the rate limiting step in the synthesis of 22:6ω-3.

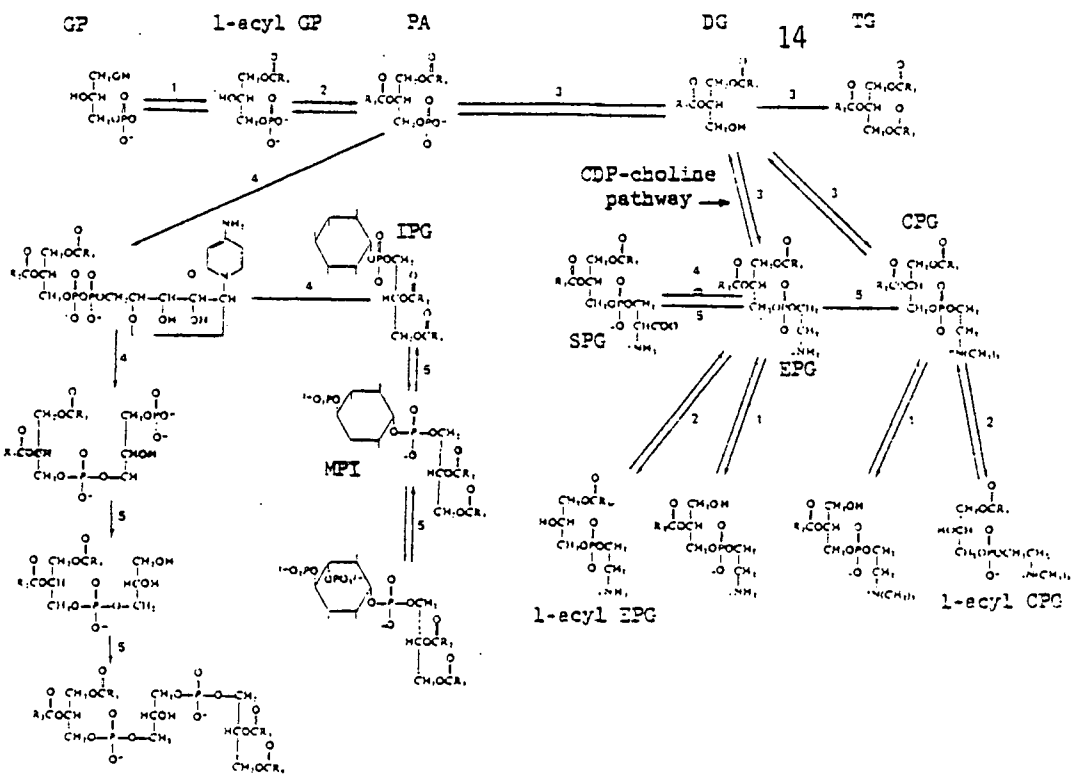

Figure 2. Specific Reactions in the Formation and Cleavage of Phospholipid Bonds. (From Lands and Crawford (10)).

GP	glycerol-3-phosphate	IPG	inositol phosphoglycerides
PA	phosphatidic acid	SPG	serine phosphoglycerides
DG	diglyceride	EPG	ethanolamine phosphoglycerides
TG	triglyceride	CPG	choline phosphoglycerides
MPI	monophosphoinositide		

The incorporation of radioactivity by liver and brain lipids of suckling rats following oral administration of ^{14}C-linoleic acid, ^{14}C-linolenic acid, ^{3}H-arachidonic acid, and ^{14}C-docosahexaenoic acid was studied by Sinclair (10). In both tissues, uptake of labelled 18:2ω-6 and 18:3ω-3 was very low compared to that of 20:4ω-6 and 22:6ω-3, agreeing with the observations of Trewella and Collins (17) and Brenner (18) that the turnover rate of linoleic acid often exceeds that of arachidonic acid in tissue lipids. This may mean that 18:2ω-6 and 18:3ω-3 are more exposed to β-oxidation than 20:4ω-6 and 22:6ω-3.

Dhopeshwarkar and Subramanian (19, 20) studied the patterns of (1-^{14}C) linolenic acid incorporation and turnover in brain of growing rats. Mammalian brains contain relatively large quantities of 22:6ω-3, especially in the ethanolamine phosphoglycerides (21). The halflife of total radioactivity in CPG was estimated to be 11.3 days while those of the other phospholipids were longer. The fatty acid 22:6ω-3 had a high specific activity at all times (up to 45 days) relative to 18:0, 18:1 and 20:4ω-6. The halflife of 22:6ω-3 estimated from rate of loss of radioactivity, was 13 days. Other ω-3 fatty acids were not examined. Similar results were obtained by Yavin and Menkes (22), employing cultured dissociated rat brain cells. This study also revealed that at intermediate temperatures (15 C) conversion of 18:3ω-3 to 20:5ω-3 was active but that further conversion to 22:6ω-3 was abolished. These latter results suggested that the conversion of 18:3ω-3 to 22:6ω-3 involved at least two distinct metabolic steps distinguishable by their temperature dependency.

Other labelled lipid precursors, namely phosphorus, acetate, and glycerol, have been used to measure the halflives of various lipids in mammalian tissues. Each of these tracers, however, tend to be incorporated into different parts of the phospholipid molecules (i.e. the base, glycerol backbone, phosphate group, or acyl groups) independent of each other (10, 17, 23). This concept was well illustrated by Landriscina et al. (24) who studied the turnover of rat liver cardiolipin using various labelled compounds including linoleic acid, the predominant fatty acid of cardiolipin. The halflife of non-essential fatty acids esterified to cardiolipin, measured with ^{14}C-acetate, was 3.1 days. When 2-^{3}H-glycerol was used as the tracer, a halflife of 5.2 days was calculated. Apparently the acyl groups of cardiolipin turn over faster than the other parts of the molecule. This may be of interest in light of the finding that cardiolipin is tightly bound to cytochrome oxidase, and could be an important structural component of the respiratory chain (25).

Lee et al. (26) used ^{14}C- and ^{3}H-acetate and glycerol to compare the turnover of components of plasma and microsomal membranes of rat liver. Although double isotope experiments indicated that radioactivity in CPG and EPG turned over more rapidly than the IPG + SPG fraction, single isotope measurements showed similar halflives of approximately 1.8 days for all three phospholipid fractions in both membrane preparations. The halflife of sphingomyelin was longer, 2.1-2.6 days, with that of plasma membranes exceeding that of microsomal membranes.

Endoplasmic reticulum membranes have been the subject of several turnover studies. Turnover of various constituents of these membranes in rat hapatocytes was examined by Omura et al. (27) by ^{14}C-acetate labelling. It was found that the total lipid halflife of these membranes was 97 hours, and total phospholipid halflife was 56 to 78 hours. Since rat hepatocytes are estimated to have halflives of 160-400 days (28), it is evident that there is considerable turnover of cellular material during the life of a single cell. Lee and Snyder (29), also using ^{14}C-acetate, found that for both choline- and ethanolamine- containing phospholipids of this fraction, fatty acids esterified at the 1-position (mostly saturated fatty acids) turned over much faster than fatty acids at the 2-position (unsaturated acids). The order of turnover of the various phospholipids was CPG>EPG, SPG>IPG>S.

Incorporation and turnover of lipid components of rat brain after injection of ^{14}C-acetate was measured by Smith and Eng (30). Inositol, choline, and serine phosphoglycerides in that order turned over most rapidly in both myelin and mitochondrial-rich fractions, followed by cerebrosides, ethanolamine phosphoglycerides, sphingomyelin, and cholesterol. Halflives of radioactivity in IPG, CPG, and SPG in the mitochondrial-rich fraction of brain were estimated to be two days, two weeks, and three weeks, respectively, during the initial few weeks. Rates of turnover in myelin were much slower.

A measure of phospholipid turnover can also be obtained by using radioactive phosphorus, ^{32}P. Trewella and Collins (17, 31), using this method of labelling, found that in rat liver the ethanolamine

phosphoglycerides, as a group, turned over at a faster rate than the choline phosphoglycerides. Rats deficient in essential fatty acids were shown to have an elevated specific activity of phospholipids relative to control animals, and that this increase was due in large part to the increased turnover rate of the 1-palmitoyl-2-oleoyl species of both the choline and ethanolamine phosphoglycerides. Similar studies in rat skeletal muscle by Shamgar and Collins (32, 33) revealed that, in contrast to liver, choline phosphoglycerides turned over faster than ethanolamine phosphoglycerides. The 1-palmitoyl-2-oleoyl and 1-oleoyl-2-linoleoyl species of choline phosphoglycerides had the fastest rate of turnover, and the 1-palmitoyl-2-arachidonyl species the slowest. In general, muscle phospholipids turned over more slowly and evenly than liver phospholipids.

Bailey et al. (34) employed both ^{32}P and ^{14}C-acetate to measure turnover of mitochondrial lipids from rat liver. In both cases the total phospholipid decay curve was bi-phasic, with an initial fast decrease in radioactivity followed by a slow decrease. The halflife of ^{32}P lipids was 1.6 and 10 days for the fast and slow rates respectively. The corresponding halflife of the ^{14}C-lipids was 2 days for the first 17 days, after which very little decrease in radioactivity was seen up to 35 days. Essential fatty acid deficiency resulted in increases in all lipid halflives.

A final method of studing rates of turnover of fatty acids is by measuring the rates of change of fatty acid composition induced by dietary manipulation. Van Golde et al. (35) examined the rates of replacement of acyl groups in the 2-position of liver choline

phosphoglycerides in essential fatty acid deficient rats following the re-feeding of these fatty acids. During essential fatty acid (ω-6) deficiency, 20:4ω-6 is replaced in the phospholipids by 20:3ω-9, which is biosynthesized from 18:1ω-9. Reversal of this trend occurs when the essential ω-6 acids (18:2ω-6 or 20:4ω-6) are supplied in the diet. Van Golde found approximate halflives of 48 and 95 hours for 20:3ω-9 and 20:4ω-6, respectively, during the initial 120 hours after feeding corn oil, which contains large amounts of 18:2ω-6.

Studies of Lipid Turnover in Fish

Most of the available information concerning lipid turnover in fish has centered on the subject of temperature adaptation. It is well known that a decrease in environmental temperature results in a general increase in unsaturation of lipids of fish and other poikilotherms (36). Knipprath and Mead (37) examined the changes in total fatty acid composition of guppies subjected to a sudden temperature decrease for two days. Marked increases occurred in 14:1, 16:1, and 18:1 fatty acids, and a slight increase in 22:6ω-3 was also evident. Content of 14:0 and 16:0 decreased, as did 20:4ω-6. No rate values could be calculated, but these results indicated that the tissues of at least this species of fish were able to respond to environmental stimuli within a relatively rapid time span. In a later study (38) the same authors measured the incorporation of ^{14}C-acetate into various fatty acids of goldfish muscle acclimated to 10 C and 30 C. Incorporation of label in the fatty acids was much greater at the lower temperature. Furthermore, comparison of activities of saturated and unsaturated fatty acids within each temperature group showed a tendency toward higher

incorporation into unsaturated acids at lower temperatures. Within the 22-carbon group, tri- and pentaenoic acids were several times as active as the other fatty acids. Label accumulated in 22:3 and 22:5 acids although their tissue content was very low. Only trace amounts of label appeared in 18:3ω-3, 18:4ω-3 and 22:6ω-3 after 4 hours of *in vivo* incubation. Since only one incubation time was used, turnover rates of the fatty acids with this label presursor could not be calculated. The authors noted that with alteration of environmental temperature, a particular fatty acid might undergo changes of varying magnitude in different types of lipids, depending on the turnover rate of the lipid. No information on halflives of fatty acids in different lipids of fish currently exist.

Anderson (39) studied the incorporation of label from ^{14}C-acetate into the phospholipids of goldfish subcellular fractions acclimated to different temperatures. He found that the pattern of incorporation among the liver mitochondrial phospholipids to be "PI-rich"$\geq$PC>>PE>SPG>CL for cold-adapted fish. SPG and EPG in this pattern were reversed in warm-adapted fish, but the rest of the pattern remained the same. No estimates of halflives could be made, but it was evident that PI and PC were more metabolically active than other phospholipids of goldfish liver.

Miller et al. (40) followed up the work of Anderson by analysing acyl composition of various species of diacyl choline and ethanolamine phosphoglycerides from intestinal microsomal fractions of goldfish adapted to low and high temperatures. Increases in 20:4ω-6 and 22:6ω-3 were observed in both phospholipids, but changes were similar

in magnitude. It was noted that acyl group substitution appeared to play a mojor role in altering the fatty acid composition, rather than de novo synthesis of new species of CPG and EPG via diglyceride. The latter mechanism of phospholipid synthesis (CDP-choline pathway) has been shown to be operative in liver microsomal fractions of rainbow trout (41), with cofactor requirements similar to that of the CDP-choline pathway in mammalian tissues. The importance of this pathway in fish to phospholipid acylgroup regulation relative to acyltransferase activity remains largely unknown.

Although dietary fatty acid requirements differ between fish and mammals, several authors have pointed out similarities in their patterns of fatty acid elongation and desaturation (42-44). The following pattern has been identified for the ω-3 fatty acids: 9,12,15-octadecatrienoic acid (linolenate)→6,9,12,15-octadecatetraenoic acid → 8,11,14,17-eicosatetraenoic acid → 5,8,11,14,17-eicosapentaenoic acid → 7,10,13,16,19-docosapentaenoic acid → 4,7,10,13,16,19-docosahexaenoic acid. In addition, Ninno et al. (45) showed that the order of liver microsomal 6-desaturation rates in catfish (α-linolenic > linoleic>oleic) was identical but 5-fold higher than that in the rat, indicating that fish do not necessarilly have reduced rates or altered patterns of lipid metabolism due to their poikilothermic nature. This study also showed that this species of fish had very low levels of 5-desaturase activity which would presumably be needed for conversion of 20:4ω-3 to 20:5ω-3.

Kayama et al. (44) injected ^{14}C-linolenic acid into mature kelp bass, and recovered label in eicosapentaenoic and docosahexaenoic

acids after 5 hours. In the only other study utilizing radioactive linolenate or linoleate, Owen et al. (46) measured the elongation and desaturation of dietary labelled fatty acids in turbot (Scophthalmus maximus) and rainbow trout (Salmo gairdneri). They found that while the trout converted considerable quantities of linolenic acid to docosahexaenoic acid in six days, very little conversion occurred in turbot. No elongation or desaturation of 18:1ω-9 or 18:2ω-6 was observed in turbot after six days--similar measurements on these acids were not made in the trout. These data are supported by the results of continuous feeding trials with rainbow trout (6-8) which showed that dietary 18:3ω-3 is effectively converted to longer chain, more unsaturated homologs. The slow rate of conversion of 18:2ω-6 and 18:3ω-3 to longer chain homologs by turbot may be analogous to that observed in the preceeding section by Sinclair for rat brain. Both studies indicate that dietary, preformed 20:4ω-6 or 22:6ω-3 may be more important in satisfying essential fatty acid needs than the shorter precursor fatty acids.

In contrast with the available evidence on polyunsaturated fatty acid metabolism, Sand et al. (47) measured the rates of turnover of wax esters in gouramis by feeding ^{14}C-oleate, a non-essential fatty acid. Results indicated that the biological halflife of 18:1ω-9 in that species was approximately four months. Whether or not this long halflife of oleic acid occurs in other species of fish is unknown.

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METABOLISM OF (1-^{14}C) LINOLENIC ACID IN COHO SALMON, ONCORHYNCHUS KISUTCH

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ABSTRACT

Juvenile coho salmon were injected intraperitoneally with (1-^{14}C) linolenic acid, and sampled at 24, 120, and 240 hours. Liver, heart, and gill lipids were extracted, analyzed, and halflives of individual liver phosphoglycerides and ω3 fatty acids determined from rates of loss of radioactivity. Incorporation of label into gill was much less than into either heart or liver. Inositol phosphoglycerides had the shortest halflife of all hepatic phospholipids. Total acyl halflife was shorter for the choline phosphoglycerides than for the ethanolamine phosphoglycerides, as were the halflives of all individual ω3 fatty acids. Eicosapentaenoic acid (20:5ω3) had the shortest halflife in both phospholipids (50-60 hours), while docosapentaenoic acid (22:5ω3) and docosahexaenoic acid (22:6ω3) had much longer halflives. Specific activities of the shorter chain ω3 fatty acids were much greater than the longer, more unsaturated homologs at all times, indicating possible differences in their mechanisms of incorporation into phospholipids. The fatty acid halflives reported here for salmon are in general agreement with those found previously in mammals, indicating that poikilotherms do not necessarily have greatly reduced levels of lipid metabolism.

INTRODUCTION

It is well established that certain species of fish require fatty acids of the ω3 family for normal growth and development. Dietary studies with rainbow trout (1-3) have shown that while linolenic acid (18:3ω3) efficiently fulfills the essential fatty acid requirement of these fish, its ultimate conversion product, docosahexaenoic acid (22:6ω3), also satisfies this requirement (4) and appears to be the fatty acid actually required at the tissue level. Although mammals have been shown to require only fatty acids of the ω6 family, acids of the ω3 family are found in large quantities in certain vital tissue lipids. Efforts to deplete ω3 content through dietary manipulation has proved difficult, indicating that these acids perform some as yet undetermined function. Several functions have been attributed to the essential fatty acids and the dynamics of the compounds, i.e. their rates and patterns of incorporation and turnover, have been studied both in vivo and in vitro (5-8).

The various pathways of phospholipid metabolism and the nature of many of the enzymes involved have been recently reviewed (9). It is evident that turnover of a membrane phospholipid may involve movement of intact molecules into or out of the membrane or metabolic transfers of only a portion of the phospholipid molecule, and that control over the microenvironment of membranes may be exercised through selective manipulations of acyl or base composition. The specificity of enzymes associated with phospholipid acylation or deacylation is thought to play an important role in preserving characteristic acyl composition

of membranes and to maintain physiochemical properties that relate to the function of enzymes and other membrane components.

Investigators have found that the level of essential fatty acids in the phospholipids of fish can be altered by diet (2, 10) or environmental temperature (11-13) and that fish differ in their capability to desaturate dietary linolenic acid (14).

However, little information is available on the incorporation and metabolism of the ω3 fatty acids in fish. It would seem that detailed studies on this subject could yield valuable information on the function of these molecules in both fish and mammals. This report describes the incorporation of (1-^{14}C) linolenic acid in the coho salmon and the half-lives of ω3 fatty acids in the major hepatic phospholipids.

MATERIALS AND METHODS

Radiochemicals

(1-^{14}C) linolenic acid, >99% pure and 50 mCi/mmole, was purchased from Amersham Corp., Arlington Heights, IL. Counting was carried out in a Nuclear-Chicago 720 liquid scintillation counter, using either toluene-PPO-POPOP (15) or Aquasol (New England Nuclear, Boston, MA) as fluors.

Animal Trials

The fish used in this study were yearling coho salmon, hatched and reared in circular tanks at the Food Toxicology and Nutrition Laboratory. Fish were fed a semi-purified diet (16), containing 6 percent salmon oil as lipid source. Water temperature was constant at 12 C. Fish fasted 48 hours, weighing 90±15 g and with noticeable parr marks were injected intraperitoneally with (1-^{14}C) linolenic acid in 4:1 DMSO-0.9% NaCl (0.10 μCi/μl) at a dose of 0.075 μCi/g fish. Three fish were used for each of the following three time periods: 24, 120, and 240 hours. All fish were fasted for the duration of the experiments. The 24 and 120 hour trials were carried out in plexiglass metabolism chambers with recirculating water maintained at 12±0.5 C by a refrigeration-pump unit (Lauda K-2/R, Brinkmann Instruments, Burlingame, CA). The sealed chambers were aerated by a 80:20 nitrogen:oxygen mixture, the effluent flow of which was bubbled through 20 percent KOH to trap radioactive carbon dioxide expired by the fish. Following termination of the trial, the chamber was acidified with 12 N HCl and the system purged for an additional hour. Ten day trials were carried out in

open isolated circular tanks at the Food Toxicology and Nutrition Laboratory.

Lipid Extraction and Analysis

At predetermined time periods, fish were killed by a blow on the head. Livers, hearts and gills were excised, perfused with 0.9% NaCl and either extracted immediately or frozen on solid CO_2 and stored at -40 C under nitrogen until analyzed. Lipid extracts were prepared by the procedure of Folch et al. (17), dried, weighed, and samples taken for liquid scintillation counting. All lipids were stored under nitrogen in benzene at -40 C.

Duplicate phosphorus analyses were done according to the method of Bartlett (18), using two dimensional TLC (19) to separate individual phospholipids. Additional plates were run in a similar fashion to obtain phospholipid samples for counting. Phospholipids were identified by comparison to standards and by specific spray reagents (20). Choline and ethanolamine phosphoglycerides (CPG and EPG) for fatty acid analysis were obtained by one dimensional preparative TLC using chloroform-methanol-water (65:25:4). Lipid bands were visualized with 2,7-dichlorofluorescein under U.V., scraped into ampoules, and transesterified under nitrogen with 4 percent sulfuric acid in methanol. Methyl esters were extracted with hexane, and samples of both aqueous and hexane fractions taken for counting.

EPG and CPG were analyzed for plasmalogen content by the procedure of Horrocks (21), and results showed that these compounds were present only in very small amounts.

Fatty acid methyl esters were analyzed using a Varian Aerograph

1200 gas chromatograph (FID), with a 6 ft stainless steel column packed with 15% ethylene glycol succinate on 80/100 mesh Gas Chrome P (Applied Science Laboratories, Inc., Inglewood, CA). Column flow was split 7:1 by a stainless steel splitter, and individual peaks of interest plus all remaining areas of the chromatogram were trapped in 25 cm x 1.2 mm I.D. glass capillary tubes packed in solid CO_2. Contents of the tubes were flushed with 1.0 ml toluene and counted in toluene fluor at an average counting efficiency of 87 percent. All trapping trials were done in duplicate and relative radioactivity percentages of the trapped fractions agreed to within 10 percent.

Calculations

Specific activities of individual fatty acids in choline and ethanolamine phosphoglycerides were calculated using the following equations (PL: phospholipid, either CPG or EPG; FA: fatty acid; Pi: inorganic phosphorus):

(1) $$\frac{(\text{dpm}/\mu\text{g PL Pi})}{25\ \mu\text{g PL}/\mu\text{g Pi}} \times 775\ \mu\text{g}/\mu\text{mole PL} \times \frac{\%\text{dpm in FA}}{2\ \mu\text{moles FA}/\mu\text{mole PL}} = \text{dpm}/\mu\text{mole total PL FA}$$

(2) $$\frac{\text{Total dpm from chromatogram}}{\text{dpm}/\mu\text{mole total PL FA}} \times \frac{\text{wt\% of indiv. FA}}{100} = \mu\text{moles indiv. FA}$$

(3) $$\frac{\text{dpm/indiv. FA trapped}}{\mu\text{moles indiv. FA}} = \text{dpm}/\mu\text{mole individual fatty acid}$$

where 775 is the average molecular weight of diacyl CPG and EPG.

RESULTS

Fatty Acid Oxidation and Incorporation

An average of 35 percent of administered (1-^{14}C) linolenic acid was recovered as $^{14}CO_2$ in 24 hours. This percentage did not increase appreciably with incubation periods longer than 24 hours.

Liver and heart lipids incorporated much more radioactivity than did gill lipids (Table I). However, while the amount of label in liver lipid decreased steadily with time, gill and heart lipids contained approximately the same amount of label at 24 and 240 hours, with a slight drop in activity at 120 hours.

Composition and Turnover of Phospholipids

Phospholipids comprised about 60 percent of the total liver lipids of coho salmon, the major components being CPG and EPG which collectively represented 80 percent of total phospholipid (Table II).

Figure 1 illustrates the relationship between the mean specific activities of the major phospholipids at the three time periods. CPG and EPG were the most highly labeled components, and sphingomyelin the least. However, calculation of halflives of these phopholipids revealed that sphingomyelin and IPG were turning over at the greatest rate, followed by CPG, SPG, EPG, and PA (Table II). All phospholipids decreased in specific activity with time except cardiolipin, in which specific activity was greater at both 24 and 240 hours than at 120 hours. All phospholipids seemed to lose activity at a rate much slower than that at which they incorporated label. Semi-log plots of specific activity of total lipid, CPG and EPG of liver (Figure 2) show linear relationships

over time, confirming that disappearance of label from the acyl portions of the phospholipids is a logarithmic (halflife) function. CPG acyl groups had a halflife of 69 hours, while that of EPG was greater (129 hours). Total liver lipid halflife was 106 hours, intermediate to all liver phospholipid values.

Composition of Acyl Groups of CPG and EPG

Acyl group analysis of CPG and EPG (Table III) revealed that CPG contained more 14:0 and 16:0 than did EPG, and that the reverse was true for 18:1 and 20:1. EPG also contained slightly greater amounts of 20:5ω3 and 22:6ω3, and in general was more unsaturated than its choline counterpart, as shown by the unsaturation indices. The ω3/ω6 ratio was considerably lower in CPG, due primarily to a higher content of 20:4ω6 in that phospholipid.

Turnover of Acyl Groups of CPG and EPG

The percentage of label found in the acyl groups of both EPG and CPG averaged ca. 96 percent of that in the total phospholipid molecules, and this amount did not change appreciably with time. Considerable radioactivity was associated with 18:3ω3 at the earlier times, but the weight percent of this fatty acid was negligible and specific activities could not be calculated.

The change in specific activity over time of the three major ω3 fatty acids of hepatic CPG and EPG (20:5ω3, 22:5ω3, and 22:6ω3) is shown in Figure 3. Variations between individual fish account for the deviations, which appear to diminish with time. At 24 hours 20:5ω3 had the highest specific activity in both CPG and EPG, but this value was equal to or less than that of 22:5ω3 at 240 hours. The specific activity

of 22:6ω3 was less than that of all other fatty acids throughout the ten day duration. All ω3 fatty acids decreased in specific activity between 120 and 240 hours, indicating that peak incorporation had passed and that halflives could be calculated from label depletion during this period. Comparison of the halflives of the ω3 fatty acids (Table IV) shows that in the choline phosphoglycerides, 20:5ω3 and 18:4ω3 had the shortest halflives, with 22:5ω3 and 22:6ω3 having the longest. The halflife of each ω3 homolog was longer in EPG than in CPG, and with the exception of 18:4ω3, which had a much longer halflife in EPG, the general pattern of fatty acid halflives relative to each other was the same in both phospholipids. A sharp break in specific activity occured between 20:4ω3 and 20:5ω3 in both phospholipids, and was still evident at 240 hours. The halflife of 20:4ω6 was not different from those of the ω3 fatty acids in CPG, but in EPG its halflife was much longer than any fatty acid measured.

DISCUSSION

Fatty Acid Oxidation and Incorporation

The recovery of significant amounts of radioactivity in expired carbon dioxide is compatible with the data of Brown and Tappel (22) who found that carp mitochondria oxidized linolenic acid at a faster rate than all other fatty acids measured, and was roughly twice that of 20:5ω3, 22:6ω3, 18:1ω9 and 16:0. The fact that most of the labeled CO_2 was recovered during the initial 24 hour period indicates that it was the initially available free (CoA ester) linolenic acid that was being oxidized, not labeled acids hydrolyzed from phospholipids or acylglycerols. Similar observations were made by Owen et al. (14), who noted that $^{14}CO_2$ expired by turbot following labeled 18:3ω3, 18:2ω6, or 18:1ω9 intake occured primarily during the first 24 hours.

The low uptake of radioactivity in gill lipids (Table I) was also observed by Anderson (24) following ^{14}C-acetate and $Na_2H^{32}PO_4$ injection. This may reflect either a slow rate of turnover of gill lipids or rapid clearance of label from the blood by heart and liver.

Turnover of Phospholipids

Choline phosphoglycerides had the highest specific activity at 24 and 120 hours, but their relatively short halflife resulted in the ethanolamine phosphoglycerides having a higher specific activity at 240 hours. The relative turnover pattern of four of the major phospholipids (IPG>CPG>SPG>PE) agrees with that observed by Smith and Eng (23) in rat myelin and by Anderson (24) in goldfish liver mitochondria following ^{14}C-acetate labelling. De Tomas and Mercuri (25) also found

that label from 1-^{14}C linolenic acid accumulated to a greater extent in CPG than in EPG in rat liver lipid. Lee and Snyder (26) measured relative rates of turnover of ^{14}C-acetate-labeled phospholipids in rat liver microsomal fractions and found the pattern to be CPG>EPG,SPG>IPG>S. In the present study IPG and S appear to be turning over at a greater rate than other phospholipids in whole liver of the salmon. The halflives of total lipid, CPG, and EPG (106, 69, and 107 hrs, respectively) are similar to those found by Omura et al. (27) for total lipid and total phospholipid of rat endoplasmic reticulum (97 and 56-78 hrs, respectively) following ^{14}C-acetate labelling. In contrast, however, calculations from the data of Poovaiah et al. (6) showed a halflife of ca. 9 hours for rat liver CPG and EPG after ^{14}C-linolenic acid injection.

The observed long halflife of label in phosphatidic acid (134 hours) does not rule out the involvement of this species in the entry of label into other phospholipids since the amount of radioactivity present at any one time in PA would be determined in part by the net effect of phosphatidate phosphatase and diglyceride kinase, i.e. forward and reverse reactions of the CDP-choline pathway. The high rate of loss of label from inositol phosphoglycerides ($t_{\frac{1}{2}}$=51 hours) may reflect either (1) acyltransferase activity or (2) stepwise hydrolysis to fatty acids, glycerol-3-P and inositol (28). The latter reaction would also contribute to the retention of label in PA noted above.

Cardiolipin specific activity was higher at both 24 and 240 hours than at 120 hours. A biphasic pattern of label loss was also noted in rat liver mitochondrial cardiolipin after ^{14}C-linoleate injection (29)

suggesting that label in cardiolipin was due principally to fatty acids esterified by two mechanisms, one rapid and the other slower.

Acyl Composition of CPG and EPG

The fatty acid composition of the two major hepatic phospholipids is different in many respects from that reported for mitochondrial and microsomal fractions of rat liver by Macfarlane et al. (30). Rat liver contains more palmitate and arachidonate, and less oleate in EPG than in CPG. The opposite of true for salmon liver. However, EPG of both rat and salmon contains more 22:6ω3 and is generally more unsaturated than CPG.

Fatty Acid Incorporation and Turnover

The data presented in Table IV shows that the incorporation and turnover patterns of the ω3 fatty acids in choline and ethanolamine phosphoglycerides is complex. The specific activity of 20:5ω3 in EPG is equal to that of CPG at 120 hours, even though its precursors (18:4ω3 and 20:4ω3) in EPG have only half the activity of their counterparts in CPG. This may indicate some transfer of 20:5ω3 from CPG to EPG, through acyl or base exchange. A second possible explanation of this occurrence is suggested by Holub and Kuksis (31), who found that the specific activities of the molecular species of CPG and EPG in rat liver following injection of labeled linoleate, linolenate, and arachidonate were not all the same. The tetraenoic species formed after injection of arachidonate indicated the arachidonate was incorporated primarily through reacylation, as found by previous authors (7, 32), and that the fatty acid at the 1-position did not appreciably affect the acylation. On the other hand, the specific activity of the 16:0-18:2 species was 2-3 times

higher than that of the 18:0-18:2 species following linoleate injection, perhaps reflecting equal incorporation by the acylation pathway in combination with de novo synthesis of the 16:0-18:2 species by the glycerophosphate pathway. The results reported here for ω3 fatty acids in coho salmon liver seem to support these observations. CPG, which contained twice the 16:0 content of EPG, also had much higher specific activities in 18:4ω3 and 20:4ω3 than did its ethanolamine counterpart. Howevever, the specific activities of 20:5ω3 and 22:6ω3 fatty acids, which in fish could be considered analogous to 20:4ω6 in the rat, were approximately the same in both phospholipids.

Hill and Lands (32) measured the rates of esterification of fatty acid-CoA esters to 1-acyl-glycerophosphate and 1-acyl-glycerophosphatidyl choline in rat liver microsomes in vitro, and found that 20:5ω3 and 22:6ω3 were much more rapidly esterified to 1-acyl-GPC than to 1-acyl-GP. However, the reverse was true for 18:3ω3 but the difference between the two rates was less and both were relatively high. These results indicated that 18:3ω3 would be incorporated into phospholipid rapidly via both de novo phosphatidate synthesis and acyltransferase activity, while the latter mechanism would be the primary route of incorporation of 20:5ω3 and 22:6ω3. The apparent dichotomy in specific activities between 18:4ω3 and 20:4ω3 on one hand, and 20:5ω3, 22:5ω3, and 22:6ω3 on the other (Table IV) seems to support the results of Hill and Lands and the suggestion by Holub and Kuksis that different (or multiple) pathways may be operating for the incorporation of the shorter, less unsaturated ω3 homologs than for those that are longer and more unsaturated. Additional evidence is offered by the observation that in the

phospholipids analyzed in this study, label appeared to be incorporated at a rate which exceeded its removal. This phenomenon can be noted in several other label depletion studies (7, 25, 29), and points out possible differences in interpretation between incorporation and depletion rates. This difference between incorporation and removal rate would not be expected if only one mechanism, e.g. acyltransferase activity, were responsible for the replacement of acyl groups in the phospholipids.

The general magnitude of the acyl halflives reported here resemble those that may be calculated from the data of van Golde et al. (33), who examined the rates of replacement of acyl groups in the 2-position of liver CPG in essential fatty acid-deficient rats following EFA feeding. The approximate halflives of 20:3ω9 and its replacement 20:4ω6 were 48 and 95 hours, respectively, during the initial 120 hours.

The halflives of the ω3 fatty acids do not always reflect their quantitative presence in the phospholipids. For example, 22:5ω3 is found only in low amounts but has the longest halflife, and 20:5ω3 has a short halflife but is more abundant. Apparently 20:5ω3 is being utilized in pathways other than that of elongation to 22:5ω3.

Continuous feeding trials with coho salmon (34) have shown that when dietary trilinolenin was increased from 1 to 5%, 18:3ω3 content in total phospholipid increased by the greatest percentage (almost 5-fold), followed by 18:4ω3 and 22:5ω3, although the latter was still relatively low (3.1%). No accumulation of 20:4ω3 occurred. Brenner and Peluffo (35) noted a decrease in reaction velocity of _in vitro_ desaturation of 18:3ω3 to 18:4ω3 through competitive inhibition by 22:6ω3, an observation which is consistent with the accumulation of dietary 18:3ω3 noted above.

From these studies plus the present data on halflives, it is evident that the desaturation of 18:3ω3 and 22:5ω3 may be key points in the control of long-chain ω3 content of phospholipids. The apparent high metabolic activity of 20:5ω3 merits further investigation as to its role in control of the desaturation-elongation sequence, and its possible involvement in other pathways involving fatty acids. The observation that the polyunsaturated acyl groups of CPG are turning over at rates different from those of EPG may reflect acyltransferase specificity not only for fatty acids but also the phospholipids. Such specificity would undoubtedly play a basic role in influencing acyl composition of fish membranes.

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TABLE I

Incorporation of Label from (1-^{14}C) Linolenic Acid into Tissue Lipids of Coho Salmon

	dpm/mg total lipid (x10^3)[a]		
Hrs after injection	Heart	Gill	Liver
24	27.7±13.6	2.8±2.0	41.0±7.5
120	18.8± 3.5	1.3±0.5	21.9±6.4
240	33.6± 8.1	2.2±1.2	8.7±1.6

[a]Each value is mean ± std. dev. of three fish.

TABLE II

Composition and Halflives of Phospholipids of Coho Salmon Liver[a]

Phospholipid	Percent[b] ± std. dev.	Halflife (hrs)
Choline phosphoglycerides	56.9 ± 3.5	69
Ethanolamine phosphoglyc.	20.4 ± 2.1	107
Inositol phosphoglycerides	6.3 ± .8	51
Sphingomyelin	4.7 ± 1.0	54
Serine phosphoglycerides	3.3 ± .6	94
Cardiolipin	2.1 ± .4	n.d.[c]
Phosphatidic acid	0.6 .3	134
Lysophosphatidyl choline	1.3 .6	n.d.
Lysophosphatidyl ethanolamine	1.2 .6	n.d.
Unknown	2.5 ± 1.7	n.d.

[a]Average of nine fish.

[b]Percent of total lipid phosphorous.

[c]Not determined.

TABLE III

Acyl Group Composition of Choline and Ethanolamine Phosphoglycerides from Liver of Coho Salmon[a]

Fatty Acid	Percent ± std. dev. CPG	EPG
14:0	1.4 ± .6	0.3 ± .1
14:1	0.2 ± .03	0.3 ± .1
16:0	20.5 ± 3.8	9.7 ± 1.1
16:1	3.7 ± .9	1.2 ± .3
18:0	7.3 ± 1.1	6.5 ± .9
18:1ω9	13.9 ± 2.6	20.3 ± 2.3
18:2ω6	0.9 ± .2	1.4 ± .4
20:1ω9	0.8 ± .3	2.2 ± .4
18:3ω3	tr[b]	tr
18:4ω3	0.6 ± .2	0.8 ± .3
20:2ω9	1.6 ± 1.0	0.8 ± .7
20:3ω6	0.2 ± .1	0.3 ± .1
20:4ω6	3.8 ± .8	1.1 ± .2
20:4ω3	0.4 ± .3	0.6 ± .3
20:5ω3	5.7 ± .8	8.2 ± 1.7
22:4ω6	tr	tr
22:4ω3, 22:5ω6	tr	tr
22:5ω3	2.3 ± .5	2.7 ± .4
22:6ω3	36.4 ± 3.6	42.8 ± 2.8
U.I.[c]	302	351
Total ω6	4.9	2.8
Total ω3	45.4	55.1
ω3/ω6	9.3	19.7

[a]Average of nine fish.

[b]Trace

[c]U.I. = unsaturation index, defined as Σ(number of double bonds in each fatty acid) x (% of each fatty acid).

TABLE IV

Comparison of Halflives and Specific Activity of Fatty Acids in Choline and Ethanolamine Phosphoglycerides of Liver[a]

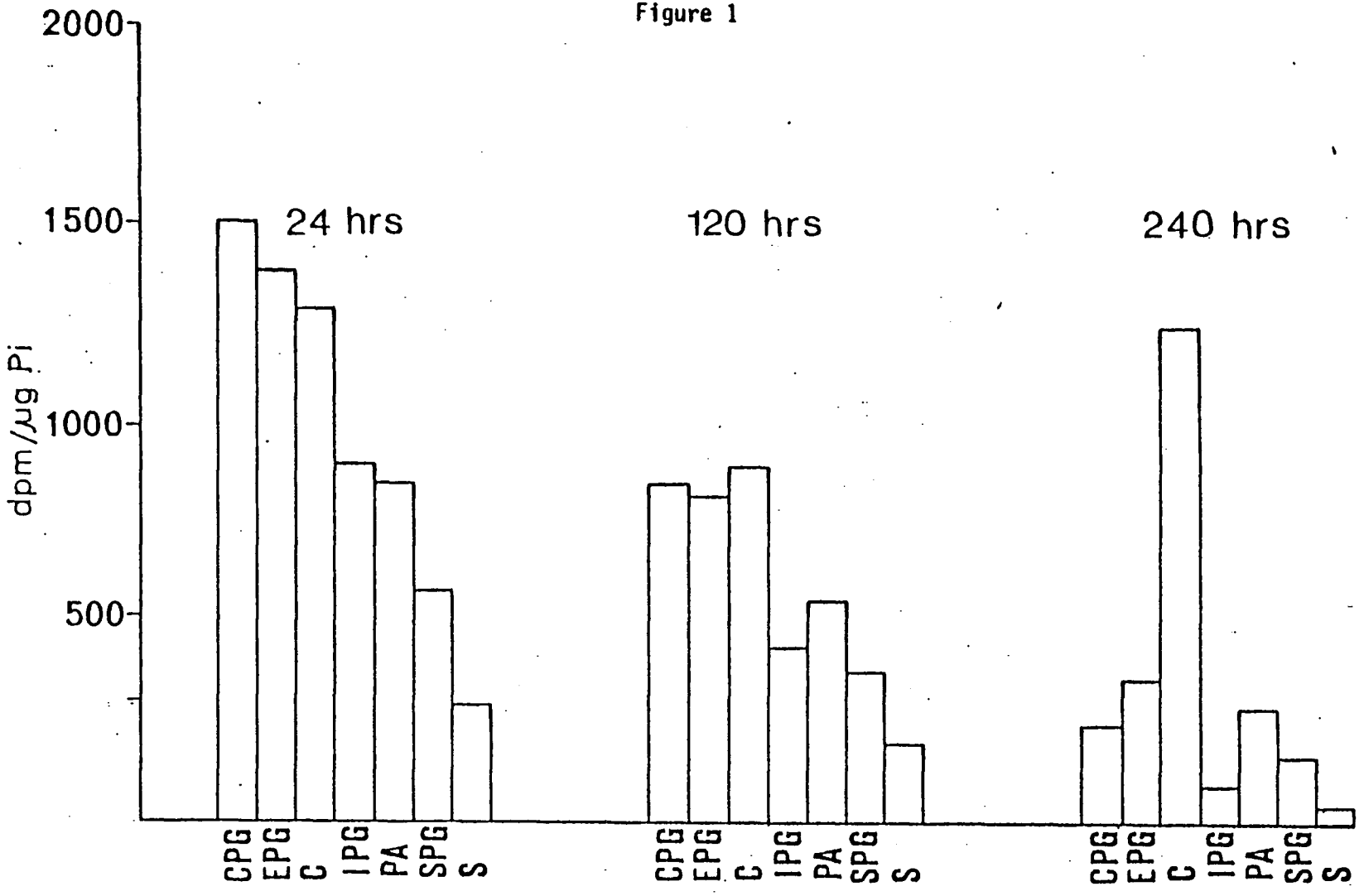
	Choline phosphoglycerides			Ethanolamine phosphoglycerides		
		dpm/μmole ($x10^3$)			dpm/μmole ($x10^3$)	
Fatty Acid	$t_{1/2}$(hrs)	120 hrs	240 hrs	$t_{1/2}$(hrs)	120 hrs	240 hrs
18:4ω3	64	237	65.5	172	138	85.0
20:4ω3	80	306	109	96	139	57.6
20:5ω3	53	40.0	7.2	65	40.5	11.5
22:5ω3	168	29.0	17.0	192	17.6	11.5
22:6ω3	100	2.2	0.9	128	1.8	0.9
20:4ω6	70	14.6	4.4	307	43.8	34.0

[a]Each value is mean of three fish.

LEGEND TO FIGURE 1

Figure 1. Distribution of radioactivity in liver phospholipids following injection of (1-^{14}C) linolenic acid. Each bar represents mean of three fish. CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides; C, cardiolipin; IPG, inositol phosphoglycerides; PA, phosphatidic acid; SPG, serine phosphoglycerides; S, sphingomyelin.

Figure 1


2000
1500
1000
500
dpm/µg Pi
24 hrs
120 hrs
240 hrs
CPG
EPG
C
IPG
PA
SPG
S

LEGEND TO FIGURE 2

Figure 2. Loss of radioactivity from liver lipids following injection of (1-^{14}C) linolenic acid. Specific activity units are as follows: total lipid, dpm/mg lipid; CPG and EPG, dpm/μmole acyl groups. Each point represents mean of three fish.

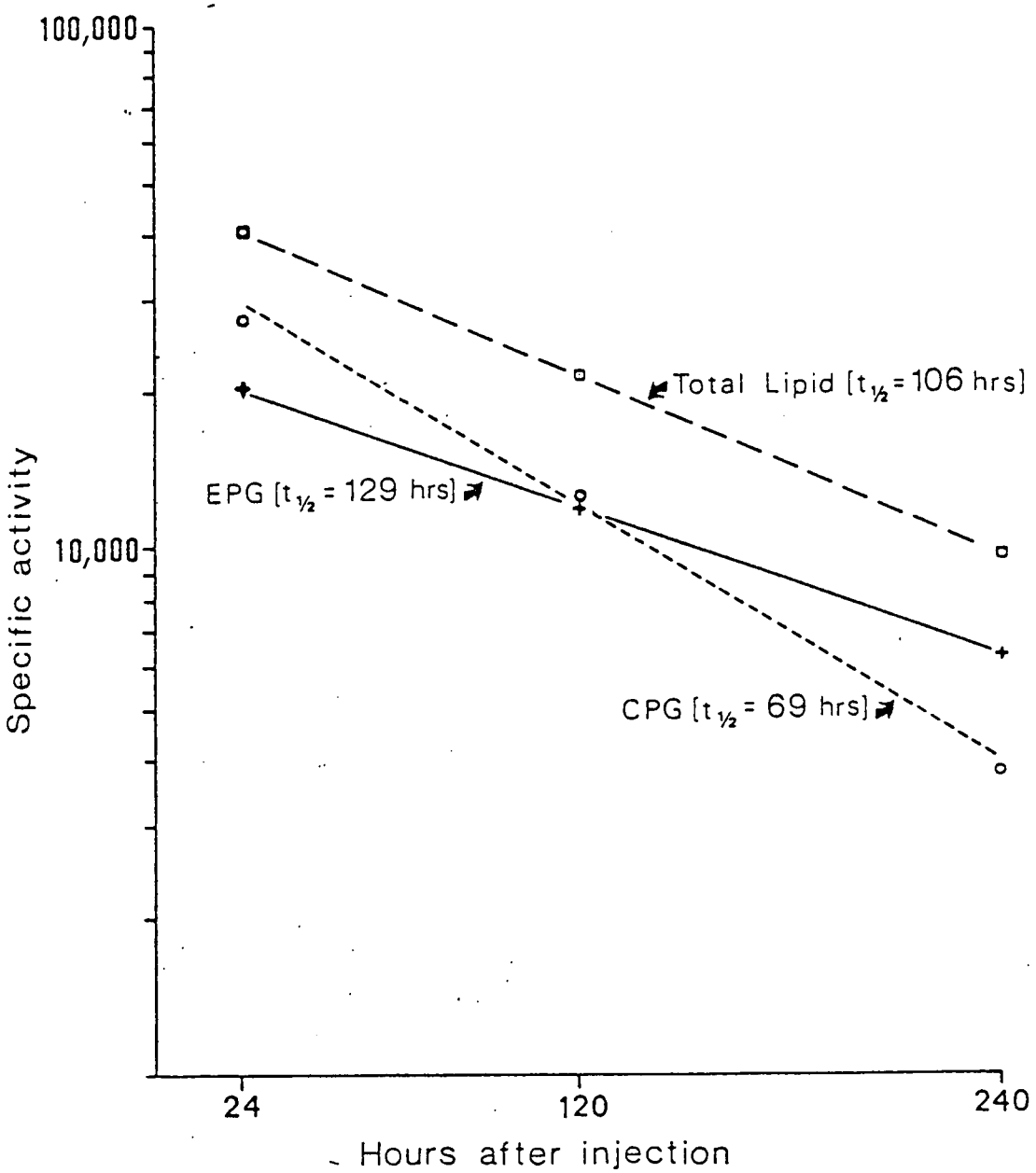
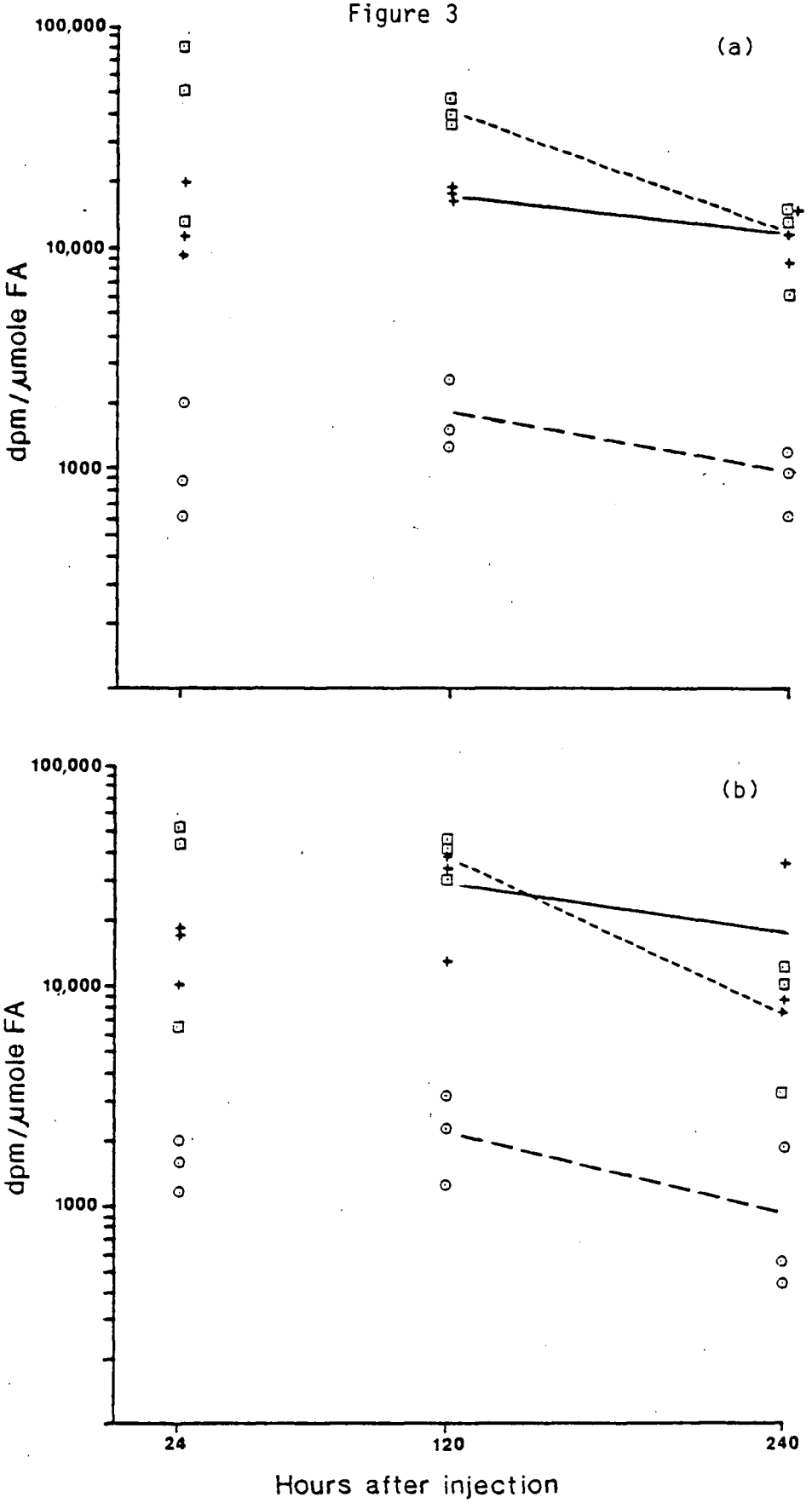

100,000
10,000
Specific activity
Total Lipid [t½ = 106 hrs]
EPG [t½ = 129 hrs]
CPG [t½ = 69 hrs]
24
120
240
Hours after injection

Figure 2

LEGEND TO FIGURE 3

Figure 3. Specific activity of fatty acids of (a) ethanolamine and (b) choline phosphoglycerides of liver at various times after injection with (1-^{14}C) linolenic acid. □ , 20:5ω3; + , 22:5ω3; ○ , 22:6ω3. Each point represents one fish.

Figure 3


(a)
100,000
10,000
1000
dpm/μmole FA
(b)
100,000
10,000
1000
dpm/μmole FA
24
120
240
Hours after injection

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Composition of Diet

Ingredient	Percent (dry wt. basis)[a]
Casein	50
Dextrin	18
Alpha-cellulose	9.8
Gelatin	8
Salmon oil	6
Mineral mix[b]	4
Vitamin mix[c]	2
Carboxymethylcellulose	1.2
Choline chloride	1.0

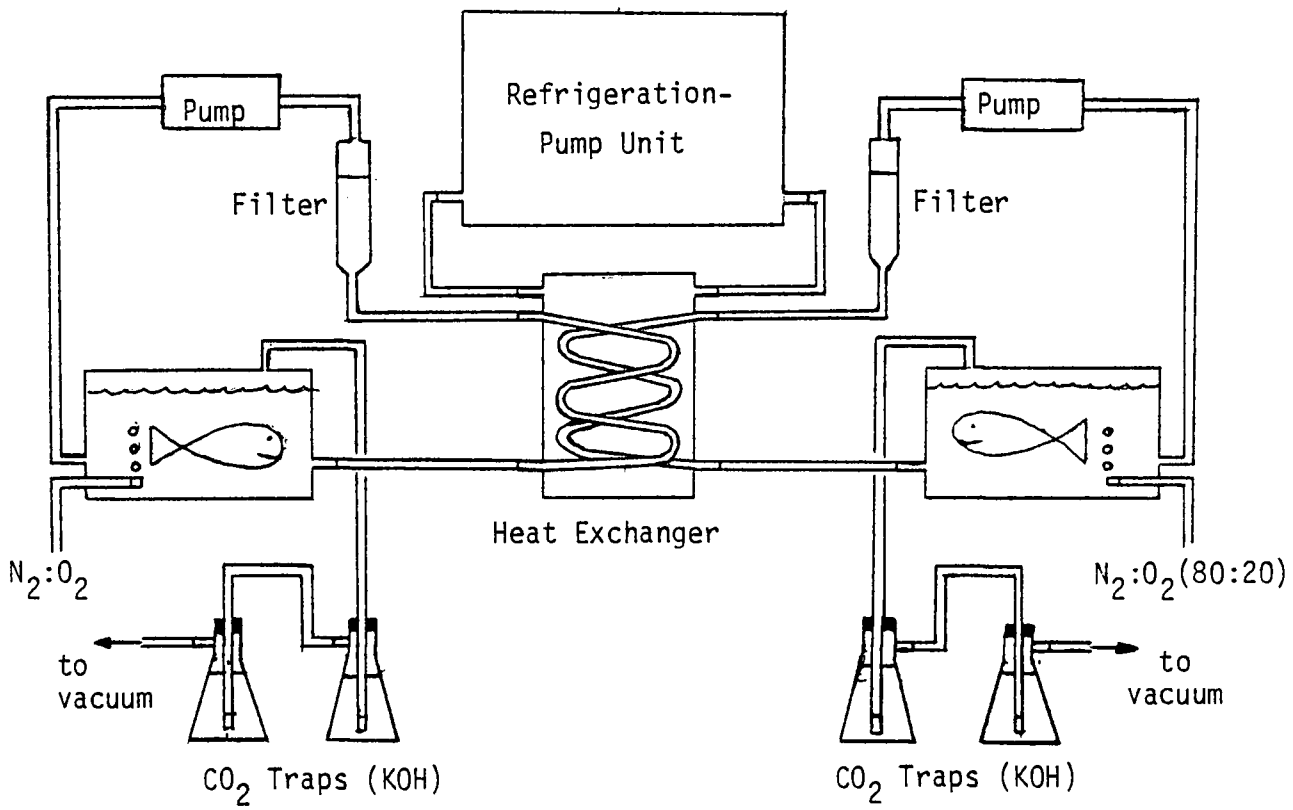
[a]Diet is 35 percent dry ingredients, 65% water.

[b]Calcium carbonate ($CaCO_3$, 2.100%), calcium phosphate ($CaHPO_4 \cdot 2H_2O$, 73.500%), potassium phosphate (K_2HPO_4, 8.100%), potassium sulfate (K_2SO_4, 6.800%), sodium chloride (NaCl, 3.060%), sodium phosphate ($Na_2HPO_4 \cdot 6H_2O$, 2.140%), magnesium oxide (MgO, 2.500%), ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$, 0.558%), manganese carbonate ($MnCo_3$, 0.418%), cupric carbonate [$2CuCO_3Cu(OH)_2$, 0.034%], zinc carbonate ($ZnCO_3$, 0.081%), potassium iodide (KI, 0.001%), sodium fluoride (NaF, 0.002%), cobalt chloride ($CoCl_2$, 0.020%), and citric acid ($C_6H_8O_7 \cdot H_2O$, 0.686%).

[c]Thiamine hydrochloride (0.3200%), riboflavin (0.7200%), niacinamide (2.5600%), biotin (0.0080%), D calcium pantothenate (1.4400%), pyridoxine hydrochloride (0.2400%), folic acid (0.0960%), menadione (0.0800%), vitamin B_{12} (cobalamine, 3000 µg/g, 0.2667%), i-inositol (meso, 12.5000%), ascorbic acid (6.0000%), p-aminobenzoic acid (2.0000%), vitamin D_2 (500,000 USP/g, 0.0400%), vitamin A (250,000 units/g, 0.50000%), dl-a-tocopherol (250 IU/g, 13.2%)k and α-cellulose (60.0293%).

Appendix II

Apparatus for CO_2 Collection



Volume of metabolic chambers: 3.5 l

Circulation rate: 0.3 l/min (controlled with variable transformer)

Temperature at metabolic chamber: 12 0.5 C

Temperature exiting filter: 15 C