The Characterisation of Docosahexaenoic Acid (22:6n-3) Biosynthesis in the Liver of Rainbow Trout *(Oncorhynchus mykiss)*

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by

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To Giselle and Giovanna

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and has not been submitted in any previous application for a degree.

The work of which it is a record has been carried out by myself. The nature and extent of any work carried out by, or in conjunction with, others has been specifically acknowledged by reference.

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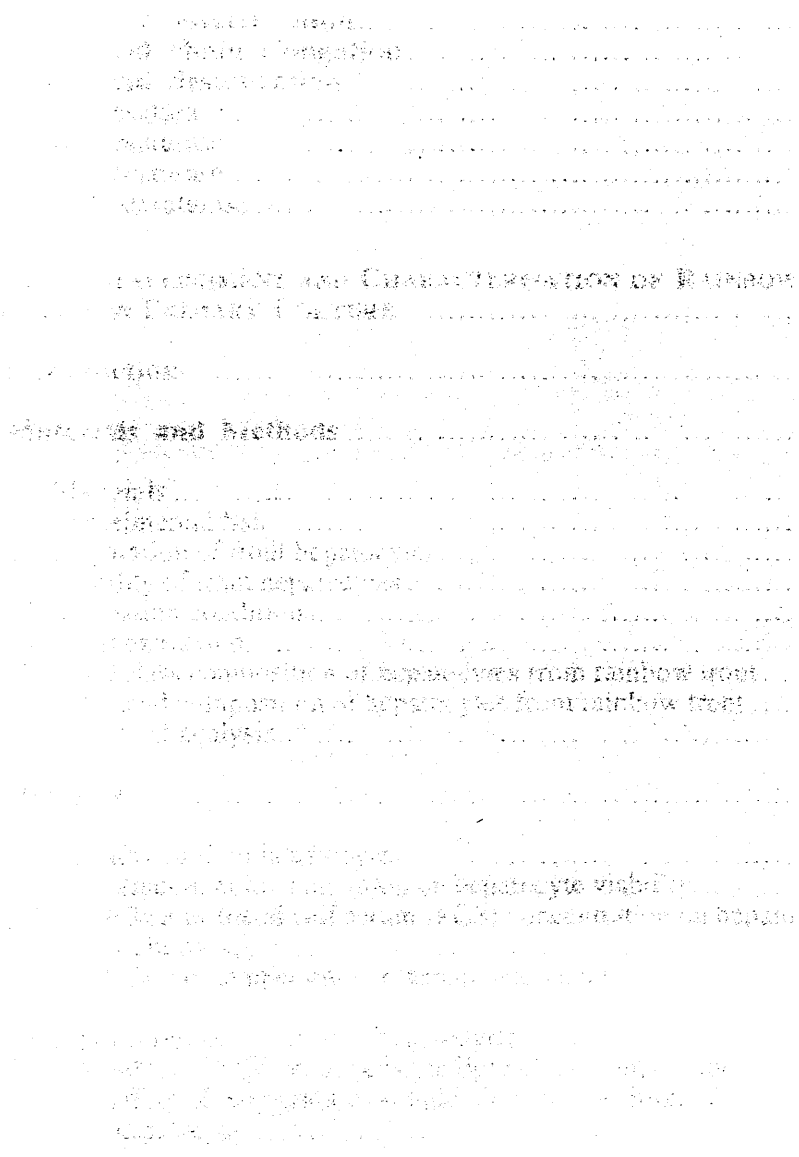
SUMMARY

The pathway for the biosynthesis of docosahexaenoic acid (22:6n-3) from linolenic acid (18:3n-3) was investigated in rainbow trout *(Oncorhynchus mykiss)* liver *in vitro*, using primary cultures of hepatocytes and liver microsomes to investigate the products of desaturation and elongation of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3. Argentation thin-layer chromatography and radio gas chromatography were employed to analyse the metabolic products of the radiolabelled fatty acid substrates and to determine the rate of conversion of 18:3n-3 to 22:6n-3. The recovery of radioactivity in various polyunsaturated fatty acids (PUFA) of trout hepatocyte lipid, including 20:5n-3 and 22:6n-3, established that juvenile trout were capable of converting 18:3n-3 to 22:6n-3. To establish the extent to which the formation of 22:6n-3 was enhanced in the absence of dietary PUFA, particularly 20:5n-3 and 22:6n-3, and therefore, facilitate the investigation of 22:6n-3 biosynthesis, rainbow trout were fed a diet based on olive oil and deficient in (n-3) PUFA. Feeding the diet deficient in (n-3) PUFA efficiently reduced the endogenous levels of (n-3) PUFA in trout hepatocytes and at the same time markedly increased the rate of 22:6n-3 formation from both [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3. When the desaturation and elongation of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 were investigated in microsomes isolated from trout liver, no radioactivity from either substrate was recovered in 22:6n-3. High proportions of radioactivity from [1-^{14}C]-20:5n-3 were, however, recovered in 24:6n-3 and 24:5n-3. These radiolabelled C_{24}-PUFA produced by the microsomal incubations were separated by argentation chromatography and used as substrates in incubations with hepatocytes isolated from trout liver. Docosahexaenoic acid (22:6n-3) was generated from both radiolabelled C_{24}-PUFA substrates by trout hepatocytes. The results establish that the biosynthesis of 22:6n-3 in rainbow trout hepatocytes does not involve Δ4-desaturation of 22:5n-3 but rather proceeds *via* the Δ6-desaturation of 24:5n-3 with the subsequent chain shortening of the 24:6n-3 produced. Cyclopropene fatty acids and curcumin significantly inhibited

the desaturation and elongation activity of hepatocytes and liver microsomes from rainbow trout. The Δ6 and Δ5-desaturase and elongase substrate specificities were investigated; it was shown that (n-3) PUFA substrates were always preferred by the enzymes over (n-6) PUFA.



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ABBREVIATIONS

ATP	adenosine triphosphate
BHT	butylatedhydroxytoluene
BSA	bovine serum albumin
CE	cholesterol ester
CHO	cholesterol
CL	cardiolipin
CoA	coenzyme A
CPFA	cyclopropane fatty acid
CPM	counts per minute
DPM	desintegration per minute
EDTA	ethylenediaminetetraacetate
EGP	ethanolamine phosphoglycerides
FAD	flavin adenine dinucleotide
FADH2	flavin adenine dinucleotide reduced form
FAME	fatty acid methyl ester
FCS	foetal calf serum
FFA	free fatty acid
FPLC	fast protein liquid chromatography
HEPES	*n*'-2-hydroxyethylpiperazine-*n*'-2-ethanesulfonic acid
HPTLC	high performance thin layer chromatography
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserime
PUFA	polyunsaturated fatty acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	sphingomyelin
TAG	triacylglycerol
TLC	thin layer chromatography
tSIE	transformed spectral index of the external standard

CHAPTER 1
GENERAL INTRODUCTION

In recent years, the beneficial effects of long chain polyunsaturated fatty acids (PUFA) of the (n-3) series, specifically docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3), in human health and development have been extensively reported (Vergroesen and Crawford, 1989; Anon., 1992). These (n-3) PUFA which are naturally abundant in fish have been shown convincingly to play a very important role in alleviating human cardiovascular disease, and are also beneficial in the treatment of other diseases including respiratory, renal and inflammatory disorders (Knapp, 1995; Enders *et al.*, 1995). Among the first evidence was the observation of a high incidence of coronary thrombosis among Greenland Eskimos who migrated to Denmark, compared to the very low rate in those that remained in their native environment and do not appear to suffer from this disorder naturally (Dyerberg 1982). This study revealed that the major difference between the populations was the amount of marine fat consumed, especially the levels of (n-3) polyunsaturated fatty acids such as eicosapentaenoic (20:5n-3) and docosahexaenoic acid (22:6n-3). Following the findings of Dyerberg, several studies were carried out on the modulating effects of 20:5n-3 on the synthesis of prostaglandins and leukotrienes from arachidonic acid (20:4n-6) and their consequences for human ischaemic heart conditions (Knapp, 1990; Kinsella *et al.*, 1990; Schmidt and Dyerberg, 1994). More recently, emphasis has been on the importance of 22:6n-3 in neural development in human infants (Innis, 1991; Carlson, 1995;). Several studies have reported that term human infants fed artificially with commercial milk formulae have less 22:6n-3 and 20:4n-6 for their biological needs than breast-fed children (Giovannini *et al.*, 1994; Decsi *et al.*, 1995; Giovannini *et al.*, 1995; Luukkainen *et al.*, 1995; Koletzko *et al.*, 1995). In fact, it has been recently proved that preterm infants have a more rapid development of visual acuity if fed human milk than fed a standard commercial formula devoided of PUFA (Jørgensen *et al.*, 1996; Makrides *et*

al., 1996; Calson and Werkman, 1996). The major source of long-chain (n-3)PUFA, both 20:5n-3 and 22:6n-3 utilised as supplements for human consumption is fish oil. Despite current interest in these marine-derived PUFA very little is known about their biosynthesis in fish, particularly, so far 22:6n-3.

1.1. Fatty acid nomenclature

Fatty acids are long chain molecules that contain a carboxyl and a methyl end group. The fatty acids have both a familiar name and a numerical identifier. In the numerical designation the number to the left of the colon indicates the number of carbons in the fatty acid. The number immediately to the right of the colon indicates the number of double bonds in the fatty acid. The numbers after the delta sign indicate the location of the double bonds counting from the carbon at the carboxyl end of the fatty acid, which is the number 1 carbon. Thus, 18:3 Δ 9, 12, 15 is a fatty acid of 18 carbons with 3 double bonds that are between the 9^{th} and 10^{th}, the 12^{th} and 13^{th}, and the 15^{th} and 16^{th} carbons in the fatty acid molecule (Table 1.1). Its familiar name is linolenic acid.

Fatty acids can be grouped in families according to the number of double bonds in the fatty acid. Fatty acids with no double bonds are known as saturated fatty acids; those with one double bond are called monounsaturated; and those with two or more double bonds are called polyunsaturated fatty acids. Another classification is the shorthand notation "c:xn-y", which means that "c" indicates number of carbons in the acyl-chain with "x" ethylenic bonds, and most importantly, the "n" value shows that the ultimate ethylenic bond is "y" carbons from and including the terminal methyl group. For example, if the double bond nearest the methyl end of the molecule is 3 carbons from the methyl end, the fatty acid is known as an n-3 or ω3 fatty acid. Thus, 18:3Δ9,12,15 can also be called 18:3n-3. For n-6 fatty acids, the final double bond is

Table 1.1. Fatty acids commonly found in fish liver.

Common name	Systematic name	Abbreviations	Melting Point (°C)	Structural formula
Stearic	octadecanoic	18:0	+70.1	$CH_3(CH_2)_{16}COOH$
Oleic	9-octadecamonoenoic	18:1Δ9 or 18:1n-9	+16.3	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
Linoleic	9,12-octadecadienoic	18:2Δ9,12 or 18:2n-6	-10	$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6COOH$
Linolenic	9,12,15-octadecatrienoic	18:3Δ9,12,15 or 18:3n-3	-10	$CH_3CH_2(CH{=}CHCH_2)_3(CH_2)_6COOH$
Stearidonic	6,9,12,15-octadecatetraenoic	18:4Δ6,9,12,15 or 18:4n-3	n.a.	$CH_3CH_2(CH{=}CHCH_2)_4(CH_2)_3COOH$
Arachidonic	5,8,11,14-eicosatetraenoic	20:4Δ5,8,11,14 or 20:4n-6	-49.5	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COOH$
Timnodonic	5,8,11,14,17-eicosapentaenoic	20:5Δ5,8,11,14,17 or 20:5n-3	-54.4	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_2COOH$
Clupanodonic	7,10,13,16,19-docosapentaenoic	22:5Δ7,10,13,16,19 or 22:5n-3	n.a.	$CH_3CH_2(CH{=}CHCH_2)_5(CH_2)_4COOH$
Cervonic	4,7,10,13,16,19-docosahexaenoic	22:6Δ4,7,10,13,16,19 or 22:6n-3	-44.5	$CH_3CH_2(CH{=}CHCH_2)_6CH_2COOH$

n.a. not available in the literature.

6 carbons from the methyl end. Four major families of unsaturated fatty acids exist in animals: n-3, n-6, n-9, and n-7 fatty acids.

1.2. Physical properties of fatty acids

Fatty acids are constituents of all cell membranes which separate the cell contents from the extracellular medium. The physical properties of every cell membrane are determined to a large extend by the fatty acid composition of phospholipids therein and their interactions with cholesterol and proteins, including proteins which are components of the cell's cytoskeleton. The appropriate environment for membrane function and fluidity is largely dependent on the chain length and the degree of unsaturation of the fatty acids (Bell *et al.*, 1986). Phospholipids containing only fully saturated acyl chains adopt a configuration in which the acyl chain carbons are in extended all-*trans* conformation. Introduction of a *cis* double bond precludes the adoption of the *trans* conformation in at least one position of the chain, disrupting the close packing that is possible with all-*trans* configuration. The presence of a even a single double bond is sufficient to exert a profound influence on the physical properties (Stubbs and Smith, 1984). For instance, the melting point of stearic acid (18:0) and oleic acid (18:1n-9) are 70°C and 16.3°C, respectively. The melting point of the octadecamonoenoic acids (18:1) isomers are highly dependent on the position of the double bond. The nearer the double bond to the centre of the chain, the lower the phase transition. For example, 18:1Δ9 melts at 10°C, whereas its isomer, 18:1Δ16, which has the double bond at the end of the chain, melts at 53°C. The situation with dienoic acids (18:2) is similar, with the Δ8,11 isomer melting at -15.5°C and the Δ14,17 isomer at 37°C (Stubbs and Smith, 1984). The melting points of fatty acids with more than two double bonds are generally below the water freezing point. For example, α-linolenic acid (18:3n-3) melts at -10°C, arachidonic (20:4n-6) at -49.5°C, eicosapentaenoic acid (20:5n-3) at -54.4°C and docosahexaenoic acid (22:6n-3) at -44.5°C. Therefore, all PUFA are fluid at temperatures well below those ever encountered by biological systems (Bell *et*

al., 1986). In fish and other poikilotherms the degree of unsaturation of membrane fatty acids is important in the process of adaptation to different environmental temperatures. In addition to their membrane structural functions, several polyunsaturated fatty acids (PUFA), including 18:3n-6, 20:4n-6 and 20:5n-3 are known to be metabolically active precursors for the production of prostaglandins and other eicosanoids.

1.3. Fatty acid biosynthesis *de novo*

The mechanism of fatty acid biosynthesis in fish is believed to be similar to that occurring in mammals. The overall process of fatty acid synthesis involves three separate enzyme systems that catalyse, respectively, the biosynthesis of palmitate from acetyl-CoA, the chain elongation of palmitate, and the desaturation of palmitate and its chain elongated products. The first pathway occurs in the cytosol, while chain elongation proceeds both in mitochondria and endoplasmic reticulum, and desaturation occurs in the endoplasmic reticulum (Mathews and Van Holde 1990).

The first step in fatty acid biosynthesis is the carboxylation of acetyl-CoA, giving rise to malonyl-CoA, which is subsequently converted to palmitoyl-CoA *via* a series of condensation, reduction and dehydration reactions by an NADPH-requiring synthetase complex called fatty acid synthase (FAS). Although salmonids can utilise carbohydrate as carbon source for fatty acid synthesis, the main carbon supply for the synthesis of fatty acids, acetyl-CoA, is derived in fish from amino acids. The acetyl-CoA is converted in the mitochondrion to citrate, which then leaves the mitochondrion to become a substrate for ATP citrate lyase in the cytosol (Walton and Cowey, 1982). Citrate lyase generates acetyl-CoA which is metabolised to malonyl-CoA by acetyl-CoA carboxylase. The formation of malonyl-CoA from acetyl-CoA and CO_2 is generally considered the first reaction of fatty acid synthesis. The reaction actually takes place in two steps catalysed by a single enzyme complex. In the first reaction, which is ATP dependent, the CO_2 (from HCO_3^-) is transferred by the biotin carboxylase portion of the acetyl-CoA carboxylase to a nitrogen of a biotin

prosthetic group attached to the ε-amino group of a lysine residue of the enzyme (Mathews and Van Holde 1990). In the second reaction, catalysed by the carboxyltransferase, the activated CO_2 is transferred from biotin to acetyl-CoA to form malonyl-CoA. Malonyl-CoA is attached to the acyl carrier protein (ACP) before condensation with acetyl-ACP. The first cycle of four reactions of the fatty acid synthetase complex generates butyryl-ACP, which reacts with further molecules of malonyl-ACP in a second cycle of two-carbon additions. A total of seven such cycles generates palmitoyl-ACP which is hydrolysed to release palmitate. McKim *et al.*, (1989) have isolated the acetyl-CoA carboxylase from rainbow trout liver and found that the enzyme is located in the cytosol and is composed of several subunits of 230,000 daltons. The biotin carboxylase portion of the enzyme is present in each subunit similar to that in mammals. Trout acetyl-CoA carboxylase is activated by citrate and inhibited by NaCl. Incubating the cytosolic fraction from trout liver with radiolabelled acetate, Eberhagen *et al.* (1969) found that the main products of fatty acid synthetase were 14:0 (myristic acid) and 16:0 (palmitic acid), followed by 12:0 (lauric acid) and 18:0 (stearic acid) in much smaller amounts. This is broadly similar to the situation in mammals.

1.4. Fatty acid esterification

The fatty acids synthesised endogenously or those arising from the diet are all potential substrates for esterification enzymes. Fatty acids which enter cells are preferably esterified into phospholipids. When provided in excess, they are incorporated into newly synthesised triacylglycerols and stored as lipid droplets in the cells. Very little of the fatty acid in cells is used to esterify cholesterol and form cholesterol ester, the storage form of cholesterol in the cells. The immediate fatty acid substrate for esterification into phospholipids, triacylglycerols, and cholesterol esters is fatty acyl-CoA. The formation of fatty acyl-CoA is catalysed by acyl-CoA synthetases present in the mitochondrial matrix of heart, kidney and skeletal muscle. In liver, the synthetase is only found in the cytosol. Long chain acyl-CoA synthetase

activities are also found in the endoplasmic reticular membranes, in the outer mitochondria membrane and in peroxisomes. Unsaturated fatty acids are activated at higher rates than saturated acids. In intact cells, the activity of synthetases is regulated both by substrate (free fatty acids, CoA) availability, and by product inhibition by acyl-CoA (Gurr and Harwood 1991).

Acyltransferase enzymes are responsible for the incorporation of fatty acyl-CoA into phospholipids and into triacylglycerols. Studies with rainbow trout hepatocytes provided evidence that exogenous C_{18} fatty acids are initially incorporated into triacylglycerols and retained for subsequent utilisation as desaturation or elongation substrates (Sellner and Hazel, 1982ab). The newly desaturated and elongated fatty acids, particularly the Δ6 and Δ5-desaturase products, are preferentially esterified into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Hazel and Prosser, 1979; Hazel, 1983; Sellner and Hazel, 1982ab). The esterification enzymes of fish remain largely uncharacterised although, in a study of phosphatidylcholine biosynthesis *de novo* in trout, Holub *et al.* (1976) identified in liver microsomes the enzyme 1-acyl-*sn*-glycerol-3-phosphorylcholine acyltransferase which is responsible for the reacylation of lysophosphatidylcholine to phosphatidylcholine during phospholipid turnover.

1.5. Fatty acid chain elongation

The elongation of fatty acids in microsomal membranes is similar to the fatty acid synthetase sequence in that it involves acyl-CoA derivatives and NADPH as the reducing coenzyme. The microsomal elongase system is, however, membrane bound unlike the soluble, cytosolic fatty acid synthetase. The preferred substrate for elongation in most cases is palmitoyl-CoA, but in contrast to the system for *de novo* fatty acid synthesis, the intermediates in subsequent reactions are CoA esters, suggesting that the process is carried out by separate enzymes rather than a complex of the fatty acid synthase type (Mathews and Van Holde 1990). The requirements for the mammalian chain elongation systems include: activated fatty acid in the form of

coenzyme A thioester; a two-carbon moiety in the form of malonyl-CoA; reducing equivalents supplied by NADPH or NADH or both (Cinti *et al.*, 1992).

The hepatic microsomal chain elongation reaction begins with the condensation of two substrates, an activated fatty acid, such as palmitoyl-CoA, and malonyl-CoA to yield ß-ketoacyl-CoA, CO_2 and coenzyme A (Figure 1.1). This step catalysed by the condensing enzyme is the rate-limiting step in the overall elongation reaction. The condensation activity is markedly stimulated by albumin (Bernert and Sprecher 1978; Berge 1979). The condensation activity for polyunsaturated fatty acids with malonyl-CoA in rat brain microsomes was investigated by Yoshida and Bourre (1991) who found that the activity for 20:5n-3 was inhibited by 18:3n-3 and 18:4n-3 *via* a non-competitive mechanism. It was suggested by Yoshida and Bourre (1991) that a non-competitive system by multiple fatty acid substrates can be an efficient system of a cell to maintain the amount of total PUFA products in phospholipids when various kinds and amounts of fatty acids are incorporated into the brain from various diets.

The second step for fatty acid chain elongation reaction is the reduction of ß-ketoacyl-CoA to ß-hydroxyacyl-CoA by the enzyme ß-ketoreductase (Figure 1.1). The reaction is faster than the condensation and requires NADPH as the source of reducing equivalent. There is some evidence that the NADPH cytochrome P_{450} reductase is also involved in the transport of electrons to the elongation system (Ilan *et al.*, 1981). The third reaction is the removal of water from ß-hydroxyacyl-CoA by a microsomal dehydrase to generate *trans*-2-enoyl-CoA. The microsomal dehydrase from mammals has been partially purified and shown to be active with several ß-hydroxy derivatives ranging from 12 to 20 carbons (Bernert and Sprecher 1979). The terminal step in the fatty acid elongation reaction is the reduction of *trans*-2-enoyl-CoA to the final product, the saturated acyl-CoA derivative. The reaction is catalysed by enoyl-CoA reductase which utilises either NADH or NADPH as the reducing agent. The electron input in this step catalysed by enoyl-CoA reductase does not involve either cytochrome b_5 or cytochrome P_{450}. Instead the enoyl

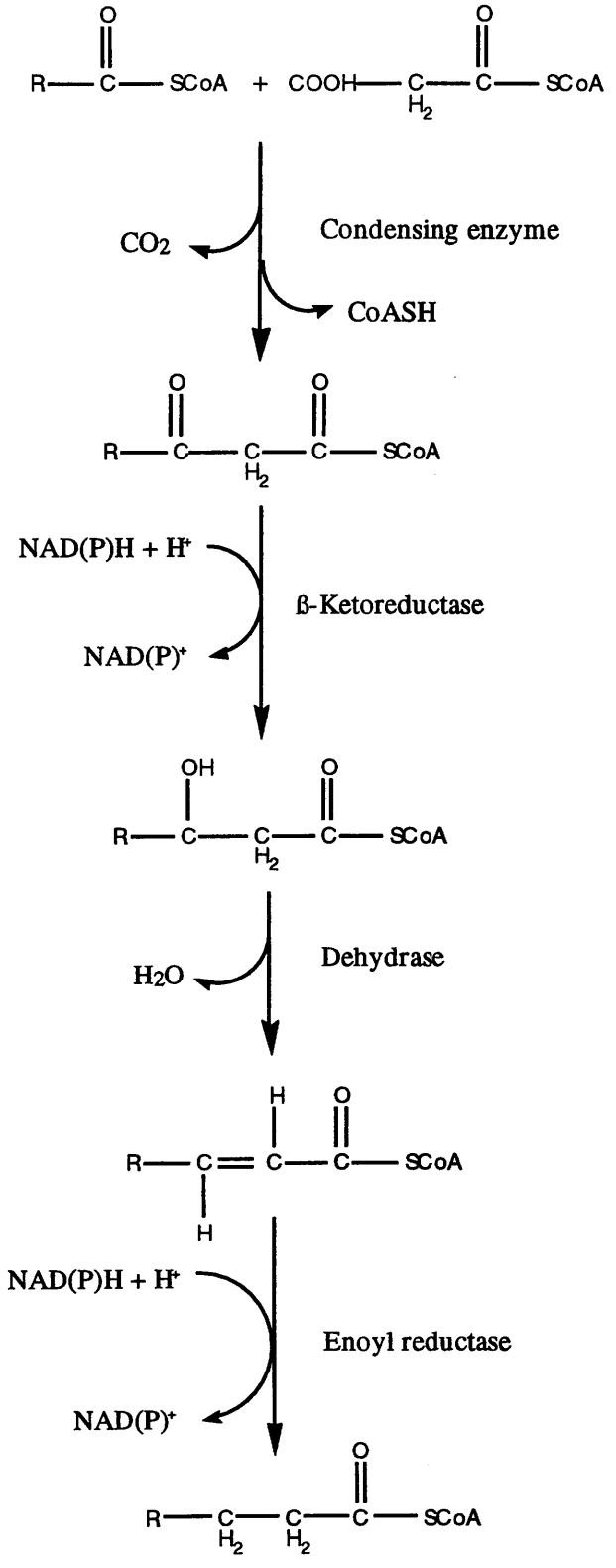

Figure 1.1. Microsomal fatty acid chain elongation of acyl-CoA derivatives

reductase involved, termed short-chain *trans*-2-enoyl-CoA reductase, utilises both NADH and NADPH (Nagi *et al.*, 1983).

To date, very few studies have been carried out on the elongation system in fish. Muci *et al.* (1992) demonstrated that fatty acid chain elongation in eel liver microsomes was remarkably active and required malonyl-CoA as the two carbon donor, as well as NADPH as the reducing agent. NADH can also be utilised by the eel liver elongation system, but NADPH is more effective. The products of the elongation reaction in eel liver microsomes were mainly esterified into neutral lipids and into phosphatidylcholine and phosphatidylethanolamine fractions. Hepatocytes isolated from rainbow trout also possess a very active fatty acid elongation system. Sellner and Hazel (1982ab) demonstrated that, when hepatocytes were incubated with [1-^{14}C]-18:3n-3, a large proportion of radioactivity was always recovered as the elongation product, 20:3n-3, which was predominantly esterified into the neutral lipid fraction. The elongation of fatty acids up to C_{24} has also been found high in astroglial cells from the turbot (Tocher, 1993).

Studying fatty acid elongation in rat liver microsomes, Sprecher (1974) found that the conversion rate of 16:0 in rats subjected to a starvation-refeeding regime using a fat free diet was significantly different from the conversion rates of the polyunsaturates 18:3n-3 and 18:2n-6. These results suggested the existence of more than one chain elongation system in rats. Further, Ludvig and Sprecher (1979) compared a series of isomeric C_{18} dienoic acids and found that only Δ6,9 and Δ7,10 isomers were suitable substrates for condensation and overall chain elongation. However, when dienoic acids with similar Δ7,10 double bond positions but increasing chain length were tested, the elongation rate increased in the following order: 14:2Δ7,10 < 16:2Δ7,10 < 18:2Δ7,10. Although several lines of evidences suggest that the elongation system in microsomes of mammals proceeds by a system of multiple condensing enzymes, its mechanism is still obscure.

1.6. Fatty acid desaturation

The introduction of double bounds into fatty acyl chains in animals is brought about by microsomal desaturation systems involving fatty acyl-CoA desaturases. The desaturases insert double bonds in specific positions along the acyl chain by an electron oxidation mechanism involving two flavin-dependent enzymes, cytochrome b_5 and cytochrome b_5 reductase (Gurr and Harwood 1991). Characterisation of the fatty acyl-CoA desaturases of animals has been difficult and slow because not only are the enzymes bound in an insoluble form to membranes, but the substrates are micellar at a concentration that is suitable for studies *in vitro*. The cytochrome b_5 electron transport protein is believed to be involved in transferring electrons from NADH to the non-haem iron of the desaturase enzyme. The reduced iron then binds molecular oxygen which, after activation by an unknown mechanism, brings about the desaturation of enzyme-bound acyl-CoA (Figure 1.2).

The most common monounsaturated fatty acids in most vertebrate lipids are oleic and palmitoleic acids which can be synthesised in vertebrates from stearate and palmitate respectively by a Δ9-desaturase. Vertebrates generally have desaturases which are capable of introducing double bonds into fatty acids between an existing Δ9 double bond and the carboxyl group. However, vertebrates are unable to introduce double bonds between the Δ9 position and the methyl terminus of the fatty acid chain and so that they cannot synthesise either linoleic acid (18:2n-6) or linolenic acid (18:3n-3). Hence these are essential fatty acid which must be provided preformed in the diet. These essential fatty acids can be further desaturated and elongated to varying degrees in animals dependent upon the relative activities of Δ6, Δ5 and "Δ4" desaturases in their microsomal membranes (see below).

The desaturases and elongases are organised in a metabolic sequence to permit synthesis of highly unsaturated and elongated fatty acyl-CoAs. This desaturation and elongation enzyme system acts commonly upon the three series of fatty acids, the oleic (n-9), the linoleic (n-6) and the linolenic series (n-3). The important desaturation/elongation/desaturation cascade for the (n-3) fatty acid linolenate is

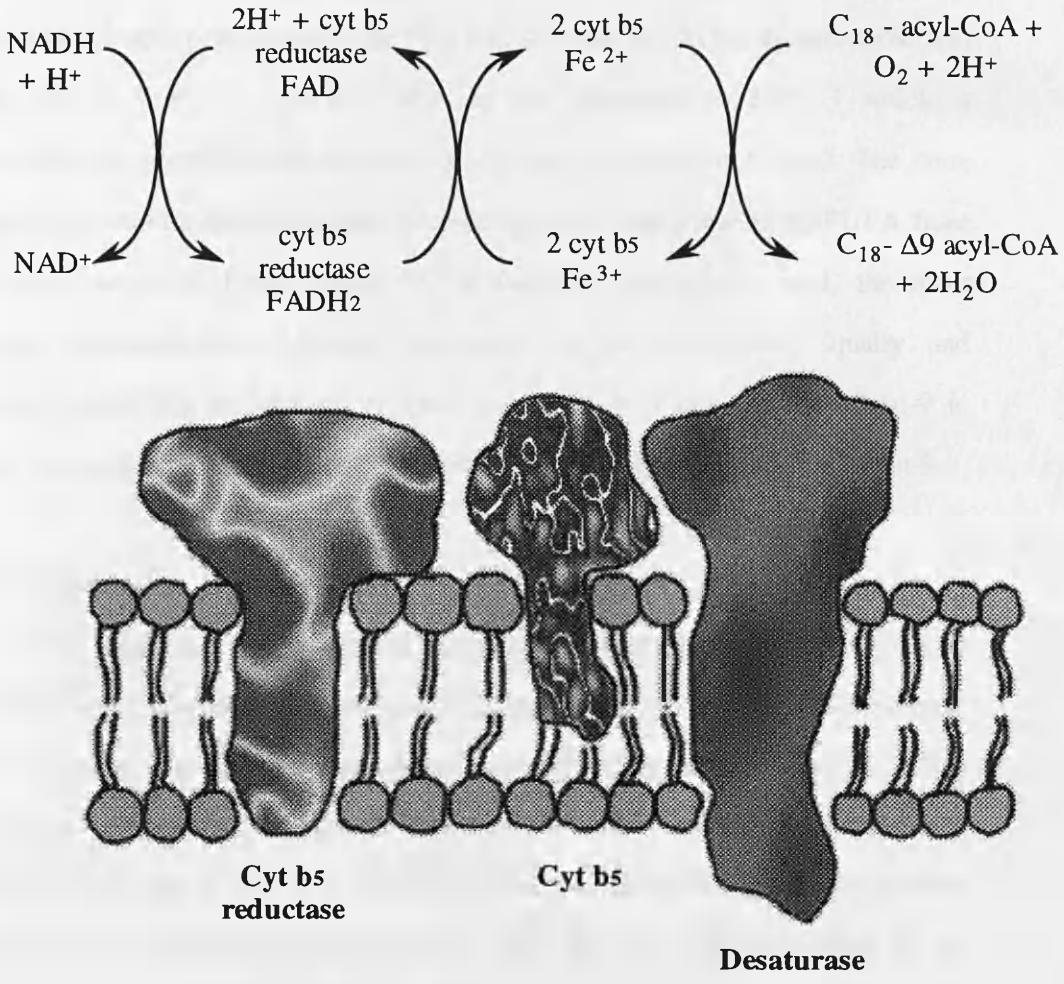

Figure 1.2. Fatty acid desaturation system of the Δ9 desaturase complex in the phospholipid bilayer of endoplasmic reticulum.

shown in Figure 1.3. Linolenate (18:3 Δ 9, 12, 15) obtained preformed in the diet is desaturated by the insertion of a double bond between the sixth and seventh carbon atom to produce 18:4n-3 (Δ 6, 9, 12, 15). This is converted by the action of the elongase, with the addition of two carbons, to a fatty acid with 20 carbons and 4 double bonds 20:4n-3 (Δ 8, 11, 14, 17). The 20:4n-3 can be transformed by the addition of a double bond between the fifth and sixth carbon to eicosapentaenoic acid (EPA, 20:5 Δ 5, 8, 11, 14, 17). EPA is then elongated to 22:5n-3 which is traditionally considered to be desaturated at position 4 to generate 22:6n-3. The same organised system of desaturases and elongases can also metabolise (n-6) PUFA from the linoleic series to longer chain PUFA including arachidonic acid, the most important precursor for eicosanoid production in all vertebrates. Equally and especially in the absence of both 18:2n-6 and 18:3n-3, it can convert 18:1n-9 to longer chain and more unsaturated derivatives, most notably the Mead acid 20:3n-9.

1.6.1. Δ9-desaturase

The Δ9-desaturation system is certainly the best studied and understood acyl-CoA desaturation system in animals. The microsomal stearyl-CoA Δ9-desaturase enzyme from rat liver was first isolated and characterised by Strittmatter *et al.* (1974) who revealed that the enzyme was composed of 458 amino acid residues with a total mass of 53,162 Da. It was also found that cytochrome b_5 was the direct electron donor to the desaturase which appeared to utilise the non-haem iron group in the oxidation and reduction reactions of the desaturation of the stearyl-CoA. Later, Enoch *et al.* (1976) demonstrated that the rate limiting step of Δ9-desaturation was the abstraction of hydrogen when acyl-CoA derivatives bind to the desaturase enzyme. It was also shown that acyl-CoA derivatives with 9 to 20 carbons were capable of binding to the Δ9-desaturase enzyme, but only derivatives from 12 to 19 carbons were acted upon by the enzyme. The activity of Δ9-desaturase in rat liver can be super-induced by subjecting the animals to a starvation-refeed regime prior to experiment (Oshino and Sato 1972). Moreover, the induction of Δ9-desaturase

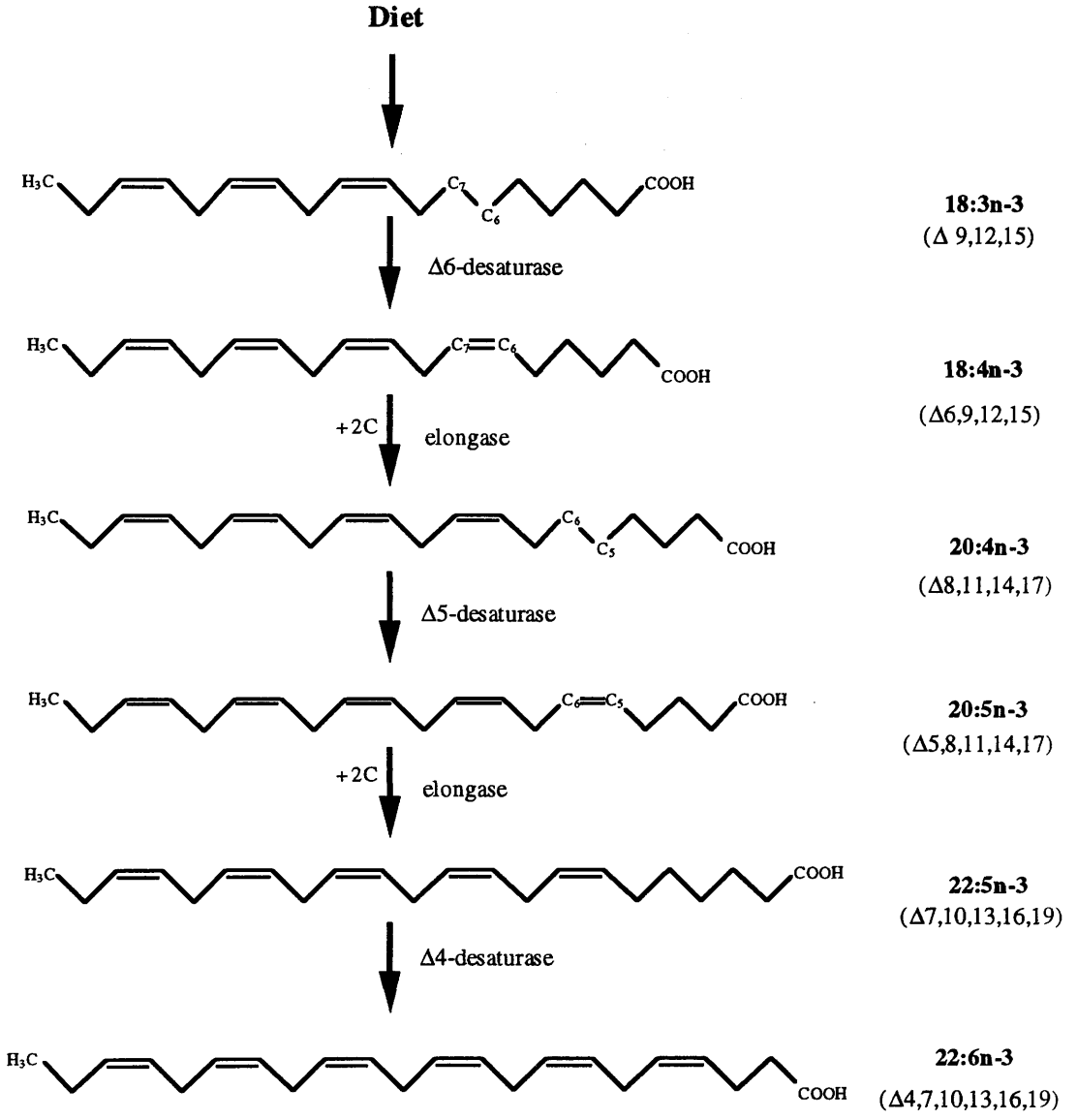

Figure 1.3. Traditional pathway for the biosynthesis of docosahexaenoic acid (22:6n-3) from linolenic acid (18:3n-3).

activity by starvation-refeeding regime can be optimised by using a fat-free diet (Strittmatter *et al.*, 1974). A recent study using rainbow trout hepatocytes showed that hepatic stearyl-CoA Δ9-desaturation can be remarkably induced by a starvation and refeeding regime in a similar manner to that described previously for mammals (Tocher *et al.*, 1996). Recent studies provided evidence that (n-3) and (n-6) PUFA exert a negative control on the expression of the Δ9-desaturase gene in rat liver (Ntambi 1992; Clarke and Jump 1993, 1994, 1996). There are two mechanisms that may explain the inhibition of Δ9-desaturase expression by PUFA; one is the interference of the translation rate of mRNA, another is the reduction in the amount of mRNA encoding the protein-enzyme (Clarke and Jump 1994). Ntambi (1992) demonstrated that when a fat-free diet was supplemented with triacylglycerols containing PUFA, the transcription of stearyl-CoA Δ9-desaturase gene 1 (SCD1) and the induction of SCD1 mRNA were both significantly depressed, whereas TAG with saturated and monounsaturated fatty acids showed no dramatic effect. It was observed recently that arachidonic acid has an inhibitory effect on expression of the stearoyl-CoA desaturase gene in mammalian lymphocytes, indicating that the feedback inhibition by highly unsaturated fatty acids on Δ9-desaturation activity is mediated at a transcriptional level (Tebbey and Buttke 1992).

The molecular biology of Δ9-stearyl-CoA desaturase of rat liver has been extensively investigated by Strittmatter and collaborators. Initially, poly (A^+)RNA containing elevated levels of Δ9-desaturase mRNA was isolated from livers of induced animals and used to construct a cDNA library. cDNAs for Δ9-desaturase were isolated by differentially screening the library with nucleic acid probes derived from reverse transcribed mRNA from induced or uninduced rat liver. Several partial cDNAs were isolated after screening library with the induced preparations that were not detected with uninduced probe preparation. These cDNAs clones were then used to isolate specific mRNAs from induced rat liver, and on *in vitro* translation of the products of these cDNAs, they were identical to the purified Δ9-desaturase enzyme (Thiede and Strittmatter, 1985). Furthermore, Northern blot analysis from these

cDNA clones revealed that the desaturase is encoded by a 4,900-base mRNA which is elevated 50-fold in induced liver (Thiede and Strittmatter, 1985). Subsequently, Thiede *et al.* (1986) derived the full cDNA length of 4900 pb by using primers to extend mRNA from induced rat liver. The cDNA contained a 1,074-base open reading frame coding for 358 amino acids with a total predicted mass of 41,400 Da which was consistent with the mass of the purified enzyme. Later these plasmids were expressed in *E. coli*, and transformed preparations showed a high Δ9-desaturase activity (Strittmatter *et al.*, 1988). The rat liver Δ9-desaturase has also been successfully expressed in yeast, *Saccharomyces cerevisiae* (Stukey *et al.*, 1990).

1.6.2. Δ6-desaturase

The conversion of the essential fatty acids 18:3n-3 and 18:2n-6 to longer chain and more unsaturated fatty acids is mainly dependent on the activity of the Δ6-desaturase. The Δ6-desaturation of fatty acids is probably the most important regulatory step in the biosynthesis of PUFA as it has been reported as being the rate limiting step in the desaturation and elongation of essential fatty acids (Brenner 1974). The solubilization of Δ6-desaturase of rat liver microsomes was accomplished by Okayasu *et al.* in 1979 using Triton X-100 or sodium deoxycholate. These authors found that the activity of the Δ6-desaturase was stimulated by the detergents whereas the activity of Δ9-desaturase was substantially inhibited. These results suggested to Okayasu *et al.* (1979) that the location of Δ6-desaturase in the membrane was different from the location of Δ9-desaturase which is believed to be deeply inserted into the bilayer structure. The Δ6-desaturation location appeared to be similar to that of cytochome b_5 with the hydrophilic part containing the active site and the hydrophobic part anchoring the enzyme into the bilayer of the endoplasmic reticulum. Further studies (Okayasu *et al.*, 1981) on the purification and characterisation of the Δ6-desaturase from rat liver microsomes revealed that the enzyme was a single polypeptide of 66,000 Da containing 49% nonpolar aminoacid residues. The Δ6-desaturase was found to be a non-heme iron protein containing one atom of iron per

molecule of enzyme which required for Δ6-desaturation, NADH, O_2, linoleoyl-CoA, lipid or detergent, NADH-cytochrome b_5 reductase, and cytochrome b_5 (Okayasu *et al.*, 1981). Since the above study, there were no significant advances in the isolation and characterisation of the mammalian Δ6-desaturase system, until recently, when a procedure using high hydrostatic pressure was used to subfractionate the components of Δ6-desaturase from rat liver microsomes (Leikin and Shinitzky, 1994). After subjecting a microsomal solution containing liposomes made from PC to a pressure of 1500 bars, total desaturase activity could be found in the liposomal fraction indicating co-extraction of NADH-cytochrome b5 reductase, cytochrome b_5 and Δ6-desaturase. Size chromatography and FPLC followed by SDS-PAGE confirmed the independent release of three components corresponding to the Δ6-desaturation system. The activity of Δ6-desaturase could be fully reconstituted by adding the aqueous dispersions of the three components without further purification. The same authors (Leikin and Shinitzky, 1995) characterised further the lipid class and fatty acid composition of the lipid surrounding the Δ6-desaturase of rat liver microsomes isolated by the same hydrostatic pressure method.

It is well established that the activity of Δ9, Δ6 and Δ5-desaturases are highly dependent on nutritional and hormonal factors (Brenner 1989). The product of Δ6-desaturation of linoleic acid (18:2n-6), gamma-linolenyl-CoA, is a product inhibitor of Δ6-desaturation that must be eliminated from rat liver microsomal membranes to restore the activity of the desaturase (Brenner *et al.*, 1969). Similarly, arachidonyl-CoA, docosapentanoyl-CoA and docosahexanoyl-CoA were found to inhibit the Δ6-desaturase activity. The Δ6-desaturation activity can therefore be, modulated by a feedback inhibition in which highest PUFA of the different series compete directly with the substrate of the reaction. The composition of the diet has a marked effect on Δ6-desaturation. The inhibitory effect of dietary highly unsaturated fatty acids on desaturation and elongation activity that is well established in mammals has also been observed in rainbow trout by Yu and Sinnhuber (1976) and by Leger *et al.*, (1980) who demonstrated that high contents of (n-3) PUFA, especially 20:5n-3

and 22:6n-3, significantly reduced the Δ6-desaturation activity not only for (n-3) fatty acid substrates but also for (n-6) and (n-9) acids.

It has also been reported that a high protein diet increases Δ6-desaturase activity in rat liver (Peluffo *et al.*, 1972) whereas a high cholesterol diet substantially decreases the enzyme activity (Garg *et al.*, 1988). Feeding a diet with a low cholesterol content, however, produces the opposite effect, demonstrating a reversible mechanism (Leikin and Brenner 1988). It was demonstrated *in vitro* that Δ6-desaturase of rat liver microsomes requires a specific lipoprotein cytosolic factor which binds the free acid produced from the desaturation reaction and, in the absence of this factor, the reaction yield is very low (Leikin and Brenner 1986).

Hormones such insulin, epinephrine, glucagon, corticoids and thyroxine are all known to modulate the activity of Δ6-desaturase in rodents (Brenner 1989). Insulin can substantially increase Δ6-desaturase activity in diabetic rats but not in normal animals (Mercuri *et al.*, 1966; Gommez Dumm *et al.*, 1985). Epinephrine and glucagon, which are antagonistic to insulin, markedly inhibit the activity of Δ6-desaturase in rat liver (Brenner 1981). The mechanism by which epinephrine inhibits the Δ6-desaturase activity is associated with an increased level of cAMP involved in glycogen breakdown. It is believed that the effect of epinephrine is at the gene expression level, since it modifies the maximal velocity of the desaturation reaction without affecting the affinity of the enzyme (Gommez Dumm *et al.*, 1978).

1.6.3. Δ5-desaturase

The enzyme Δ5-desaturase converts eicosatrienoic acid (20:3n-6) into arachidonic acid (AA or 20:4n-6) in the (n-6) series and eicosatetraenoic acid (20:4n-3) to eicosapentaenoic acid (EPA or 20:5n-3) in the (n-3) series. AA and EPA are very important since they are substrates for the biosynthesis of eicosanoids, a diverse family of biologically potent compounds. The Δ5-desaturase from rat liver microsomes was solubilized with detergents and partially purified from microsomal pellets (Pugh and Kates 1979). The Δ5-desaturase activity for converting

2-eicosatrienoyl-PC to 2-arachidonyl-PC as well as eicosatrienoyl-CoA to arachidonyl-CoA was reconstituted in the presence of deoxycholate, cytochrome b_5, NADH-cytochrome b_5 reductase. The activity of the solubilized enzyme was enhanced by the addition of PC, which provided evidence for the direct desaturation of the PC substrate without prior conversion to the acyl-CoA thioester (Pugh and Kates 1979).

Similarly to the Δ6-desaturase, Δ5-desaturation *in vitro* of 20:3n-6 to 20:4n-6 requires the presence of cytosolic lipoprotein bound to the microsomes to produce full activity (Leikin and Brenner 1989). Moreover, the nutritional and hormonal factors that modulate Δ6-desaturation activity also affect Δ5-desaturase. The activity of Δ5-desaturase is depressed by high cholesterol and high carbohydrate diets, dietary PUFA, epinephrine, glucagon and cAMP, but is increased by insulin and an essential fatty acid-deficient diet (Brenner 1981).

Rainbow trout efficiently convert dietary C_{18}-PUFA to highly unsaturated fatty acids such as 20:5n-3 and 22:6n-3 (Kanazawa *et al.*, 1979). Marine fish, conversely, have a limited ability to convert C_{18}-substrates into longer chain PUFA. This inability has been attributed to a deficiency in Δ5-desaturation activity in marine fish species (Owen *et al.*, 1975; Linares and Henderson 1991). Because of these differences in desaturation activities, freshwater and marine fish are different in terms of their qualitative and quantitative requirements for essential fatty acids. There is considerable evidence that freshwater fish require 18:2n-6 and 18:3n-3 whereas marine fish have a dietary requirement for the longer chain more highly unsaturated 20:5n-3, 22:6n-3 and presumably 20:4n-6 (Sargent *et al.*, 1995).

1.6.4. "Δ4"-desaturase

Although many studies have sought to clarify the metabolism and the interconversions of polyunsaturated fatty acids in animals, it has been difficult to established the definite existence of a specific Δ4-desaturase activity in liver. It has been generally assumed that Δ4-desaturase is the enzyme responsible for the

conversion of 22:5n-3 to 22:6n-3 but there has been little or no direct evidence to prove this. In fact, many studies investigating desaturation metabolism in mammalian liver microsomes have not reported any data on the formation of 22:6n-3 by the alleged Δ4-desaturase (Brenner and Peluffo, 1966; Brenner, 1974; Brenner, 1981; Nino *et al.*, 1974; Garg *et al.*, 1988; Christiansen *et al.*, 1991; Voss *et al.*, 1991). A few studies, (Kanazawa *et al.*, 1993; Kanazawa and Fujimoto, 1993) conversely, have reported that mammalian liver microsomes were able to generate 22:6n-3 from 20:5n-3, but the conversion rates were extremely low.

In addition to mammalian primary hepatocytes, only a few cell lines such as human Y79 retinoblastoma cells (Hyman and Spector 1981; Yorek *et al.*, 1984) and foetal skin fibroblasts (Rosenthal *et al.*, 1991) were able to form 22:6n-3. Hyman and Spector (1981) demonstrated that radiolabelled 22:6n-3 formed from [1-^{14}C]-18:3n-3 accumulated substantially in phospholipids of cultured human retinoblastoma cells.

Studies using human epidermal keratinocytes demonstrated that cells were capable of converting [1-^{14}C]-18:2n-6 to 18:3n-6, 20:2n-6, 20:3n-6 and 20:4n-6, but unable to form the Δ4-desaturation product, 22:5n-6 (Marcelo and Dunham 1993). Several other studies carried out with the human intestinal CaCo-2 cell line (Chen and Nilsson 1993; Dias and Parsons 1995), human SK-Hep 1 hepatoma cell (Marra and Dealaniz 1992) and leukaemia K562 cells (Naval *et al.*, 1993) also reported the absence of 22:6n-3 formation. Studying the metabolic conversions of linoleic and linolenic acid in six mammalian cultured cell lines, Maeda *et al.* (1978) found that only one mammalian cell line (FM3A- mouse mammary tumour cell) could generate 22:6n-3 from 18:3n-3, and that was at a low level. Weithmann *et al.*, (1989) found that although Chinese hamster lung fibroblasts incorporated radiolabelled 20:4n-6 and 20:5n-3 into phospholipids intensively, no further conversion of those to longer and more unsaturated fatty acids occurred. Several other studies performed with rat myocytes (Mohammed *et al.*, 1990), macrophages (Chapkin and Miller 1990), neuroblastoma and glioma cells (Robert *et al.*, 1978; Cook *et al.*, 1983; Cook and Spence 1987; Cook and Spence 1987ab; Robert *et al.*, 1983) have also reported the

lack of 22:6n-3 formation. The situation in fish is not far from that in mammals as many authors have reported that no significant conversion of 22:5n-3 to 22:6n-3 by Δ4-desaturase could be detected either *in vivo* or *in vitro* (Owen *et al.*, 1975; Tocher *et al.*, 1989; Olsen *et al.*, 1990; Tocher and Sargent 1990; Linares and Henderson 1991).

In a study of the metabolism of PUFA in skin fibroblasts from Zellweger patients known to inherit a peroxisomal genetic defect, Martinez (1989) observed that the liver content of 22:6n-3 in these patients was 17-fold lower than in controls and suggested that the desaturation at position 4 might be a peroxisomal event. Furthermore, Mimouni *et al.* (1991) suggested that both C_{20} elongation and Δ4-desaturation reactions were located at the peroxisomes.

The most recent advances in the elucidation of the mechanisms of 22:6n-3 biosynthesis have came from the work of Sprecher and collaborators. Studies in Sprecher's laboratory showed that liver microsomes isolated from rats and incubated with [1-^{14}C]-22:5n-3 were unable to produce significant amounts of 22:6n-3 under conditions where 18:2n-6 was readily desaturated to 18:3n-6 (Voss *et al.*, 1991), an outcome that had previously been the experience of most researchers in this field. In the presence of malonyl-CoA, however, [1-^{14}C]-22:5n-3 was sequentially chain elongated to 24:5n-3 and this was further desaturated at position 6 to generate 24:6n-3, at a similar rate to that observed for the Δ6-desaturation of 18:2n-6 to 18:3n-6. When rat hepatocytes were incubated with [1-^{14}C]-22:5n-3, radiolabelled 22:6n-3 was readily formed, but in addition, it was also possible to detect small amounts of esterified [^{14}C]-24:5n-3 and [^{14}C]-24:6n-3 in phospholipids, which is in agreement with their role as intermediates in the 22:6n-3 formation. When hepatocytes were incubated with radiochemically synthesised [3-^{14}C]-24:5n-3 or [3-^{14}C]-24:6n-3, only a minor proportion of the radioactive substrates were found esterified. The distribution of radioactivity from [3-^{14}C]-24:5n-3 in hepatocyte PUFA showed that 24:5n-3 was metabolised both by ß-oxidation to [1-^{14}C]-22:5n-3 and by serving as a precursor for the biosynthesis of 24:6n-3 and thence 22:6n-3. The primary metabolic

fate of [3-^{14}C]-24:6n-3 was ß-oxidation to [1-^{14}C]-22:6n-3 and acylation into phospholipids. These results indicated to Voss *et al.* (1991) that the formation of 22:6n-3 from 22:5n-3 in rat hepatocytes proceeds by a pathway independent of a microsomal Δ4-desaturase, involving instead microsomal chain elongation to 24:5n-3, followed by a Δ6-desaturation to 24:6n-3, which is then chain shortened to 22:6n-3 (Figure 1.4).

This pathway for the formation of 22:6n-3 via Δ6-desaturation of C_{24}-PUFA intermediates has subsequently been demonstrated in mouse cerebral microvascular endothelia (Moore *et al.*, 1993), in amphibian retina (Wang and Anderson 1993) and human skin fibroblasts (Moore *et al.*, 1995). The latter authors have also demonstrated that fibroblasts from Zellweger's patients, who are genetically defective in peroxisomes, were unable to synthesise 22:6n-3 from either [1-^{14}C]-18:3n-3, [3-^{14}C]-22:5n-3, [3-^{14}C]-24:5n-3 or [3-^{14}C]-24:6n-3, which reinforces the fact that peroxisomes are involved in the 22:6n-3 formation. A further study showed that the formation of 22:5n-6 in rat liver microsomes proceeds by the same pathway involving 24:4n-6 and 24:5n-6 as intermediates which probably require the intracellular communication between microsomes and the site for ß-oxidation (Mohammed *et al.*, 1995).

The recent metabolic pathway proposed for the conversion of 22:6n-3 from 22:5n-3 which questions the existence of a Δ4-desaturase emphasises the importance of retroconversion and partial ß-oxidation in the biosynthesis of long chain polyunsaturated fatty acids. Relevant to this, is the knowledge that retroconversion of 22:6n-3 itself is known to occur. Although the retroconversion of 22:6n-3 to 22:5n-3 and 20:5n-3 has been demonstrated in the rat (Schlenk *et al.*, 1969), little is known of the degradation of C_{22} polyunsaturated fatty acids in fish. The mechanism of fatty acid oxidation in mammals as well as in fish proceeds *via* ß-oxidation which occurs mainly in the mitochondria. Peroxisomal ß-oxidation is generally found to play a minor role in the overall fatty acid oxidation(Mathews and Van Holde, 1990). Nevertheless, experiments carried out with isolated peroxisomes from rat showed that this organelle

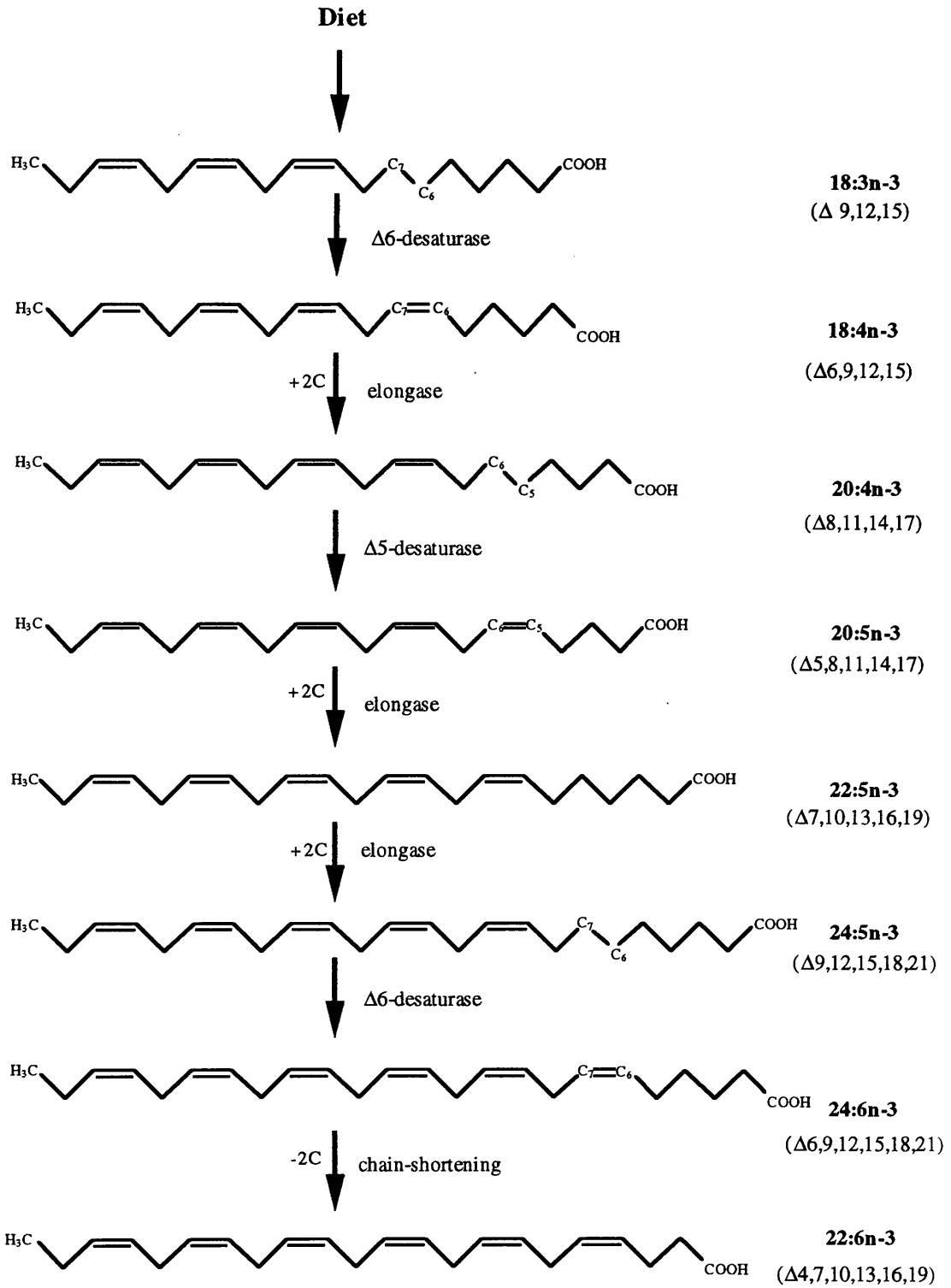

Figure 1.4. Pathway of desaturation and elongation of linolenic acid (18:3n-3) to docosahexaenoic acid (22:6n-3) according to the hypothesis proposed by Voss *et al.* (1991).

plays an important part in the oxidation of long chain polyunsaturated fatty acids (Hiltunen *et al.*, 1986; Hovik and Osmundsen 1987). Crockett and Sidel (1993ab) reported that the liver peroxisomal ß-oxidation in marine teleost fish is an important catabolic pathway responsible for ß-oxidising the major share of PUFA, such as 20:5n-3 and 22:6n-3 which are present in high concentrations in all tissues. The high ß-oxidative capacities in the marine teleost has been suggested as a catabolic pathway adapted ultimately for anabolic processes.

In view of the lack of information on the mechanism of 22:6n-3 biosynthesis in fish, the aim of the present study was to elucidate the biosynthetic pathway of 22:6n-3 from its precursor, 18:3n-3. The initial objectives were the development of a cell system suitable for this purpose and to investigate the use of dietary manipulation to maximise the rate of conversion of 18:3n-3 to 22:6n-3. Having developed the *in vitro* system it was intended to use it to establish in details the pathways involved on the formation of 22:6n-3, particularly the last desaturation step which has been traditionally attributed to a Δ4-desaturase.

CHAPTER 2

ESTABLISHMENT AND CHARACTERISATION OF RAINBOW TROUT HEPATOCYTES IN PRIMARY CULTURE

2.1 INTRODUCTION

The culture of primary hepatocytes is an extensively used technique because it provides for study, under defined conditions *in vitro*, cells that exhibit a wide range of metabolic functions characteristic of hepatocytes in intact liver.

Primary culture of isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) has been extensively used to investigate metabolic processes of the liver such as lipogenesis (Hazel and Prosser 1979; Hazel and Sellner 1979; Voss and Jankowsky 1986; Segner *et al.*, 1994), gluconeogenesis (Walton and Cowey 1979; Morata *et al.*, 1982; Mommsen and Suarez 1984), hormonal responses (Morata *et al.* 1982; Segner *et al.* 1994) and the study of toxicity and carcinogenicity (Klaunig *et al.*, 1985; Kocal *et al.*, 1988; Baksi and Frazier 1990; Pesonen and Andersson 1991).

For studies on lipogenesis in hepatocytes over a period of a few hours, hepatocyte suspensions provide a suitable system essentially because of their simplicity. An advantage of using hepatocyte suspensions is that they are convenient for sampling frequently from uniform population of cells and for providing a metabolic system that mostly resembles the intact liver (Berry *et al.*, 1991). Studies on lipogenesis in isolated hepatocytes from rainbow trout showed that meaningful results were obtained up to 12 hours of incubation using suspension culture as the metabolic system (Hazel and Prosser 1979; Hazel and Sellner 1979; Voss and Jankowsky 1986; Segner *et al.* 1994).

The establishment of an appropriate procedure of cell isolation and culture medium depends on the phenomena under investigation (Baksi and Frazier 1990; Berry *et al.* 1991). Previous reports described that the most appropriate media for perfusing liver to obtain hepatocytes are those that mimic closely the plasma

composition of the animal in question, so that one should obtain the maximum yield of viable hepatocytes while minimising biochemical changes in the cells and contamination with non-hepatocytes (Wolf and Quimby 1969; Moon *et al.*, 1985; Nicholson 1989; Baksi and Frazier 1990). The use of balanced salt solution buffered, with HEPES for instance, is appropriate for fish systems where the pH of fish plasma *in vivo* is higher than in mammals (Moon *et al.* 1985; Baksi and Frazier 1990). Enzymatic disaggregation using microbial collagenase (*Clostridium sp.*) has been shown to be the simplest and most efficient procedure to obtain isolated viable cells from intact livers of mammals (Berry *et al.* 1991; Freshney 1992) and fish (Moon *et al.* 1985; Baksi and Frazier 1990). In experiments using hepatocyte suspensions where the incubation period does not extend beyond 24 hours, a simple culture medium such as Krebs-Henseleit physiological saline or medium 199 with low amino acid and glucose contents is satisfactory to meet the cell's minimum requirements (Wolf and Quimby 1969; Berry *et al.* 1991). Fish hepatocyte suspensions are usually kept in a shaking water bath, continuously gassed with a mixture of 5% CO_2/ 95%O_2 and cell concentrations varying from 1.10^5 to 1.10^7 cells.ml^{-1} (Baksi and Frazier 1990).

For most of the cell cultures, foetal calf serum (FCS) is usually the incubation medium of choice due its superior growth-enhancing properties, commercial availability and for its ability to reduce the tendency of hepatocytes to aggregate at longer incubation times (Wolf and Quimby 1969; Nicholson 1989; Berry *et al.* 1991). The effect of serum concentration on the viability of trout hepatocytes was previously investigated by Klaunig *et al.* (1985).They concluded that a concentration of 5% foetal bovine serum (FBS) was optimal for maintenance of trout hepatocytes in suspension culture. In short-term cultures the use of FCS at low concentrations can be utilised to preserve the culture against excessive mortalities and provide the cells with hormones important for their metabolism.

Environmental temperature affects a variety of physiological and biochemical processes in poikilothermic animals such as fish. Previous studies with suspension

cultures of isolated trout hepatocytes have demonstrated that the survival of hepatocytes is dependent on the temperature of incubation (Hazel and Prosser 1979; Hazel and Sellner 1979; Sellner and Hazel 1982ab; Klaunig *et al.* 1985). Klaunig *et al.* (1985) reported that the highest survival rate of a primary culture of trout hepatocytes was achieved when cells were incubated between 4°C to 10°C.

The lipid class and fatty acid compositions of rainbow trout liver have been reported previously by several authors (Castell *et al.*, 1972a; Watanabe *et al.*, 1974; Hazel 1979ab; Leger *et al.*, 1980; Henderson and Sargent 1981, 1983, 1984; Sellner and Hazel 1982a). In the neutral fraction of trout liver, triacylglycerols are the major lipid class followed by cholesterol and cholesterol esters. Free fatty acids, diacylglycerols and wax esters are quite minor components (Hazel 1979b). Among phospholipids, PC is the major class usually accounting for more than 60%. Phosphatidylethanolamine is the second most abundant phospholipid followed by PI and SM. Phosphatidylglycerol, PS, CL and PA are all minor phospholipid components of trout liver lipid (Hazel 1979a). The fatty acid composition of rainbow trout liver is characterised by a high proportion of polyunsaturated fatty acids, particularly the (n-3) series, although this can be markedly influenced by dietary lipids. Docosahexaenoic acid is usually the major fatty acid in trout liver total lipids, followed by 18:1 and 16:0. Among the (n-6) PUFA, 18:2n-6 and 20:4n-6 are the most abundant. Eicosapentaenoic acid is the second important fatty acid among the (n-3) PUFA after 22:6n-3. Other fatty acids such as 14:0, 16:1, 18:0, 20:1, 24:1, 18:3n-3, and 22:5n-3 are minor components and range between 1% and 10% (Leger *et al.* 1980; Sellner and Hazel 1982a; Henderson and Sargent 1981, 1983, 1984).

In studies on lipid metabolism it is important to adjust experimental parameters such as culture media composition, incubation time and temperature, all of which might affect the metabolism of the system in question. The culture media used for studying polyunsaturated fatty acid metabolism in fish cells are essentially devoid of fat and therefore cells in culture usually derive their lipids and fatty acids from the lipid present in the serum component. The use of serum in fish tissue culture is

important to provide the cells with essential fatty acids, growth factors and hormones necessary for the metabolism, avoiding mortalities. Due to the difficulties in obtaining large quantities of marine and freshwater fish serum, mammalian serum became the most common serum used in fish cell culture. One of the problems of using mammalian serum in fish tissues is the balance of (n-3) and (n-6) PUFA in serum. Studies carried out on PUFA metabolism using fish cell lines in the presence of foetal calf serum (FCS) revealed that fish cells, which were originally rich in (n-3) PUFA, became gradually depleted from the (n-3) series and enriched in (n-6) PUFA which reflects a typical PUFA balance in mammalian sera and cells (Tocher *et al.*, 1988; Tocher *et al.*, 1995). The depletion of (n-3) PUFA in fish cells would certainly change the entire fatty acid metabolism pattern of the cells used in studies.

The effect of temperature on fatty acid and lipid metabolism in trout liver has been extensively investigated by several studies (Hazel 1979ab; Hazel and Prosser 1979; Hazel and Sellner 1979; Sellner and Hazel 1982ab; Voss and Jankowsky 1986; Williams and Hazel 1995). The work of Hazel and his group using liver isolated from thermally acclimated rainbow trout reported that liver lipid from cold-acclimated trout was characterised by increased levels of (n-3) PUFA and low levels of monoenes and saturated fatty acids (Hazel 1979ab; Hazel and Prosser 1979). Cold exposure also changes the lipid class composition by increasing the proportion of PE (Hazel 1979a). *In vitro* experiments using isolated hepatocytes from trout (Voss and Jankowsky 1986), carp (Farkas and Csengeri 1976) and liver slices of eel (Abraham *et al.*, 1984) demonstrated that incubation temperature substantially affected the incorporation and distribution of ^{14}C-acetate into both lipid class and fatty acids.

The aim of the work described in the present chapter was to investigate the effect of medium serum (FCS) concentration and temperature of incubation on cell viability, so as to determine optimal culture conditions for studying fatty acid biosynthesis in suspensions of trout hepatocytes. The effects of serum and incubation temperature on the lipid metabolism of trout hepatocyte were determined by analysing the changes in lipid class and fatty acid composition over 24 hours incubation.

2.2. Materials and Methods

2.2.1. Materials

Foetal calf serum was obtained from ICN Biochemicals (Thame, Oxfordshire, UK). Collagenase Type IV and all chemicals used in the culture medium and lipid analysis were purchased from SIGMA Chemical Co. Ltd (Poole, Dorset, UK). Solvents were HPLC grade and were purchased from Rathburn Chemicals (Walkerburn, Peebleshire, UK).

2.2.2. Experimental fish

Rainbow trout, *Oncorhynchus mykiss,* were obtained from a commercial fish farm (College Mill Trout Farm, Almondbank, Perthshire) and kept in the aquarium facilities of the Department of Biological and Molecular Science, University of Stirling. The fish were maintained in 500 litres self-cleaning circular tanks supplied with recirculated flowing dechlorinated freshwater at 12°C and fed a commercial diet (EWOS Aquaculture Ltd, West Lothian, UK) at a daily rate of 2% of the body weight. The rainbow trout used to prepare hepatocytes for the viability experiments weighed around 400g.

2.2.3. Preparation of trout hepatocytes

Trout hepatocytes were prepared by modification of the methods of Moon *et al.* (1985) and Klaunig *et al.* (1985). A rainbow trout (*Oncorhynchus mykiss*) was placed in a plastic bucket containing fresh water and anaesthetic (3-aminobenzoic acid ethyl ester) (1:2000 w/v). After being anaesthetised the fish was injected *via* the caudal vein with 1000U/kg heparin in 0.9% NaCl. After 5 minutes the fish was killed by a sharp blow to the head. A ventral incision was made and the liver rapidly removed onto the perfusion tray (Figure 2.1). The hepatic portal vein was cannulated and the blood cleared using a well-oxygenated (95% O_2/5%CO_2) Hank's Balanced Salt Solution (HBSS) containing 0.6mM EDTA, 25mM $NaHCO_3$, and 10.0 mM

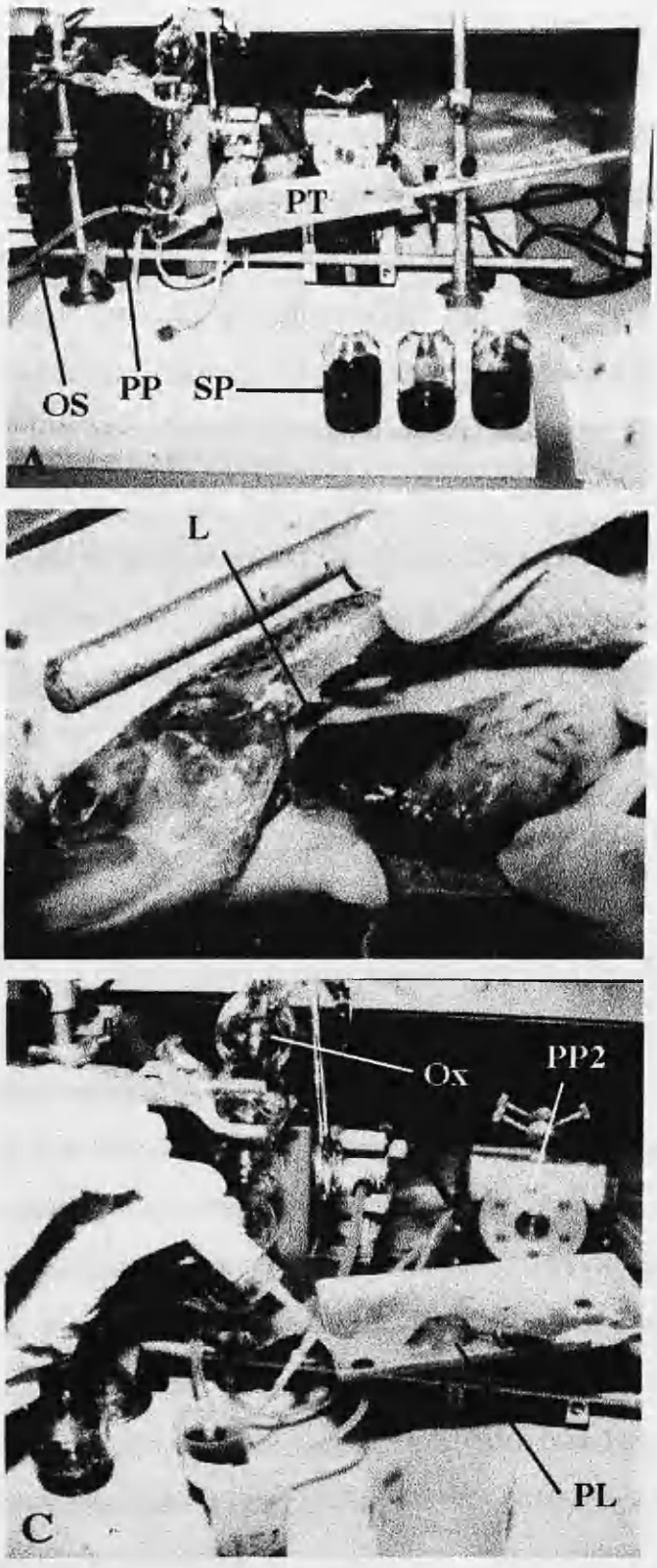

Figure 2.1. Perfusion apparatus used in isolation of hepatocytes from trout. A, perfusion equipment: peristaltic pump (PP), perfusion tray (PT), oxygen supply (OS), solutions for perfusion (SP). B, incision performed ventrally on a trout exposing the liver (L). C, liver digestion: the solution containing collagenase was gassed with O_2/CO_2 in the oxygenator and perfused through the liver (PL) by the second peristaltic pump (PP2).

HEPES (*N*-2-hydroxyethylpiperazine-*N'* -2-ethanesulfonic acid)(pH 7.4). The hepatic portal vein was severed to prevent build up of back pressure. A gentle massage was applied to the liver to enhance blood clearance and the perfusion flow rate adjusted to $3ml.min^{-1}.g\ liver^{-1}$. After the blood had been cleared, digestion was carried out by continuing perfusion with $1mg.ml^{-1}$ collagenase Type IV in well-oxygenated HBSS solution containing 4.0 mM $CaCl_2$, 10.0mM HEPES and 25mM $NaHCO_3$, until the liver showed signs of disintegration (usually after 30-45 minutes). The disrupted liver was placed on a watch glass, minced with scissors and passed through a nylon screen of plankton netting (mesh size 250μm) to obtain the isolated cells and to filter off extrahepatic tissue. The isolated hepatocytes were collected by centrifugation (100 x *g* min) and washed twice with the same HBSS-BSA solution and dispersed in HBSS solution containing 10mg/ml BSA, 10.0mM HEPES and 25mM $NaHCO_3$.

2.2.4. Viability of trout hepatocytes

The viability of rainbow trout hepatocytes was assessed by dye exclusion using Trypan Blue. The method is based in the concept that viable cells do not take up Trypan Blue whereas damaged or dead cells are permeable to this dye. A volume of 0.4ml of 0.4% Trypan Blue in HBSS was transferred into a 1ml Eppendorf tube containing 50μl of the hepatocyte suspension (dilution factor of 8). The solution was mixed thoroughly and allowed to stand for 5 to 15 minutes. With the cover-slip of a haemocytometer well positioned, a small volume of Trypan Blue-cell suspension was transferred carefully by touching the edge of the cover-slip with the pipette tip allowing each chamber to fill by capillary action. Both viable (non stained) and non viable cells (stained in blue) were counted to determine the viability and total number of viable cells/ ml suspension.

Viable cells/ml suspension = average count per square * dilution factor * 10^4

(10^4 represents each square of the hemocytometer with the total volume of $0.1mm^3$ or 10^{-4} cm^2).

Cell viability (%) = [total viable cells (unstained) ÷ total cells (Σ stained and non stained)] * 100

2.2.5. Incubation conditions

Isolated hepatocytes were incubated as suspensions with (orbital shaking, 50 oscillations/min) and without agitation in 10ml sterile plastic petri dishes (Sterilin Stone, UK). The cells were kept at a concentration of 2.5 . 10^6 cells ml^{-1} in 5 ml Medium 199 (SIGMA) supplemented with 2% foetal calf serum (FCS), 10mM lactate, 5000Um/5mg.ml^{-1} penicillin/streptomycin and glutamine (5mg.ml^{-1}). Hepatocytes were incubated in suspension with and without agitation (static suspension culture) at 12°C to test the culture viability over the period of 24 hours. Subsequently, in another experiment, the cells (static cultures) were incubated for 24 hours to test cell viability at different temperatures and different serum concentration. For the temperature experiments, four temperatures (5°C, 10°C, 15°C and 20°C) were used. For the serum experiments two concentrations (2% and 10%) were used. Cell viability and the number of viable cells were determined after 1, 3, 6, 12, and 24 hours of culture.

In time course experiments, lipid class and fatty acid composition were determined after 1, 3, 6 and 24 hours of culture. To test the effect of serum concentration on the hepatocyte lipid class composition and fatty acid composition, cells were incubated for 24 hours at 12°C in medium containing 0%, 2% or 10% of foetal calf serum. In experiments on temperature effects, cells were incubated for 24 hours at 5°C, 12°C or 20°C.

2.2.6. Lipid extraction

After incubation the hepatocytes were aspirated into test tubes and washed twice with Medium 199. The lipids were extracted by homogenisation in 10 ml chloroform/ methanol (2:1 by vol.) as described by Christie (1982). The cell homogenate in chloroform/methanol was filtered through a paper filter (Whatman N°1, Maidstone, England) which had been pre-washed with chloroform/methanol 2:1(v/v). Aqueous KCl (0.88%, w/v) was added to the filtrate at 1/4 of the standing volume to separate the organic phase. The upper phase was aspirated and discharged. The organic solvent removed under a stream of nitrogen and the total lipid desiccated under vacuum for 2 hours, and subsequently weighed.

2.2.7. Lipid class composition of hepatocytes from rainbow trout

The class composition of trout hepatocytes were determined by high performance thin layer chromatography (HPTLC). The dry lipids were redissolved in chloroform/methanol (2:1, by vol.) and 10μg of total lipid was spotted on a 10cm x 10cm Merck HPTLC silica Gel 60 plates (Darmstadt, Germany) which had been pre-run in a diethyl ether/hexane (1:1 by vol.). The plates were developed half way with methyl acetate/isopropanol/chloroform/ methanol/ 0.25% KCl (25:25:25:10:9 by vol.), and then developed fully with hexane/diethyl ether/glacial acetic acid (80:20:2 by vol.) after being desiccated for 30 min. The bands of the various lipid classes were visualised by spraying the chromatogram with 3% copper acetate (w/v) in 8% H_3PO_4 (v/v) followed by charring of the plate for 20 minutes at 160°C. The separated lipid classes were quantitated by scanning densitometry using a Shimadzu CS 9000 densitometer equipped with computer integrator and recorder (Henderson and Tocher, 1992).

2.2.8. Fatty acid composition of hepatocytes from rainbow trout

The fatty acid composition of total hepatocyte lipids was determined by capillary gas chromatography. Trout hepatocyte total lipids were subjected to acid-

catalysed transesterification as described by Christie (1982) in which procedure the fatty acid methyl esters (FAME) were prepared by incubating the hepatocyte total lipids overnight at 50°C in the presence of 1% (by vol.) H_2SO_4 in methanol. After incubation, the methyl esters were extracted twice using hexane/diethyl ether (1:1 by vol.) and purified by HPTLC using hexane/diethyl ether/acetic acid (85:15:1 by vol.) containing 0.05% (w/v) BHT as developing solvent. The recovered FAME were dissolved in a small volume of hexane containing 0.05% (w/v) BHT and analysed in a Packard 436 gas chromatograph equipped with a capillary column of fused silica (50m x 0.32mm) coated with CP Wax 52CB (Chrompack, The Netherlands). Hydrogen was used as carrier gas and samples were injected directly on to the column. The oven temperature was programmed to rise from 50°C to 150°C at a rate of 39C° min^{-1} and then at 2.5C° min^{-1} to 230°C. Individual FAME were identified by reference to a well characterised fish oil and to authentic standards.

2.2.9. Statistical analysis

All results are means of three experiments ± standard deviation. To determine the cell viability and the number of viable cells, aliquots of medium containing cells were taken from a single dish or flask over the period of incubation. The significant differences in time course incubations were determined by Repeat-Measure ANOVA Single Factor performed by StatView 4.0 for Apple Macintosh computer. In experiments with fixed incubation times, significant differences were determined by OneWay Factorial ANOVA. The percentages were normalised by arcsin transformation prior to the statistical analysis. Differences in incubation treatments were evaluated by Scheffé's F and Bonferroni/Dunn post hoc tests and reported in tables if $P<0.05$.

2.3. Results

2.3.1. Viability of trout hepatocytes

Examination by light microscopy showed that freshly isolated hepatocytes from rainbow trout were predominantly spherical. The cells contained a clear round nucleus centrally located within the cytoplasm (Figure 2.2).

2.3.1.1. Effect of culture agitation on hepatocyte viability

Hepatocytes that had been incubated in suspension with constant agitation were usually attached to each other forming large clumps of cells which were difficult to disperse. Cells that had been incubated without agitation formed a weak attachment to the plastic substrate and were easy to dislodge from the culture surface when the dish was gently shaken. Substantially more damaged cells and debris were observed in suspension cultures with agitation than in static cultures. The reason for subjecting a suspension culture to constant shaking (50 oscillation/min) was to ensure an adequate oxygen and nutrient supply and to increase the interaction between the hepatocyte membrane and compounds present in the medium to be tested. The results in Table 2.1 showed that hepatocyte viability decreased significantly (Repeat Measure ANOVA, $P< 0.01$) over the incubation period, irrespective of the treatment applied. The data show that cell viability in suspension culture with constant shaking decreased (from 97.3% to 15.6%) progressively, and significantly more (Repeat Measure ANOVA, $P< 0.01$) over the period of 24 hours than in cells incubated with no agitation (from 97.3% to 78.8%). At the end of the incubation period of 24 hours, the population of viable cells in the static suspension culture ($1.98 \ .10^6$ cells.ml^{-1}) was significantly higher than in the culture with constant agitation ($0.39 \ .10^6$ cells.ml^{-1}).

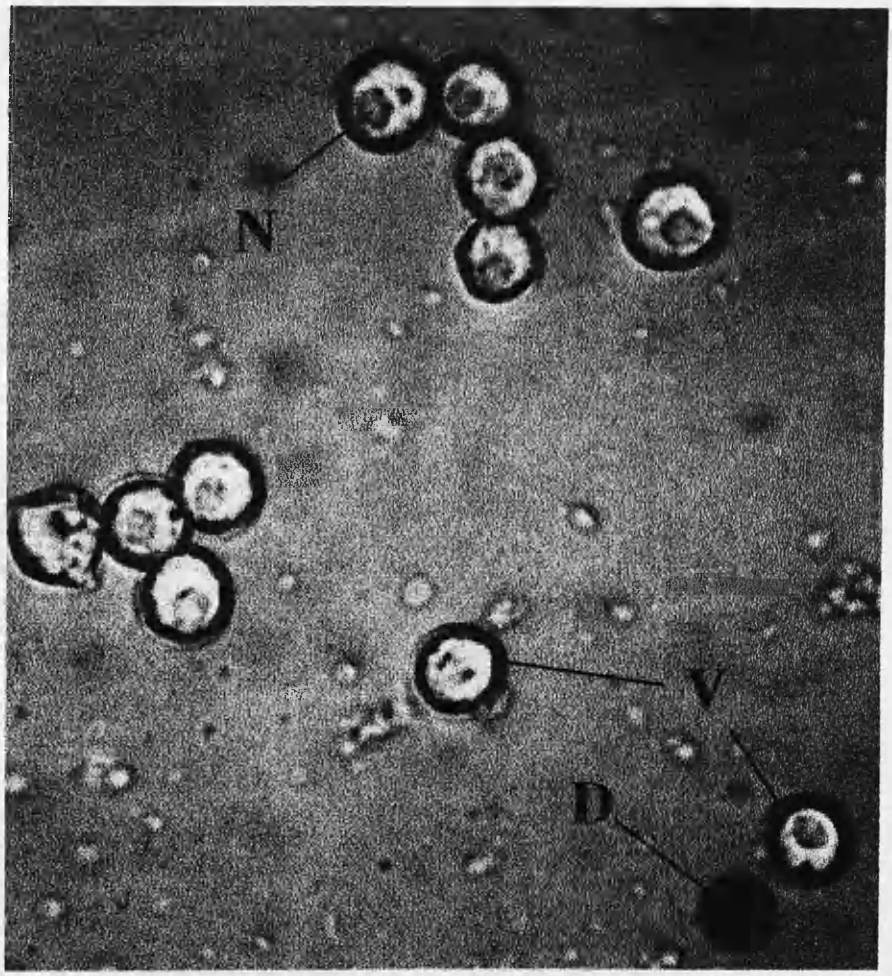

Figure 2.2. Freshly isolated hepatocytes from rainbow trout. The letter "N" indicates hepatocyte nucleus, "D" dead cell and "V" viable cells. The cell viability was assessed by trypan blue exclusion.

Table 2.1. Viability of hepatocytes from rainbow trout incubated at 12°C as suspension with (50 oscillations.min^{-1}) and without agitation

	Time (hours)							
Culture method	0	1	3	6	12	18	24	
	Hepatocyte Viability (%)							
Agitation	97.3 ± 1.3	93.4 ± 2.1	85.4 ± 1.5	71.2 ± 1.6	57.7 ± 1.5	40.4 ± 2.3	15.6 ± 2.7	a
Static	97.3 ± 1.3	96.7 ± 1.7	93.2 ± 1.8	90.7 ± 2.3	86.5 ± 2.4	82.9 ± 2.5	78.8 ± 2.1	b
	N° Viable Hepatocytes.ml^{-1} (x 10^6)							
Agitation	2.51± 0.12	2.34 ± 0.11	2.14 ± 0.09	1.78 ± 0.10	1.44 ± 0.07	1.01 ± 0.10	0.39 ± 0.06	a
Static	2.51 ± 0.12	2.42 ± 0.10	2.34 ± 0.11	2.28 ± 0.08	2.17 ± 0.09	2.08 ± 0.10	1.98 ± 0.06	b

Data are means ± SD (n=3). Different letters represent significant different ($P < 0.05$) according to the multiple comparision tests of Scheffé's F and Bonferroni/Dunn.

2.3.1.2. Effect of foetal calf serum (FCS) concentration on hepatocyte viability

Table 2.2 shows the effect of foetal calf serum (FCS) concentration on the viability of hepatocytes isolated from trout. The results show that hepatocyte mortality decreased with an increase in FCS concentration. At the end of 24 hours the viability of cells incubated with 10% FCS was significantly higher than control cells incubated with no FCS. Although the viability of hepatocytes incubated with 10% FCS after 24 hours (81.3%) was higher than in cells incubated with 2% FCS (79.7%), the results were not statistically different (Repeat Measure ANOVA, $P< 0.05$). Furthermore, although the concentration of 10% FCS in the medium resulted in larger number of viable hepatocytes at the end of 24 hours incubation, the number was not statistically different from that of viable cells in the medium containing 2%FCS. Overall the concentration of viable cells in the medium with 10% FCS was significantly higher than in the medium with no FCS (control).

2.3.1.3. Effect of temperature on hepatocyte viability

The effect of temperature on the viability of primary hepatocytes from rainbow trout is presented in Table 2.3. The results show that the decrease in cell viability was substantially less at 5°C (11.5%) than at higher temperatures. Incubations carried out at 20°C had the lowest percentages for hepatocyte viability and the smallest population of viable cells at the end of the incubation period. Cell viability at 20°C decreased by 28.5% over 24 hours and the number of viable hepatocytes decreased from $2.62 .10^6$ to $1.82 .10^6$ cells.ml^{-1}. This decrease was significantly different (Repeat Measure ANOVA, $P< 0.05$) from those observed with cells incubated at 5°C and 10°C. Statistical analysis of the data show that the decrease in viability with time between cells incubated at 5°C, 10°C and 15°C was not significantly different ($P<0.05$).

Table 2.2. Effect of serum concentration on the viability of trout hepatocytes incubated at 12°C over the period of 24 hours.

	Time (hours)							
FCS (%)	0	1	3	6	12	18	24	
	Viability (%)							
0	96.5 ± 1.2	95.7 ± 2.1	93.3 ± 1.5	90.7 ± 1.6	85.2 ± 1.5	80.4 ± 2.3	75.5 ± 2.7	a
2	96.5 ± 1.2	95.5 ± 1.8	93.4 ± 1.7	91.6 ± 2.4	87.7 ± 2.5	83.8 ± 2.2	79.7 ± 1.9	ab
10	96.5 ± 1.2	95.6 ± 2.5	93.7 ± 2.4	92.4 ± 2.2	88.2 ± 1.8	85.1 ± 1.5	81.3 ± 2.3	b
	Number viable cells.ml^{-1} (x 10^6)							
0	2.52 ± 0.02	2.41 ± 0.01	2.35 ± 0.02	2.29 ± 0.04	2.15 ± 0.05	2.03 ± 0.02	1.90 ± 0.03	a
2	2.52 ± 0.02	2.41 ± 0.01	2.35 ± 0.02	2.31 ± 0.04	2.21 ± 0.04	2.11 ± 0.02	2.01 ± 0.10	ab
10	2.52 ± 0.02	2.41 ± 0.02	2.36 ± 0.02	2.33 ± 0.03	2.22 ± 0.07	2.14 ± 0.12	2.05 ± 0.11	b

Data are means ± SD (n=3). Different letters represent significant different ($P < 0.05$) according to the multiple comparision tests of Scheffé's F and Bonferroni/Dunn

Table 2.3. Effect of temperature on the viability of trout hepatocyte incubated over the period of 24 hours.

Temperature (°C)	Time (hours) 0	1	3	6	12	18	24	
	Hepatocyte viability (%)							
5	97.0 ± 1.0	96.1 ± 1.4	94.4 ± 2.5	93.3 ± 2.6	90.9 ± 1.6	88.1 ± 1.7	85.8 ± 2.3	a
10	97.0 ± 1.0	96.2 ± 1.2	93.4 ± 2.7	91.5 ± 3.1	89.3 ± 2.3	87.3 ± 2.1	84.9 ± 1.6	a
15	97.0 ± 1.0	95.5 ± 2.3	92.7 ± 2.5	90.4 ± 2.8	86.5 ± 2.3	84.3 ± 2.2	83.1 ± 2.7	ab
20	97.0 ± 1.0	92.3 ± 2.3	90.2 ± 2.6	87.6 ± 2.6	83.5 ± 3.3	78.5 ± 2.8	69.4 ± 3.2	b
	Number of viable hepatocytes.ml^{-1} (x 10^6)							
5	2.62 ± 0.13	2.52 ± 0.05	2.47 ± 0.06	2.44 ± 0.07	2.38 ± 0.10	2.31 ± 0.09	2.25 ± 0.05	a
10	2.62 ± 0.13	2.52 ± 0.18	2.45 ± 0.05	2.40 ± 0.06	2.34 ± 0.13	2.29 ± 0.13	2.22 ± 0.09	a
15	2.62 ± 0.13	2.41 ± 0.21	2.43 ± 0.19	2.37 ± 0.04	2.27 ± 0.13	2.21 ± 0.09	2.17 ± 0.06	ab
20	2.62 ± 0.13	2.42 ± 0.19	2.36 ± 0.25	2.30 ± 0.06	2.19 ± 0.09	2.06 ± 0.07	1.82 ± 0.10	b

Data are means ± SD (n=3).Different letters represent significant ($P < 0.05$) effect of temperature according to the multiple comparision tests of Scheffé's F and Bonferroni/Dunn.

2.3.2. Lipid composition of trout hepatocytes

The average total lipid content of freshly isolated trout hepatocytes was 0.77mg/ml of hepatocyte culture medium containing 2.5 x 10^6 cells (Table 2.4).

The total lipid from hepatocytes was analysed by high performance thin-layer chromatography. A typical lipid class profile from trout hepatocytes as found by HPTLC is presented in Figure 2.3. The solvent system for separating the phospholipids resolved firstly sphingomyelins (SM), followed by phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), a non resolved mixture containing phosphatidylglycerol, cardiolipin, phosphatidic acid, (PG/CL/PA) and, lastly, ethanolamine phosphoglycerides (EGP) which contains phosphatidylethanolamine (PE) and plasmalogens. The second development solvent system for neutral lipids resolved cholesterol (CHO), followed by free fatty acids (FFA), triacylglycerols (TAG) and cholesterol esters (CE).

Densitometrical analysis showed that PC (28.0%), TAG (19.2%) and EGP (18.1%) were the major components from freshly isolated trout hepatocytes (Table 2.4). Cholesterol and PI accounted for 14.0% and 5.3% of the total lipid respectively, whilst minor components such as SM, PS, FFA and CE comprised in total approximately 11%. The remaining unresolved fraction containing PG/CL/PA accounted for less that 5% of the total lipid. The data in Table 2.4 show that there were no significant changes in the content of hepatocyte SM, PI, PG/CL/PA and cholesterol over the incubation period of 24 hours. The level of TAG increased slightly from 19.2% to 23.0%, but the results were not statistically different. Conversely, the levels of major phospholipids decreased significantly (Repeat Measure ANOVA Single Factor, $P<0.05$) over 24 hours. EGP had a 35.4% decrease and PC 15.7% relative to their initial values. The percentage amount of PS increased significantly from 2.4% to 3.6% for the initial 6 hours and then decreased to 2.6% at the end of incubation period. Free fatty acids changed in the opposite manner over the incubation period initially decreasing from 2.6% to 0.7% over 6 hours and finally increasing to 3.5% after 24 hours. The results indicate that most of the lipid classes of

Table 2.4. Percentage lipid class composition of trout hepatocyes incubated at 12°C for 24 hours.

	Time (hours)				
	0	1	3	6	24
Total lipid*	0.77 ± 0.06	0.73 ± 0.11	0.76 ± 0.13	0.77 ± 0.10	0.75 ± 0.10
Class					
SM	2.4 ± 0.4	2.4 ± 0.2	2.3 ± 0.3	2.2 ± 0.2	2.6 ± 0.3
PC	28.0 ± 1.8 $_{a}$	27.0 ± 1.4 $_{ab}$	23.8 ± 1.2 $_{ab}$	23.5 ± 1.8 $_{b}$	23.6 ± 1.0 $_{b}$
PS	2.4 ± 0.4 $_{a}$	2.9 ± 0.2 $_{b}$	3.3 ± 0.2 $_{bc}$	3.6 ± 0.2 $_{b}$	2.6 ± 0.2 $_{ac}$
PI	5.3 ± 0.7	5.7 ± 0.6	5.8 ± 0.5	6.0 ± 0.2	4.8 ± 0.3
PG/CL/ PA	4.6 ± 0.1	5.4 ± 0.5	5.4 ± 0.5	5.0 ± 0.3	5.5 ± 0.3
EGP	18.1 ± 0.7 $_{a}$	18.1 ± 0.6 $_{a}$	18.0 ± 0.6 $_{a}$	15.6 ± 1.3 $_{a}$	11.7 ± 0.6 $_{b}$
CHO	14.0 ± 0.8	13.9 ± 0.2	13.8 ± 0.6	13.2 ± 0.5	13.9 ± 0.7
FFA	2.6 ± 0.3 $_{a}$	1.8 ± 0.3 $_{a}$	1.0 ± 0.1 $_{b}$	0.7 ± 0.2 $_{b}$	3.5 ± 0.4 $_{c}$
TAG	19.2 ± 1.8	19.5 ± 2.2	22.8 ± 0.7	24.0 ± 1.8	23.0 ± 1.7
CE	3.4 ± 0.4 $_{a}$	3.3 ± 0.3 $_{a}$	3.7 ± 0.7 $_{a}$	6.4 ± 1.1 $_{b}$	8.9 ± 0.4 $_{c}$

* Total lipid content (mg) of 1 ml of trout hepatocyte culture medium containing 2.5 x 10^6 cells. Data are means ± SD (n=3 fish). The subscript letters denote significant differences ($P<0.05$, Repeat Measures ANOVA, Scheffé's F test). Absence of a subscript indicates not significantly different. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters

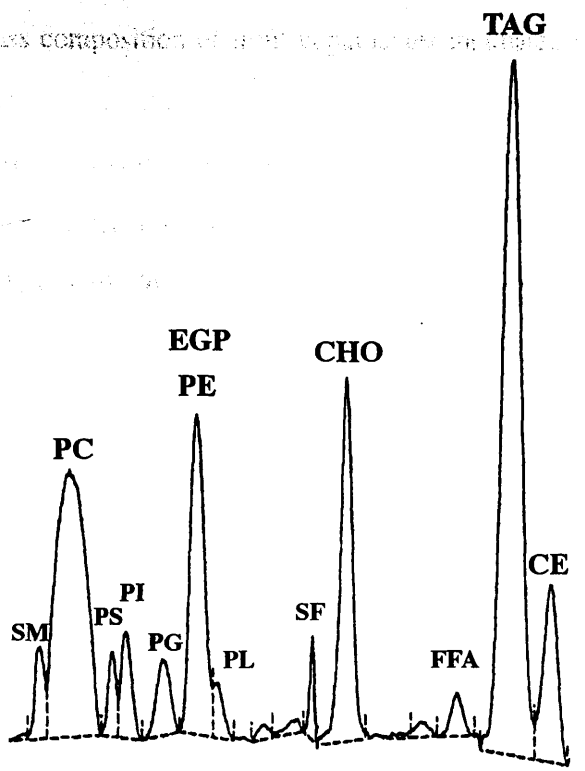

Figure 2.3. Typical lipid class profile of hepatocytes isolated from rainbow trout. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol/cardiolipin/phosphatidic acid; EGP, ethanolamine phosphoglycerides (PE, phosphatidylethanolamine/PL, plasmalogens); SF, solvent front; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerol; CE, cholesterol ester. Lipid classes were separated by HPTLC and the bands of variuos classes were visualized by charring the plates with copper acetate in H_3PO_4. The lipid classes were quantitated by densitometry.

trout hepatocytes, with the exception of FFA, showed no significant changes in composition within the first 3 hours of incubation.

2.3.2.1. Effect of FCS on hepatocyte lipid class composition

The lipid class composition of trout hepatocytes incubated for 24 hours with FCS at concentrations of 0%, 2% and 10% is given in Table 2.5 along with that of the FCS used. The lipid class composition of FCS showed that 86% of the lipids were found in the neutral fraction. The major component was CE accounting for 56.1% followed by TAG with 26.4% of the total. A low content of polar lipids was found in foetal calf serum. The most abundant polar lipid was PC (8.6% of the total lipid) followed by SM (4.5%). Polar lipids accounted for less than 14% of the total calf serum lipids. No significant changes in hepatocyte lipid class composition occurred in any of the incubations with different serum concentrations. The TAG content of cells incubated with 10% FCS increased by 6.9% of the value obtained with cells incubated without FCS. The levels of CE increased by 5.4% and 13.7% when cells were incubated at 2% and 10% FCS respectively.

2.3.2.2. Effect of temperature on lipid class composition of trout hepatocytes

The results in Table 2.6 show that no significant effect of the incubation temperature on the lipid class composition was observed in trout hepatocytes incubated for 24 hours at temperatures of 5°C, 12°C and 20°C. The TAG content of hepatocytes at 20°C increased slightly compared to cells at 12°C but the results were not statistically different. No significant changes in the proportions of phospholipid classes were observed in hepatocytes incubated at the three different temperatures. Overall, there was no change in the amounts of total polar and neutral lipids in hepatocytes incubated at the three different temperatures.

Table 2.5. Percentage lipid class composition of FCS and trout hepatocytes after 24 hours incubated with different concentration of FCS at 12°C.

		Trout hepatocytes incubated with serum		
	FCS	0% FCS	2% FCS	10% FCS
Total lipid*	0.37%	0.75 ± 0.10	0.78 ± 0.16	0.87 ± 0.21
Class				
SM	4.5 ± 0.2	2.6 ± 0.3	2.5 ± 0.2	2.7 ± 0.2
PC	8.6 ± 0.4	23.6 ± 1.0	23.2 ± 0.4	21.1 ± 1.4
PS	0.3 ± 0.0	2.6 ± 0.2	2.5 ± 0.2	3.0 ± 0.2
PI	0.1 ± 0.0	4.8 ± 0.3	4.8 ± 0.6	5.3 ± 0.1
PG/CL/ PA	0.0 ± 0.0	5.5 ± 0.3	5.4 ± 0.4	5.2 ± 0.2
EGP	0.1 ± 0.0	11.7 ± 0.6	12.2 ± 0.6	11.9 ± 0.2
CHO	3.5 ± 0.3	13.8 ± 0.7	13.3 ± 0.5	13.1 ± 0.5
FFA	0.4 ± 0.1	3.5 ± 0.4	3.1 ± 0.2	2.8 ± 0.3
TAG	26.4 ± 1.7	23.1 ± 1.6	23.6 ± 1.0	24.8 ± 1.0
CE	56.1 ± 2.4	8.8 ± 0.4	9.3 ± 0.3	10.2 ± 0.8

* Total lipid content (mg) in 1 ml of trout hepatocyte culture medium containing 2.5 x 10^6 cells. Data are means ± SD (n=3 fish). The subscript letters denote significant differences ($P<0.05$, Repeat Measures ANOVA, Scheffé's F test). Absence of a subscript indicates not significantly different. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters

Table 2.6. The effect of incubation temperature on the percentage lipid class composition of hepatocytes from rainbow trout.

	Temperature of incubation		
	5°C	12°C	20°C
Total lipid*	0.75 ± 0.14	0.75 ± 0.10	0.74 ± 0.12
Class			
SM	2.5 ± 0.2	2.6 ± 0.3	2.8 ± 0.6
PC	24.1 ± 0.5	23.6 ± 1.0	23.8 ± 1.0
PS	2.8 ± 0.3	2.6 ± 0.2	2.7 ± 0.7
PI	4.6 ± 0.3	4.8 ± 0.3	4.6 ± 0.7
PG/CL/ PA	5.3 ± 0.3	5.5 ± 0.3	5.1 ± 0.6
EGP	11.5 ± 0.2	11.7 ± 0.6	11.7 ± 0.7
CHO	13.9 ± 0.4	13.8 ± 0.7	13.5 ± 1.1
FFA	2.9 ± 0.2	3.5 ± 0.4	2.9 ± 0.5
TAG	23.8 ± 0.4	23.1 ± 1.6	24.0 ± 1.1
CE	8.7 ± 0.1	8.8 ± 0.4	8.9 ± 0.8

* Total lipid content (mg) in 1 ml of trout hepatocyte culture medium containing 2.5 x 10^6 cells. Data are means ± SD (n=3 fish). The subscript letters denote significant differences ($P<0.05$, Repeat Measures ANOVA, Scheffé's F test). Absence of a subscript indicates not significantly different. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters

2.3.3. Fatty acid composition of trout hepatocytes

The percentage fatty acid composition of freshly isolated trout hepatocytes is given in Table 2.7 in comparison with that of cells incubated for various lengths of time. The percentage composition of total hepatocyte lipids was characterised by the high content of docosahexaenoic acid (22:6n-3), which accounted initially for 31.6% of the total fatty acids. Other major fatty acid components were the saturated fatty acid 16:0 (17.5%) and the monounsaturated fatty acid 18:1n-9/7 (12.8%). Stearic acid (18:0) and eicosapentaenoic acid (20:5n-3) accounted for 6.5% and 6.2%, respectively. The percentage of arachidonic acid (20:4n-6) was around 3.7%. The data show that hepatocyte lipid was rich in PUFA, especially those of the (n-3) series which accounted in total for 43%. The content of (n-6) PUFA was lower and only comprised 10% of the total fatty acids. The data in Table 2.7 also show that there were no significant changes in the content of the individual fatty acid from trout hepatocyte total lipid when cells were incubated for 24 hours, with the exception of 22:6n-3 whose proportion increased from 31.6% to 35.2%. The total (n-3) PUFA content had a small but significant increase over the 24 hours, whereas total content of (n-6) PUFA decreased slightly within the same incubation period. No significant changes in trout hepatocyte fatty acid composition were observed within the first 3 hours of incubation.

2.3.3.1. Effect of FCS on hepatocyte fatty acid composition

The fatty acid compositions of FCS and trout hepatocytes incubated over 24 hours in the presence of foetal calf serum (FCS) are given in Table 2.8. Fatty acid composition of the FCS showed that this serum batch was rich in monoenes (40.7%) and saturated (37.4%) fatty acids. Palmitic acid (22.5%) was the most abundant saturated fatty acid followed by 18:0 which accounted for 12.0% of the total fatty acids. Oleic acid, 18:1n-9 was the most common among the monoenoic fatty acids

Table 2.7. Fatty acid composition (wt%) of trout hepatocytes incubated at 12°C for 24 hours.

	Time (hours)				
Fatty acid	0	1	3	6	24
14:0	1.6 ± 0.1	1.7 ± 0.0	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.0
15:0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
15:1	n.d.	n.d.	n.d.	0.2 ± 0.0	0.2 ± 0.0
16:0	17.5 ± 0.1	17.9 ± 0.4	17.5 ± 0.6	17.3 ± 0.1	17.4 ± 0.2
16:1	3.0 ± 0.5	2.7 ± 0.1	2.9 ± 0.2	2.8 ± 0.3	2.8 ± 0.1
16:2	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:3 n-3	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:4 n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
17:0	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	6.5 ± 0.3	6.4 ± 0.3	6.3 ± 0.3	6.2 ± 0.1	6.3 ± 0.3
18:1n-9/7	12.8 ± 0.5	13.3 ± 0.2	13.2 ± 0.4	12.9 ± 0.3	12.8 ± 0.4
18:2 n-6	3.6 ± 0.3	3.2 ± 0.1	3.4 ± 0.2	3.3 ± 0.2	3.3 ± 0.4
18:3 n-6	n.d.	n.d.	n.d.	n.d.	n.d.
18:3 n-3	0.4 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.7 ± 0.0
18:4 n-3	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:0	0.1 ± 0.0	n.d.	n.d.	0.1 ± 0.0	0.1 ± 0.1
20:1	3.5 ± 0.1	3.4 ± 0.2	3.1 ± 0.4	3.2 ± 0.3	2.8 ± 0.3
20:2 n-6	0.8 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
20:3 n-6	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
20:4 n-6	3.7 ± 0.7	3.5 ± 0.1	3.7 ± 0.5	3.7 ± 0.1	3.6 ± 0.3
20:4 n-3	1.6 ± 0.2	1.4 ± 0.1	1.3 ± 0.3	1.3 ± 0.1	1.2 ± 0.3
20:5 n-3	6.2 ± 0.7	6.0 ± 0.1	6.1 ± 0.2	5.8 ± 0.1	5.4 ± 0.1
22:1n-11	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
22:5 n-6	0.2 ± 0.1	n.d.	n.d.	n.d.	n.d.
22:5 n-3	2.7 ± 0.3	2.7 ± 0.1	2.5 ± 0.3	2.4 ± 0.2	2.3 ± 0.2
22:6 n-3	31.6 ± 0.5 $_{a}$	32.8 ± 0.7 $_{a}$	33.1 ± 0.4 $_{ab}$	33.7 ± 0.3 $_{bc}$	35.2 ± 0.5 $_{c}$
24:1n-9	1.5 ± 0.2	1.5 ± 0.1	1.9 ± 0.3	1.5 ± 0.1	1.3 ± 0.2
Σ Saturates	26.1 ± 0.4	26.3 ± 0.7	25.7 ± 0.8	25.5 ± 0.2	25.7 ± 0.3
Σ Monoenes	21.1 ± 0.6 $_{a}$	21.1 ± 0.1 $_{a}$	21.2 ± 0.2 $_{a}$	20.9 ± 0.1 $_{ab}$	20.1 ± 0.4 $_{b}$
Σ (n-6)	10.0 ± 0.6 $_{a}$	9.1 ± 0.1 $_{ab}$	9.4 ± 0.2 $_{ab}$	9.5 ± 0.2 $_{ab}$	9.3 ± 0.0 $_{b}$
Σ (n-3)	42.8 ± 0.6 $_{a}$	43.5 ± 0.6 $_{a}$	43.7 ± 0.8 $_{a}$	44.1 ± 0.3 $_{ab}$	44.9 ± 0.7 $_{b}$
Σ PUFA	52.8 ± 0.2 $_{a}$	52.5 ± 0.7 $_{a}$	53.1 ± 1.0 $_{a}$	53.6 ± 0.1 $_{ab}$	54.3 ± 0.7 $_{b}$

Data are means ± SD (n=3 fish). The notation "n.d." represent not detected. The subscript letters denote significant differences ($P<0.05$, Repeat Measures ANOVA, Scheffé's F test). Absence of a subscript indicates not significantly different.

and comprised 32.0% of the total fatty acids in FCS. The levels of (n-3) and (n-6) PUFA in FCS total lipid were much lower than the saturated and monoenoic acids and together accounted for less than 22% of the total hepatocyte fatty acids.

The addition of either 2% or 10% FCS into the hepatocyte culture medium resulted in no alterations to the hepatocyte fatty acid composition. Docosahexaenoic acid content decreased slightly when the cells were incubated with 10% FCS, but the results were not statistically different from 0% or 2% FCS. No significant changes were observed in the content of total (n-3) and (n-6) PUFA in hepatocytes incubated with FCS. Total content of (n-3) PUFA decreased whereas (n-6) PUFA increased in hepatocytes incubated with 10% FCS.

2.3.3.2. Effect of temperature on hepatocyte fatty acid composition

The fatty acid composition of trout hepatocytes incubated for 24 hours at different temperatures is shown in Table 2.9. The data here show that there were no significant changes in the proportions of individual fatty acids in hepatocytes incubated at 5°C, 12°C or 20°C. Nevertheless, significant differences on the total content of monoenoic fatty acids and (n-3) PUFA were observed between hepatocytes incubated at the three temperatures. The total proportion of monoenoic fatty acid increased from 19.1% at 5°C to 20.1% at 12°C. Conversely, the (n-3) PUFA were higher in hepatocytes incubated at 5°C (46.8%) than at 12°C (44.9%) or at 20°C (45.6%). No changes were observed in either the levels of total saturated fatty acids or (n-6) PUFA.

2.4. Discussion

Trout hepatocytes with a high viability were readily isolated by the perfusion method reported in this section. The average viability of freshly isolated hepatocytes was over 96% as assessed by Trypan Blue exclusion. This demonstrated that the method for isolation of trout hepatocytes was acceptable since it produced results

Table 2.8. Fatty acid composition (wt%) of FCS and trout hepatocytes incubated at 12°C for 24 hours in different concentration of mammalian sera.

		Hepatocytes incubated with FCS		
Fatty acid	FCS	0% FCS	2% FCS	10% FCS
14:0	2.4 ± 0.1	1.7 ± 0.4	1.7 ± 0.1	1.6 ± 0.3
15:0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
15:1	n.d.	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
16:0	22.5 ± 0.1	17.4 ± 0.2	17.3 ± 0.5	17.9 ± 0.4
16:1	8.0 ± 0.3	2.8 ± 0.1	3.0 ± 0.1	2.8 ± 0.3
16:2	n.d.	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:3 n-3	n.d.	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
16:4 n-3	n.d.	0.4 ± 0.0	0.4 ± 0.2	0.4 ± 0.1
17:0	n.d.	n.d.	n.d.	n.d.
18:0	12.0 ± 0.3	6.3 ± 0.3	6.4 ± 0.1	6.2 ± 0.1
18:1n-9/7	32.0 ± 0.2	12.8 ± 0.7	13.4 ± 0.6	14.1 ± 0.5
18:2 n-6	5.0 ± 0.2	3.3 ± 0.2	3.3 ± 0.2	3.6 ± 0.2
18:3 n-6	0.2 ± 0.0	n.d.	n.d.	n.d.
18:3 n-3	0.4 ± 0.2	0.7 ± 0.2	0.5 ± 0.3	0.4 ± 0.1
18:4 n-3	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
20:0	n.d.	n.d.	n.d.	n.d.
20:1	0.7 ± 0.2	2.8 ± 0.3	3.0 ± 0.1	2.8 ± 0.1
20:2 n-6	n.d.	0.9 ± 0.1	1.7 ± 1.1	1.2 ± 0.1
20:3 n-6	1.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.2	0.6 ± 0.2
20:4 n-6	8.2 ± 0.4	3.6 ± 0.1	4.0 ± 0.3	4.4 ± 0.4
20:4 n-3	n.d.	1.2 ± 0.2	1.3 ± 0.2	1.0 ± 0.1
20:5 n-3	0.7 ± 0.1	5.4 ± 0.1	5.3 ± 0.1	5.0 ± 0.1
22:1n-11	n.d.	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
22:5 n-6	n.d.	n.d.	n.d.	n.d.
22:5 n-3	3.1 ± 0.2	2.3 ± 0.4	2.1 ± 0.2	1.9 ± 0.1
22:6 n-3	2.9 ± 0.3	35.2 ± 0.5	33.3 ± 1.5	33.4 ± 0.5
24:1n-9	n.d.	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.2
Σ Saturates	37.4 ± 0.2	25.7 ± 0.3	25.7 ± 0.3	26.1 ± 0.4
Σ Monoenes	40.7 ± 0.2	20.1 ± 0.4	21.1 ± 0.3	21.3 ± 0.5
Σ (n-6)	14.7 ± 0.4	9.3 ± 1.0	10.3 ± 0.9	10.6 ± 0.7
Σ (n-3)	7.2 ± 0.5	44.9 ± 0.7	42.8 ± 1.2	42.0 ± 0.5
Σ PUFA	21.9 ± 0.5	54.2 ± 0.7	53.1 ± 0.4	52.6 ± 0.5

Data are means ± SD (n=3 fish). The notation "n.d." represent not detected

Table 2.9. The effect of incubation temperature on the fatty acid composition (wt%) of hepatocytes isolated from rainbow trout

	Temperature of Incubation		
Fatty acid	5°C	12°C	20°C
14:0	1.7 ± 0.1	1.7 ± 0.4	1.8 ± 0.2
15:0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
15:1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
16:0	17.2 ± 0.3	17.4 ± 0.2	17.1 ± 0.5
16:1	2.4 ± 0.1	2.8 ± 0.1	3.0 ± 0.1
16:2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:3 n-3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:4 n-3	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.1
17:0	n.d.	n.d.	n.d.
18:0	6.0 ± 0.2	6.3 ± 0.6	6.1 ± 0.1
18:1n-9/7	12.5 ± 0.5	12.8 ± 0.5	12.6 ± 0.3
18:2 n-6	3.3 ± 0.2	3.3 ± 0.3	3.3 ± 0.1
18:3 n-6	n.d.	n.d.	n.d.
18:3 n-3	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
18:4 n-3	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.1
20:0	n.d.	n.d.	n.d.
20:1	2.7 ± 0.1	2.8 ± 0.2	2.7 ± 0.2
20:2 n-6	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
20:3 n-6	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
20:4 n-6	3.7 ± 0.1	3.6 ± 0.3	3.5 ± 0.4
20:4 n-3	1.3 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
20:5 n-3	5.7 ± 0.2	5.4 ± 0.1	5.5 ± 0.2
22:1n-11	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
22:5 n-6	n.d.	n.d.	n.d.
22:5 n-3	2.5 ± 0.1	2.3 ± 0.4	2.4 ± 0.1
22:6 n-3	36.0 ± 1.1	35.2 ± 0.5	35.6 ± 0.6
24:1n-9	1.1 ± 0.1	1.3 ± 0.2	1.3 ± 0.2
Σ Saturates	25.1 ± 0.6	25.7 ± 0.3	25.2 ± 0.6
Σ Monoenes	19.1 ± 0.6 a	20.1 ± 0.4 b	20.0 ± 0.1 b
Σ (n-6)	9.1 ± 0.3	9.3 ± 0.3	8.9 ± 0.5
Σ (n-3)	46.8 ± 0.9 a	44.9 ± 0.7 b	45.6 ± 0.6 b
Σ PUFA	55.9 ± 1.2 a	54.2 ± 0.7 b	54.5 ± 0.9 b

Data are means ± SD (n=3 fish). The notation "n.d." represent not detected. The subscript letters denotes significant differences (P<0.05) OneWay Anova, Scheffé's F test. Absence of a subscript indicates not significantly different

consistent with previous hepatocyte isolation methods (Klaunig *et al.* 1985; Kocal *et al.* 1988; Baksi and Frazier 1990; Pesonen and Andersson 1991). The suspension culture of trout hepatocytes exhibited a gradual decrease in cell survival over time. The results here showed that nearly 80% of the trout hepatocytes remained viable after 24 hours in a defined condition of serum-free medium and 12°C. A previous study with suspension culture of trout hepatocytes in serum-free medium demonstrated that survival decreased from 96.3% to 75.7% after 24 hours of incubation, which is consistent with the present findings (Klaunig *et al.* 1985). Another recent study using trout liver cells reported that cells maintained in free-serum medium were able to perform central functions of hepatocellular metabolism such as gluconeogenesis, lipogenesis and responsiveness to hormones for up to 20 hours (Segner *et al.* 1994).

Constant agitation of 50 oscillation.min^{-1} applied to suspension cultures resulted in a dramatic increase of trout hepatocyte mortality. Previous work using rat hepatocytes in suspension culture has shown that reciprocal agitation tends to break up more cells than orbital agitation (Crane and Miller 1977). Although the agitation applied to the cultures was gentle and followed an orbital pattern, the hepatocyte survival observed here was five-fold lower than in static suspensions. The higher content of debris and damaged cells found in suspension culture with agitation might have contributed to the increase of cell mortality. A possible explanation for the increase of damaged cells and debris might be based on the fact that the geometry of the incubation dish was not appropriate for the culture volume and the shaking conditions may not have been able to keep the cells evenly suspended. In fact, a substantial amount of dead and damaged hepatocytes were found stuck to the plastic around the air-liquid interface. Kreamer *et al.*, (1986) reported that damaged cells can adversely affect the remaining viable cells in a suspension culture of rat hepatocytes by the release of proteases into the medium.

The addition of mammalian sera (foetal calf serum-FCS) to the medium of trout hepatocyte suspensions resulted in a slight increase of cell survival. The best hepatocyte survival was achieved when cells were incubated in medium containing

10% FCS, although no significant differences were found between this concentration and cells incubated with 2% FCS. Likewise, previous work using mammalian serum to improve cell viability in primary suspensions of trout hepatocytes showed that no statistical differences were found between cells grown in 5, 10 and 20% of foetal bovine serum after the first 24 hours of incubation (Klaunig *et al.* 1985). Studies carried out with fish cell lines have demonstrated that growth rate is not significantly different between cells incubated with 0, 1, 2, 5 or 10% FCS after the first day of culture (Shea and Berry 1983; Tocher and Dick 1991). The purpose of using FCS in short-term suspension culture of trout hepatocytes was not necessarily to improve culture growth rate but to provide a suitable condition for preservation of hepatocyte integrity. Foetal calf serum is a source of hormones such as insulin which stimulates protein synthesis and inhibits cell degradation. Berry *et al.* (1991) reported that in long-term incubations the use of 10% of FCS reduced the tendency of rat hepatocytes to aggregate thus improving the culture viability.

The optimal growth temperature over which a particular cell culture will grow usually reflects the animal species form which its cells were derived and its natural environment. Temperatures of 15°-20°C are usually optimal for cells from "cold-water" fish species such as trout and salmon (Nicholson 1989). The rainbow trout used in the present work were kept constantly in well-oxygenated freshwater at 12°C throughout the period of study. The choice for incubating the trout hepatocytes at 12°C was essentially to avoid temperature variations which would eventually increase stress affecting cell metabolism. Trout hepatocytes were incubated at 5°, 10°, 15° and 20°C in order to study the effect of temperature on the cell survival. The results here demonstrated that hepatocytes incubated at lower temperatures showed higher survival rates, although no significant difference ($P<0.05$) was found in survival between temperatures of 5°C, 10°C and 15°C. The present results are similar to those of Klaunig *et al.* (1985) who reported that trout hepatocytes incubated at temperatures of 10°C, 15°C, 20°C and 25°C showed no significant difference ($P<0.01$) in survival during the first 6 days of culture. It is possible that the lower mortality rate displayed

by the hepatocytes incubated at 5°C is a direct result of the effect of temperature on the inhibition of the culture degradation. For instance, proteolytic enzymes are less active at 5°C than in higher temperatures. Studying the process of temperature acclimation on primary teleost hepatocyte suspensions, Koban (1986) indicated that protein degradation was highest at 25°C and lowest at 7°C. It is known that cells exposed to a temperature lower than normal tend to slow down their metabolism, since enzyme kinetic activity varies directly with temperature at a molecular level. Rainbow trout gonadal cell line (RTG-2) proliferates at temperatures ranging from 4°C to 26°C (Wolf and Quimby 1969). At 4°C the RTG-2 cells takes 13 days for the population to double in size, whereas at 20°C (near optimum) the number of cells in the culture double in only 2 days.

The lipid class composition of freshly isolated hepatocytes from trout used in the present work was characterised by possessing high amount of TAG, PC and EGP, which resembles the composition of trout liver tissues reported previously (Castell *et al.* 1972b; Hazel 1979a; Leger, Fremont *et al.* 1980). The results obtained here showed that the content of hepatocyte CE and TAG increased by 61.7% and 16.5% of their initial values, respectively, within 24 hours of incubation, although the differences in TAG levels were not statistically significant. As a result, the relative proportion of EGP and PC decreased reciprocally. The increased content of these neutral lipids in the hepatocytes might be related to the fish's nutritional condition, since metabolic functions of freshly isolated hepatocytes mostly resemble the intact liver. The rainbow trout used in the experiments were fed on a high caloric diet, rich in fish oil, which presumably had induced esterification enzymes towards the formation of storage lipid such as TAG. In addition, lactate present in the hepatocyte culture medium might have boosted the formation of TAG since it is known to be one of the most effective substrates for lipogenesis (Voss and Jankowsky 1986). Time course incubations demonstrated that no significant changes in fatty acid composition occurred within the first 3 hours of incubation.

The results here showed that the total lipid content increased more in freshly isolated hepatocytes incubated with 10% than with 2% FCS. Tocher and Dick (1990) likewise found that the total lipid content in Atlantic salmon cell line incubated with 10% FCS was 24% higher than in cells incubated with 2% FCS. Regardless of lipid content, trout hepatocytes incubated for 24 hours in the presence of 2% and 10% FCS showed no significant changes in lipid class composition, although neutral lipids tended to increase in cells incubated in the presence of FCS. This may be related directly to the fact that serum lipids with high contents of TAG and CE were the only available lipid source in the medium. Moreover, a study using the cultured salmon cell line demonstrated that the percentage of neutral lipids such as TAG and cholesterol were higher in cells incubated at 10% than at 2% FCS (Tocher and Dick 1990).

Shifting the incubation temperature of hepatocytes isolated from rainbow trout maintained at 12°C to 5°C and 20°C evoked no detectable significant changes on the total lipid content and lipid class composition of the cells. Although it is known that the relative proportion of EGP increases in hepatocytes when trout is submitted to cold exposure (Hazel 1979a), the present results showed that no significant changes in PE content were observed when hepatocytes where incubated either at cold (5°C) or at warm (20°C) temperatures. Although TAG content in cells incubated at 20°C for 24 hours increased compared to cells at 12°C, the results were not statistically different. Overall, the results are in keeping with temperature studies on lipogenesis in rainbow trout hepatocytes which demonstrated that the incorporation of ^{14}C-acetate into the lipid classes was not affected by the assay temperature with the exception of TAG which increased at 20°C (Voss and Jankowsky 1986). In addition, long term incubations of a rainbow trout gonadal cell line (RTG-2) showed no significant differences in the incorporation of radiolabelled (n-3) and (n-6) PUFA into lipid classes either at 10°C or 22°C (Tocher and Sargent 1990a). Liver slices of European eel (*Anguilla anguilla*) incorporate ^{14}C-acetate into lipid classes at a rate independent of incubation temperature (Abraham *et al.* 1984).

The fatty acid composition of freshly isolated hepatocytes from rainbow trout was characterised by a high content of 22:6n-3. Several previous studies have reported that 22:6n-3 is the major constituent in rainbow trout hepatocyte and liver tissue lipids (Hazel 1979ab; Leger *et al.* 1980; Sellner and Hazel 1982ab; Henderson and Sargent 1981, 1983, 1984). The trout hepatocytes used in the present experiment showed a significant increase in 22:6n-3 content over 24 hours, but the other individual fatty acids were not significantly affected. These results indicate that there was active synthesis of 22:6n-3 in the trout intact liver tissue and are in keeping with the general belief that among all tissues in fresh water fishes, including trout, the liver is the most active tissue involved in fatty acid biosynthesis (Henderson and Tocher 1987).

To examine the effect of FCS lipid on trout hepatocyte fatty acid composition cells were incubated with medium containing 2% and 10%FCS. The addition of FCS to the hepatocyte medium caused no significant changes in the cells fatty acid composition. The slight decrease of (n-3) PUFA, particularly 22:6n-3, with the increase of (n-6) PUFA in cells incubated in 10%FCS is evidence that the hepatocytes could up take serum lipid from the medium, which was typically rich in (n-6) PUFA and poor in (n-3) PUFA. The effect of mammalian serum on the fatty acid composition of fish cell line was investigated by Tocher *et al.* (1988, 1990, 1995) who demonstrated that cultured fish cell line had a fatty acid composition which reflected the composition of the foetal calf serum in the media rather than their fish tissue origins. In fish cells cultured in FCS the levels of (n-6) PUFA tended to increase whereas the (n-3) PUFA content became depleted to the point of deficiency (Tocher *et al.* 1988, 1995; Tocher and Dick 1990).

The fatty acid composition of hepatocytes and liver tissue of thermally acclimated rainbow trout has been extensively investigated by Hazel and his group (Hazel 1979ab; Hazel and Prosser 1979; Hazel and Sellner 1979; Sellner and Hazel 1982ab). In early studies Hazel demonstrated that liver phospholipids of trout exposed to 5°C exhibited an increased quantity of polyunsaturated fatty acids,

particularly those of the (n-3) series, and a lower amount of saturated fatty acids compared to fish exposed to 20°C (Hazel 1979ab). During acclimation to 5°C the proportion of 22:6n-3 increased in PC, while that of 20:5n-3 increased in PE, SM and PS. The levels of 20:4n-6 increased in PI followed cold exposure (Hazel 1979a). The increase of unsaturation of the (n-3) series was also observed in the TAG fraction of liver tissue from cold acclimated trout (Hazel 1979b). In contrast, incubating isolated hepatocytes at 5°C or 20°C from cold-acclimated rainbow trout Hazel and Prosser (1979) showed that fatty acid compositions did not change significantly during assay, although hepatocytes from cold-acclimated trout exhibited higher levels of (n-3) PUFA and lower levels of monoenoic fatty acids than hepatocytes from warm-acclimated fish. In agreement with the studies of Hazel and Prosser (1979), the present experiments demonstrated that no significant differences in the content of individual fatty acids were observed in trout hepatocytes incubated at 5°C or 20°C. Nevertheless, the levels of total (n-3) PUFA increased significantly in cells incubated at 5°C, whereas total monoenoic fatty acids decreased. These results suggest that the exposure of trout hepatocytes to 5°C lead to an increase of (n-3) PUFA formation as a response of cell adaptation.

Studying the incorporation of ^{14}C-acetate in carp livers, Farkas and Csengeri (1976) showed that more radioactivity was distributed into long-chain PUFA when livers were incubated at 5°C than at 20°C. These authors concluded that the distribution of radioactivity among different fatty acids was dependent on the experimental temperature rather than the temperature to which the fish were adapted, suggesting that incubation temperature could act directly upon the fatty acid metabolism of isolated cells adjusting the biosynthesis pattern to assure the proper physico-chemical properties for the functional cell membrane. The effect of temperature acclimation on several enzyme activities in isolated hepatocytes from catfish was investigated by Koban (1986) who showed that cultured hepatocytes exhibited only a partial temperature acclimation *in vitro*, suggesting that other chemical factors such as hormones are required to mediate the full process of

acclimation. A recent study on the process of acclimation in rainbow trout hepatocyte membrane concluded that the plasma membrane of trout hepatocytes is a highly dynamic structure characterised by continuous lipid restructuring/turnover which can be rapidly alterated upon acute cold exposure to adjust membrane phospholipid composition to the prevailing thermal environment (Williams and Hazel 1995).

The modified method for isolation of trout hepatocytes described in this section proved to be appropriate for the present study since it delivered high rates of viable hepatocytes (>96%). At the same time, the culture medium and conditions for hepatocyte suspensions were also established. As a result of preliminary experiments and discussion it was decided to incubate the hepatocytes using low concentration of mammalian sera (2%) and temperature adjusted to 12°C.

The data presented here also indicated that the use of either 2% or 10% FCS in primary suspensions of trout hepatocytes did not affect the lipid class and fatty acid compositions of the cells during the period of 24 hours incubation. A medium concentration of 2% FCS was considered the most advisable to adopt, since it is sufficient to sustain the cells with hormones while keeping the lipid and fatty acid composition unaltered. The data also indicated that the temperature of 12°C (holding temperature of the fish) is the most appropriate for hepatocyte incubations as it removes any problems that might result from temperature acclimation when significant shift of temperature occurs.

CHAPTER 3

BIOSYNTHESIS OF DOCOSAHEXAENOIC ACID (22:6N-3) BY HEPATOCYTES ISOLATED FROM RAINBOW TROUT

3.1. Introduction

The lipids of fish in general are characterised by high contents of eicosapentaenoic acid 20:5n-3 and especially docosahexaenoic acid (22:6n-3) (Henderson and Tocher 1987). Vertebrate animals are incapable of synthesising these polyunsaturated fatty acids (PUFA) *de novo* although they can, depending on species and to greater or lesser extents, readily convert dietary 18:3n-3 to 20:5n-3 and thence to 22:6n-3.

Several authors have investigated the conversion of 18:3n-3 to 22:6n-3 in the liver of rainbow trout (Owen *et al.*, 1975; Kanazawa *et al.*, 1979; Sellner and Hazel 1982ab; Hagve *et al.*, 1986). *In vivo* studies have shown that when rainbow trout are injected with radiolabelled linolenic acid (18:3n-3) radioactivity is recovered in a wide range of PUFA acids including 20:5n-3 and 22:6n-3 (Owen *et al.*, 1975; Kanazawa *et al.*, 1979). Owen *et al.* (1975) examined the distribution of radioactivity from [1-^{14}C]-18:3n-3 in the body lipid of rainbow trout for 6 days and found that 22:6n-3 was the most labelled PUFA comprising 70% of the total radioactivity, followed by 18:3n-3, 20:5n-3, 18:4n-3, 20:4n-3 and 22:5n-3 which shared the remaining proportion of radioactivity. Another *in vivo* study demonstrated that when [1-^{14}C]-18:3n-3 was injected into rainbow trout, 10% and 12.5% of the total radioactivity present in the polar fraction of trout lipid were recovered after 24 hours in 22:6n-3 and 20:5n-3, respectively (Kanazawa *et al.*, 1979). Hepatocytes isolated from rainbow trout can efficiently incorporate radiolabelled 18:3n-3 and rapidly desaturate and elongate this C_{18} PUFA into longer chain and more unsaturated fatty acids including 20:5n-3 and 22:6n-3 (Sellner and Hazel 1982ab; Hagve *et al.*, 1986).

The study of Sellner and Hazel (1982ab) using isolated hepatocytes from thermally acclimated trout demonstrated that 22:6n-3 is always produced when cells are incubated with [1-^{14}C]-18:3n-3, albeit 22:6n-3 is only produced in small quantities. Similarly, Hagve *et al.*, (1986) demonstrated that although the desaturation and elongation rate of [1-^{14}C]-18:3n-3 into 22:6n-3 in trout hepatocytes was higher than in rats, the proportion of radioactivity present in trout 22:6n-3 never exceeded 2% of the total incorporated radioactivity.

The rates of formation of 22:6n-3 have invariably been low in this system and details of the conversion pathway(s) have not been established in this, or in any other fish species. The activity of fatty acid desaturases in mammals is known to be affected by dietary and hormonal factors (Brenner 1981) and studies carried out with rats showed that prolonged fasting decreases Δ6 desaturation activity and that the re-administration of a fat free diet based on glucose to a pre-fasted animal reactivates the desaturation enzymes (Brenner 1981). Fish can withstand long periods of starvation, but it can be expected that the rate of lipogenesis decreases in response to the reduction of the substrate available for fatty acid synthesis. It has been shown in coho salmon that hepatic lipogenic activity is markedly reduced by starvation (Lin, *et al.* 1977). The strategy of inducing Δ9-desaturation activity by a fast-refeeding regime has long been shown to be effective in mammals, but has only recently been demonstrated in fish (Tocher *et al.*, 1996).

Several *in vitro* studies with rat liver microsomes have indicated that Δ6-desaturase activity is reduced in animals fed diets rich in long chain (n-3) polyunsaturated fatty acids (PUFA) (Brenner and Peluffo 1966; Garg *et al.*, 1988; Christiansen *et al.*, 1991; Kanazawa and Fujimoto 1993). There is some evidence from *in vivo* studies with rainbow trout that dietary 20:5n-3 and 22:6n-3 inhibit the Δ6-desaturation of both 18:2n-6 and 18:3n-3 (Owen *et al.*, 1975; Yu *et al.*, 1977; Leger *et al.*, 1980). Since fish oil rich in 22:6n-3 is the major lipid component of most commercial fish diets, it is inevitable that the formation of 22:6n-3 from 18:3n-3 will be low in fish fed these diets.

The biosynthesis of PUFA in liver tissue of mammals (Gurr and Harwood 1991) as well as in fish (Ninno *et al.*, 1974; De Torrengo and Brenner 1976) is a microsomal process that proceeds *via* alternating position-specific acyl-CoA desaturases and malonyl-CoA-dependent chain elongation reactions. It has traditionally been accepted that 18:3n-3 is converted to 22:6n-3 by a pathway combining the sequential action of Δ6, Δ5 and Δ4 desaturases with chain elongation reactions (Henderson and Tocher 1987). However, recent studies with rat hepatocytes have established that a Δ4 desaturase may not be obligatory in the pathway and that 22:6n-3 can be biosynthesised by the sequential chain elongation of 20:5n-3 to 22:5n-3 and thence to 24:5n-3, followed by Δ6 desaturation of 24:5n-3 to yield 24:6n-3, which is finally chain shortened by peroxisomes to yield 22:6n-3 (Voss *et al.*, 1991; Voss *et al.*, 1992). Moreover, other studies performed with frog retina tissue (Wang and Anderson 1993) and rat brain tissue demonstrated that 22:6n-3 biosynthesis proceeds via C_{24} intermediates. Nevertheless, the conversion of 24:5n-3 and 24:6n-3 to 22:6n-3 remain to be demonstrated in fish. The hypothesis proposed recently by (Voss *et al.*, 1991) suggests that the process of 22:6n-3 biosynthesis, involving C_{24}-PUFA as intermediates, is a conjugation of anabolic and catabolic processes (desaturation/elongation and ß-oxidation) which occur separately in distinct compartments within the cell, the microsomes and peroxisomes respectively. The extent to which this pathway proposed by Voss *et al.*, (1991) exists in species other than mammals and amphibian retina is not known. In particular, it is not known whether the pathway exists in species that are naturally rich in 22:6n-3 and can readily biosynthesise this fatty acid, either *de novo* as occurs in certain single cell eukaryotes, or from 18:3n-3 as occurs in certain lower vertebrates (Tinoco, 1982).

The aim of the present study was to characterise the steps involved in the biosynthesis of 22:6n-3 from 18:3n-3 in hepatocytes isolated from rainbow trout. The distribution of radioactivity from [1-^{14}C]-18:3n-3 into lipid class of trout hepatocytes was determined and the desaturation and elongation products of 18:3n-3 characterised

and identified. In the present study, rainbow trout were subjected to starvation regimes as well as fed a diet deficient in 22:6n-3 in an effort to enhance the rate of conversion of 18:3n-3 to 22:6n-3 in hepatocytes isolated from these fish, and hence facilitate investigation of whether the pathway which excludes direct Δ4 desaturation exists in this species. In addition, the desaturation and elongation of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 in trout liver microsomes were examined.

3.2. Materials and Methods

3.2.1. Materials

[1-^{14}C]Linolenic acid (specific activity 54mCi/mmol) and [1-^{14}C]eicosapentaenoic acid (specific activity 58mCi/mmol) were purchased from Amersham International plc (Bucks., UK). Foetal calf serum and casein were obtained from ICN Biochemicals (Thame, Oxfordshire, UK). Fish meal was obtained from Avebe (Veendam, Holland), fish oil from Seven Seas (Hull, UK) and olive oil from a local supermarket (Tesco Stores, UK). Ecoscint A was purchased from National Diagnostics (Sigma, Poole, Dorset, UK). Solvents were of HPLC grade and were purchased from Rathburn Chemicals (Walkerburn, Peebleshire, UK). All the reagents used in the hepatocytes and microsomal preparation and incubations were purchased from SIGMA Biochemicals (Poole, Dorset, UK).

3.2.2. Experimental fish and dietary regimes

Rainbow trout, *Oncorhynchus mykiss*, (400-450g) were kept in a 500 litre aquarium and fed a commercial diet at a daily rate of 2% of their body weight. Fish from this stock tank were divided into 3 groups each of 12 fish and transferred to 150 litre circular tanks at 12°C. In one group the fish were fed 30 days on a commercial diet at the normal feeding rate (Treatment A). Another group of trout were starved for 30 days (Treatment B). In the third group the fish were starved for 25 days and refed

for 5 days on a commercial diet at the same feeding rate (Treatment C). After 30 days all the trout were killed to prepare hepatocytes (section 2.2.3).

In the time course desaturation experiments, juvenile rainbow trout weighing circa 70g were used to prepare hepatocytes. Juvenile rainbow trout (circa 70 g), were divided into two groups, both maintained in 150 litre self-cleaning, circular tanks supplied with recirculated flowing water at 12°C. One group was maintained on a control diet containing fish oil, while the other group was fed an experimental diet based on olive oil and deficient in 20:5n-3 and 22:6n-3 (Table 3.1, Table 3.7). Both groups were fed at a daily rate of 2% of their body weight for 120 days then sacrificed to prepare hepatocytes (section 2.2.3).

3.2.3. Preparation of trout liver microsomes

After trout were sacrified, livers were rapidly removed, finely minced and washed twice in 0.25M sucrose containing 2.5 mM EDTA and 100mM potassium phosphate buffer, pH 7.0. The livers were homogenised in the same medium (1g liver in 4ml) using a Teflon-in-glass homogenizer (Glas-col, Indiana, USA), and the homogenate centrifuged at 3.6 $.10^3$ x g.min to remove intact cells and debris, and then at 6.8 $.10^4$ x g.min to pellet the mitochondria. The post-mitochondrial supernatant was centrifuged at 3.75 $.10^5$ x g.min to pellet the peroxisomal-rich fraction and the resulting supernatant then centrifuged at 6.3 $.10^6$ x g.min to obtain microsomes and cytosol. Protein content was determined by the method of (Lowry *et al.*, 1951) using essential fatty acid - free BSA as standard.

3.2.4. Analysis of trout liver cell fractions

The trout liver cell fractions obtained by differential centrifugation were tested routinely for purity. Three diagnostic enzymes were assayed to estimate contamination: succinate dehydrogenase for mitochondria, catalase for peroxisomes and NADH-cytochrome c reductase for microsomes.

Table 3.1. Composition of the control and (n-3)-PUFA deficient diets fed to rainbow trout

Component	Control (g/kg)	n-3 deficient (g/kg)
Casein	450.0	450.0
Fish meal	50.0	50.0
Starch	150.0	150.0
Fish oil (Fosol)	150.0	
Olive oil		150.0
Mineral mix[1]	47.1	47.1
Vitamin mix[2]	10.0	10.0
Antioxidant mix[3]	0.4	0.4
Choline chloride (40% w/v)	10 ml	10 ml
Arginine	4.0	4.0
Methionine	3.0	3.0
Cystine	2.0	2.0
Leucine	4.0	4.0
α –Cellulose	125.0	125.0

1. Supplied (g/100g): $CaCO_3$ 1.8; $Ca_3(PO_4)_2$ 41.7; K_2HPO_4 20.6; NaH_2PO_4 13.0; NaCl 6.6; KCl 5.0; $MgSO_4$ 9.1; $FeSO_4$ 3.0; $ZnSO_4$ 0.4; $CuSO_4$ 0.1; $MnSO_4$ 0.4; KI 0.02; $CoSO_4$ 0.1.

2. Supplied (mg/kg): all-racemic-α-tocopheryl acetate, 40; menadione, 10; ascorbic acid, 1000; thiamine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate, 7.3; cholecalciferol, 0.06.

3. Dissolved in propylene glycol and contained (g/L): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40.

The succinate dehydrogenase activity in cell fractions of trout liver was assessed according to the method of Clark and Porteous (1964). Succinate dehydrogenase is found in mitochondria and it oxidises succinate to fumarate in the tricarboxylic acid cycle. The enzyme appears to form an integral part of the mitochondrial membrane. The flavin adenine dinucleotide (FAD) is the H^+ acceptor in the reaction.

$$\text{Succinate} + FAD^+ \Leftrightarrow \text{fumarate} + FADH + 2\varepsilon$$

The hydrogen acceptor for this assay is iodonitrotetrazolin violet which is reduced to red formazan. A 3.0 ml solution of sodium phosphate (pH 7.2) containing 0.5M sodium succinate, 0.1% p-iodonitrotetrazolium violet, and 100 µl of the cell fraction was incubated at 30°C for 30 minutes. The reaction was terminated by the addition of 300 µl of 50% trichloroacetic acid (TCA) and the red formazan extracted with 4.0 ml ethyl acetate. The tubes were centrifuged at 1200 rpm for 5 minutes to remove the precipitate. The organic layer containing the formazan was measured spectrophotometrically at 490 ηm. The specific activity for succinate dehydrogenase was expressed as nmoles $FADH_2$ mg protein $^{-1}$ min^{-1} (Figure 3.1).

Catalase activity was determined according to the method of Baudhuin *et al.*, (1964).The enzyme catalase present in the peroxisomes is involved in the detoxification of hydrogen peroxide (H_2O_2). It catalyses the conversion of H_2O_2 to water and oxygen.

$$2\ H_2O_2 \Leftrightarrow 2\ H_2O + O_2$$

The method consisted in measuring the residual H_2O_2 after the incubation with the enzyme using the yellow titanyl sulphate-H_2O_2 complex. A solution of 200 µl of 20mM imidazole buffer (pH 7.0) was made and divided in two 100 ml aliquots. BSA (0.2g) was added to one of the aliquots to prepare the catalase blank solution (CBS),

and 30% H_2O_2 added to the other aliquot to make the catalase substrate solution (CSS). All the incubations were kept on ice and were carried out by adding 100 µl of the cell fraction containing the enzyme, 100 µl of 2.2% (w/v) Triton-X-100 and 5.0 ml of the CBS or CSS. The control blank contained 100 µl of distilled water instead of enzyme, 5.0 ml of CSS and 100 µl Triton-X-100. After 1 minute the reaction was terminated by adding titanium oxysulphate (2.25g/L) in 1M H_2SO_4 and the solution measured spectrophotometrically at 405 ηm against distilled water. The final absorbance was calculated according to the equation:

$$\text{Final Abs} = \text{Abs control blank} - (\text{Abs CSS} - \text{Abs CBS})$$

The specific activity for catalase was expressed as units/mg protein. One unit of the enzyme was defined as the amount of destroying 90% H_2O_2 present in 50 ml reaction volume per minute (Figure 3.1).

The activity of NADH-cytochrome *c* reductase was estimated according to the method of Edelhoch *et al.*, (1952). Cytochome *c* reductases are enzymes that transfer electrons from the pyridine nucleotide-linked dehydrogenase to cytochrome *c*. In microsomal membranes NADH-cytochrome *c* reductase is involved in the reduction of cytochrome c and in the transport of electrons from NADH to cytochrome P_{450}.

$$\text{NADH} + \text{ferricytochrome } c \Leftrightarrow NAD^+ + H^+ + 2 \text{ ferrocytochrome } c$$

The enzyme activity can be measured spectrophotometrically at 30°C by the increase in optical density at 550.5 ηm in the presence of excess NADH. The rate of increase is associated with the rate of reduction of the α-band of cytochrome *c*. The cell fractions containing enzyme (2-4µg) were incubated at 30°C in 1 ml quartz cuvette placed in the spectrophotometer (Uvikon model 940, Kontron Instruments, Milan, Italy) slot. The incubation medium contained 1.4 mM NADH, 1.0% cytochrome *c*, 0.1M sodium azide in 600 µl of 0.2M K^+ phosphate buffer (pH 7.5). The cuvette

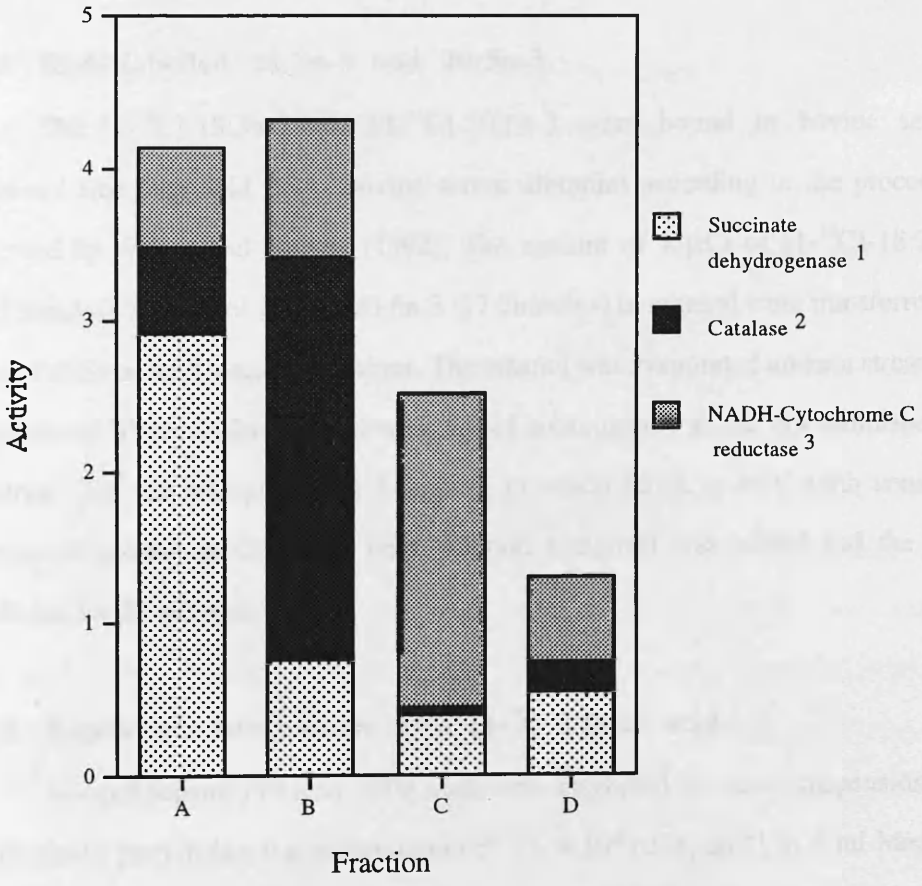

Figure 3.1. Typical distribution of subcellular fractions of trout liver separated by differential centrifugation using a 0.25M sucrose gradient. A, mitochondrial fraction; B, peroxisomal-rich fraction; C, microsomal fraction; D, cytosol.1, nmoles $FADH_2$ /mg protein/min ; 2, units/mg protein/min; 3, nmoles NADH produced/mg protein/min.

volume was made up to 1 ml with distilled water. The cell fractions were solubilised in 10% sodium cholate in phosphate buffer prior to the incubation to obtain maximal activity. The rate of reduction of cytochrome *c* was measured at 550 nm. Optical density readings were taken at 15 sec. intervals against a blank containing cytochrome *c* in phosphate buffer. Specific activity was expressed as μmoles of cytochrome *c* reduced per minute per milligram of cell fraction protein (Figure 3.1).

3.2.5. Radiolabelled 18:3n-3 and 20:5n-3

The [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 were bound to bovine serum albumin (free fatty acid BSA- bovine serum albumin) according to the procedure described by Wilson and Sargent (1992). The amount of 1 μCi of [1-^{14}C]-18:3n-3 (18.5 nmoles) or 1μCi of [1-^{14}C]-20:5n-3 (17.2nmoles) in ethanol were transferred to a 2ml reacti-vial with a magnetic stirrer. The ethanol was evaporated under a stream of nitrogen and 50 μl of 22mM KOH were added subsequently to the dry radiolabelled substrate. The vial was placed for 5 minutes in a heat block at 36°C with constant stirring. A volume of 20 μl of BSA solution (1mg/ml) was added and the vial incubated for 30 minutes.

3.2.6. Hepatocyte incubations with [1-^{14}C]-fatty acids

Isolated hepatocytes from 400g trout were incubated as static suspensions in sterile plastic petri dishes at a concentration of 2.5 x 10^6 cells. ml $^{-1}$ in 5 ml Medium 199 supplemented with 2% foetal calf serum and 10mM lactate. The cells were incubated for 3 hours at 12°C with 18.5 nmoles (1μCi) of [1-^{14}C]linolenic acid adsorbed onto albumin. In the time course experiments, the hepatocytes from juvenile trout (circa 70g) were incubated in 100ml Falcon sterile flasks (Primaria, Oxnard, CA, U.S.A.) containing 30ml of the cell suspension. Five millilitres of cell suspension were taken from the incubation flasks after 1, 1.5, 3, 6, 12, and 24 hours.

In dietary experiments, isolated trout hepatocytes were incubated as primary suspensions (orbital shaking, 50 oscillations/min.) in 10ml sterile plastic petri dishes (Bibby Sterilin Ltd., Stone, Staffs, UK) containing 5 ml of the cell suspension in the same medium and at the same cell concentration as above. The cells were incubated for 3 hours at 12°C with 9.3 nmoles (0.5μCi) of [1-^{14}C]linolenic acid or 8.6 nmoles (0.5μCi) of [1-^{14}C] eicosapentaenoic acid. The radiolabelled fatty acids were added to the medium as their potassium salts bound to BSA.

Trout hepatocytes were also incubated for 3 hours with 0.3 μCi [^{14}C]-24:5n-3 and 0.3μCi of [^{14}C]-24:6n-3 (prepared as described below) at 12°C for 3 and 6 hours, respectively.

3.2.7. Microsomal incubations with [1-^{14}C]-fatty acids

Microsomal incubations were performed in a 2 ml Eppendorf vial containing 3.0 mg of microsomal protein in a total volume of 1.2 ml. The standard desaturation and elongation assay system contained: 0.15 M KCl, 10 mM $MgCl_2$, 0.3 mM nicotinamide, 1.5 mM glutathione, 1.5 mM NADH, 1.5 mM NADPH, 3.5 mM ATP, 0.2 mM coenzyme A, 0.2 mM acetyl-CoA and 0.2 mM malonyl-CoA in 100mM potassium phosphate buffer (pH 7.0). The microsomes were incubated with 9.3 nmoles (0.5μCi) of [1-^{14}C]-linolenic acid or 8.6 nmoles (0.5μCi) of [1-^{14}C]-eicosapentaenoic acid for 3 hours at 12°C. All radiolabelled fatty acids were added to the incubation medium as their potassium salts bound to BSA.

3.2.8. Preparation of radiolabelled 24:5n-3 and 24:6n-3

Radioactive 24:6n-3 was synthesised biochemically by incubating [1-^{14}C]-20:5n-3 in the presence of microsomes isolated from trout by differential centrifugation as described above. Incubations with microsomes were performed in 100ml sterile polypropylene Falcon culture bottles containing 75mg of microsomal protein, 0.15 M KCl, 10 mM $MgCl_2$, 0.3 mM nicotinamide, 1.5 mM glutathione, 1.5 mM NADH, 1.5 mM NADPH, 3.5 mM ATP, 0.2 mM coenzyme A, 0.2 mM

acetyl-CoA and 0.2 mM malonyl-CoA in 100mM potassium phosphate buffer (pH 7.0) in a total volume of 67.5ml. After the addition 511 nmoles (30μCi) of [1-^{14}C]-20:5n-3 as the potassium salt bound to BSA, the microsomes were incubated for 3 hours at 12°C. At the end of this time the incubation system was aspirated into test tubes and the microsomal lipids extracted with chloroform/methanol (2:1 by volume) (section 2.2.6). The organic solvent was removed under a stream of nitrogen and the total lipid extract desiccated under vacuum for 2 hours. The dried lipids were then subjected to acid-catalysed transesterification as described previously (section 2.2.8) to generate fatty acid methyl esters (FAME), which were separated by degree of unsaturation and chain length by argentation thin-layer chromatography ($AgNO_3$-TLC) (section 3.2.11). The radiolabelled 24:5n-3 and 24:6n-3 were located on the TLC plate, marked and scraped into test tubes for extraction using ice-cold chloroform/methanol (2:1 by volume) The samples were washed with 20% NaCl (w/v) to precipitate the remaining silver. The identities of ^{14}C-24:5n-3 and ^{14}C-24:6n-3 were confirmed using a gas chromatograph coupled to a radio-detector as described below (section 3.2.12). To remove the remaining traces of radioactive C_{20} substrate and other radioactive FAME, the isolated ^{14}C-24:5n-3 and ^{14}C-24:6n-3 were re-subjected to argentation high performance thin-layer chromatography ($AgNO_3$-HPTLC) on 10cm x 20cm x 0.15mm HPTLC plates using toluene/ acetonitrile (97:3 by vol). Unresolved or partially separated radioactive $AgNO_3$-HPTLC FAME bands were scraped, extracted and re-run on a 10cm x 20cm x 0.15mm HPTLC plate using a more polar toluene/acetonitrile (95:5 by vol.) solvent system. The final purity of radiolabelled 24:5n-3 and 24:6n-3 was in excess of 95% as determined by analysis with $AgNO_3$-HPTLC and radio gas chromatography. Contaminants were mainly composed of oxygenated products and the substrate [1-^{14}C]-20:5n-3. The identification of 24:5n-3 and 24:6n-3 was aided by radio GLC analysis of the samples before and after catalytic hydrogenation (section 3.2.12) and also by comparison of the retention times on a radio GLC with those of 24:5n-3 and 24:6n-3 previously identified by GC-mass spectrometry (Henderson *et al.*, 1995).

The yield of radiolabelled PUFA generated from [1-^{14}C]20:5n-3 is presented in Table 3.2. The purified C_{24} methyl esters were hydrolysed to free fatty acids by saponification with 1M KOH in 95% ethanol (Christie 1982).

3.2.9. Incorporation of [1-^{14}C]-fatty acids into lipid classes

The incorporation of [1-^{14}C]-18:3n-3 into lipid classes was determined by high performance thin-layer chromatography (HPTLC). Total lipid (30μg) was applied as a 3mm streak to a 10cm x 10cm HPTLC plate coated with silica gel G60 and which had been pre-run in diethyl ether : hexane (1:1 by vol.). The plates were developed half way with methyl acetate-isopropanol-chloroform-methanol-0.25% KCl (25:25:25:10:9 by vol) and then developed fully with hexane : diethyl ether : glacial acetic acid (80:20:2 by vol.). The developed chromatograms were exposed to iodine vapour to visualise the components. The bands of adsorbent containing lipid classes were marked and, after sublimation of the iodine, scraped into scintillation vials containing 2.5ml Ecoscint A. Radioactivity was determined using a Packard 2200CA Tri-Carb liquid scintillation analyser and the data obtained calibrated using a quench standard curve.

Total polar lipids were separated from neutral lipids by thin-layer chromatography (TLC) on 20 cm x 20 cm glass plates coated with silica gel G60 using hexane : diethyl ether : glacial acetic acid (80:20:2 by vol.) as the developing solvent. Developed chromatograms were sprayed with 2',7'-dichloroflourescein in methanol containing 0.01 % BHT and the lipids visualised under UV light. The bands of adsorbent containing polar and neutral lipids were scraped off the plates into test tubes and subjected to acid-catalysed transesterification as described by Christie (1982) to produce fatty acid methyl esters (FAME). Bands of adsorbent containing cholesterol esters, triacylglycerols and free fatty acids were combined as a single neutral lipid fraction. A portion of total lipid was also subjected to transesterification. FAME were quantified by gas chromatography (section 2.2.8).

Table 3.2. Preparation of radioactive (n-3) PUFA substrates by trout liver microsomes incubated with 30μCi (511 ηmoles) [1-^{14}C]20:5n-3.

Fatty acid	η moles [^{14}C]-PUFA recovered
20:5n-3	114.8
22:5n-3	28.7
24:5n-3	10.3
24:6n-3	51.6
Total incorporated*	205.1

*Total radioactivity incorporated in liver microsomal lipid. Incubation was performed during 3 hours at 12°C

3.2.10. Preparation of quench calibration curves

The method used to measure quench was the transformed Spectral Index of the External Standard (tSIE) which was calculated from the Compton spectrum induced in the scintillation sample by an external ^{133}Ba gamma source. Calibration curves were prepared to estimate radioactive counting in terms of disintegration per minute (DPM) from radiolabelled fatty acid substrates used in the experiments and their metabolic products.

An amount of 0.05μCi (110000 DPM) of [1-^{14}C]-18:3n-3 in methanol was transferred to scintillation vials and the solvent evaporated under stream of nitrogen. Incremental amounts of dry silica Gel 60 (1,2,4,6....30mg; quench range) were scraped from a TLC plate (20cm x 20cm) previously impregnated with 10% (w/w) silver nitrate and added subsequently into the vials. Ecoscint A (2.5ml) was added to the silica and the vials well mixed and left at room temperature for 24 hours. The radioactivity counting (CPM) was performed by a Packard 2200 Tri-Carb liquid scintillation analyser. To determine the absolute radioactivity (DPM) of radiolabelled fatty acids incorporated into lipid classes, the above procedure was followed without impregnating the HPTLC plate with $AgNO_3$. The counting efficiency was calculated from recorded CPM values using the following relationship:

$$\text{Efficiency (\%)} = (\text{CPM} \div \text{DPM}) * 100$$

A correlation was made using the tSIE values on axis (X) and the % efficiency on the other axis (Y).The curves were fitted to the standard points and the DPM values calculated from the regression equation (Figure 3.2).

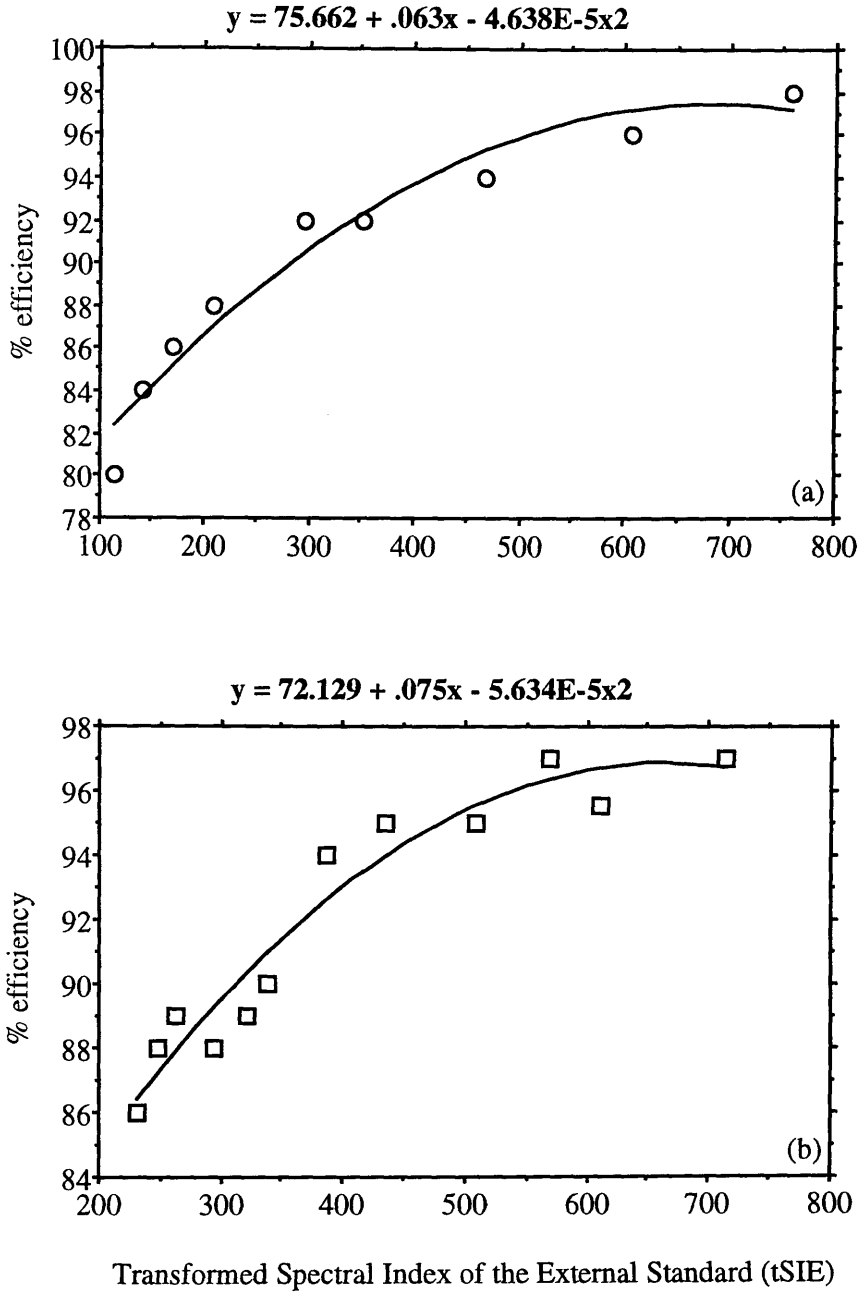

Figure 3.2. External standard tSIE quench curve for ^{14}C. The standard was prepared using [1-^{14}C]-18:3n-3 as radioactive source. (a) curve to correct quench of radioactivity distributed into lipid class. (b) curve to correct quench of radioactivity distributed in PUFA.

3.2.11. Incorporation of radioactivity from [1-^{14}C]-fatty acid into PUFA

The FAME prepared as described above were separated by the degree of unsaturation and chain length by argentation thin-layer chromatography ($AgNO_3$-TLC) according to the method of Wilson & Sargent (1992). Briefly, 20ml of acetonitrile containing 2g of $AgNO_3$ were applied with a syringe over the surface of a glass plate (20cm x 20cm) precoated with a layer of silica gel 60 of thickness 0.25mm until saturated. The plates were dried with a stream of nitrogen before being activated in the oven at 110°C for 30 minutes. FAME were streaked on to $AgNO_3$-TLC plates using a maximum loading of 1mg. cm^{-1}. The $AgNO_3$-TLC plates were developed with toluene : acetonitrile (97:3 by vol.) to 1.5 cm from the top in a standard TLC tank placed in the dark. After the development of the autoradiography plates the developed plates were dried in the desiccator and marked with radioactive ink before being subjected to autoradiography for 6 days with Konica A2 X-Ray film. The individual radioactive bands were located, marked and scraped into scintillation vials containing 2.5 ml Ecoscint A. Radioactivity was determined using a Packard 2200CA Tri-Carb liquid scintillation analyser and the data obtained calibrated using the quench standard curve from Figure 3.2.

3.2.12. Identification of radiolabelled PUFA

The identification of radiolabelled PUFA derived from the desaturation and elongation of [1-^{14}C]-18:3n-3 was performed by radio gas chromatography. After marking and locating the individual radioactive bands on a $AgNO_3$-TLC plate the bands of adsorbent were scraped into test tubes. The FAME were extracted from the silica using chloroform : methanol (2:1 by vol) containing 0.01% BHT. Aqueous KCl (0.88%w/v) was added to separate the organic phase which was subsequently dried, redissolved in hexane and washed with 20% NaCl to remove traces of silver. The FAME were identified using a gas chromatograph Chrompack model CP-9000 fitted

with a capillary column of fused silica (25m x 0.53mm) coated with CP Wax 52 CB and coupled to a Lab Logic (Sheffield, UK) GC-RAM radio detector. A mixture of argon / 10% CO_2 was used as a carrier gas. Sample application was by on-column injection and the oven temperature was programmed to rise from 50°C to 250°C during each analysis.

3.2.13. Statistical analysis

All results are means of three experiments ± standard deviation. The significant differences between dietary regimes were tested by OneWay Factorial ANOVA. The percentages were normalised by arcsin transformation prior to the statistical analysis. Differences in incubation treatments were evaluated by Scheffé's F and Bonferroni/Dunn post hoc tests and reported in tables if $P<0.05$.The significant differences in time course incubations were determined by Repeat-Measure ANOVA Single Factor. All the calculations were performed by StatView 4.0 for Apple Macintosh computers.

3.3. Results

3.3.1. Induction of desaturation and elongation activity in trout hepatocytes by dietary manipulation.

3.3.1.1. Effect of starvation and starvation/refeeding regimes on the hepatocyte lipid class and fatty acid composition

The percentage lipid class composition of hepatocytes from rainbow trout starved for 30 days is presented in Table 3.3. The data show that no significant differences were observed in the percentage of individual lipid class of hepatocytes from starved rainbow trout (treatment B) compared to those from trout fed on a commercial diet at a normal feeding rate (treatment A). The levels of neutral lipids in hepatocytes of starved trout were in general slightly lower than in those from fed trout. The re-introduction of commercial diet to the trout that had starved for 28 days (treatment C) did not alter the hepatocyte lipid class composition significantly in relation to the hepatocytes from 30-day starved trout.

Table 3.4 shows the percentage fatty acid composition of hepatocytes from rainbow trout subjected to the starvation regimes and trout fed the commercial diet. No significant changes in the individual fatty acid content were detected between the different regimes. The levels of 22:6n-3 in starved trout hepatocytes were slightly higher than in hepatocytes from fed trout, although the difference was not in the significant range.

3.3.1.2. Effect of starvation and starvation/refeeding regimes on the distribution of radioactivity from [1-^{14}C]-18:3n-3 into hepatocyte lipid class

The distribution of radioactivity incorporated from [1-^{14}C]-18:3n-3 into individual lipids of trout hepatocytes is presented in Table 3.5. The results show that approximately 2nmol of [1-^{14}C]-18:3n-3 /mg protein was incorporated into hepatocyte

Table 3.3. Effect on starvation and refeeding after starvation on lipid class composition (wt%) of hepatocytes isolated from rainbow trout.

Lipid class	Treatment A	Treatment B	Treatment C
SM	2.8 ± 0.2	2.4 ± 0.2	2.2 ± 0.1
PC	24.2 ± 1.7	25.0 ± 3.2	25.5 ± 3.3
PS	2.8 ± 0.7	3.6 ± 0.1	3.2 ± 0.3
PI	5.2 ± 0.5	5.9 ± 0.4	5.7 ± 0.4
PG/CL/PA	5.4 ± 0.7	5.3 ± 0.7	4.9 ± 0.3
EGP	15.6 ± 3.9	17.3 ± 1.6	16.9 ± 2.3
CHO	13.8 ± 0.2	13.3 ± 0.6	13.8 ± 0.7
FFA	2.3 ± 1.2	1.3 ± 0.7	1.4 ± 0.8
TAG	22.7 ± 1.6	21.1 ± 4.4	21.5 ± 3.8
CE	5.3 ± 2.7	4.7 ± 2.1	4.9 ± 1.9
Σ Polar	56.0 ± 4.3	59.5 ± 5.1	58.4 ± 5.3
Σ Neutral	44.0 ± 4.5	40.5 ± 4.9	41.6 ± 5.0

Treatment (A): trout fed on a commecial diet; (B): trout starved for 30 days; (C): trout starved for 28 days and fed 2 days on a commercial diet, prior to analysis. Data are means ± SD (n=4 fish). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters.

Table 3.4. Effect of starvation and refeeding after starvation on fatty acid composition (wt%) of hepatocytes isolated from rainbow trout

Fatty acid	Fed (a)	Starved (b)	Starved/fed (c)
14:0	1.6 ± 0.1	1.7 ± 0.0	1.7 ± 0.0
15:0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
15:1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
16:0	17.7 ± 0.4	17.5 ± 0.1	17.3 ± 0.2
16:1	3.0 ± 0.5	2.8 ± 0.1	2.8 ± 0.1
16:2 n-3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:3 n-3	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
16:4	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
17:0	n.d.	n.d.	n.d.
18:0	6.5 ± 0.2	6.3 ± 0.1	6.4 ± 0.4
18:1	12.9 ± 0.3	13.1 ± 0.3	12.7 ± 0.3
18:2 n-6	3.4 ± 0.2	3.3 ± 0.0	3.5 ± 0.3
18:3 n-6	n.d.	n.d.	n.d.
18:3 n-3	0.5 ± 0.2	0.5 ± 0.2	0.6 ± 0.1
18:4 n-3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
20:0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:1	3.3 ± 0.4	3.2 ± 0.4	3.1 ± 0.4
20:2n-6	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.0
20:3 n-6	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:4 n-6	3.9 ± 0.5	3.6 ± 0.1	3.5 ± 0.3
20:4 n-3	1.5 ± 0.3	1.4 ± 0.2	1.3 ± 0.1
20:5 n-3	5.6 ± 0.5	5.9 ± 0.5	6.2 ± 0.7
22:1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
22:5 n-6	n.d.	n.d.	n.d.
22:5 n-3	2.6 ± 0.3	2.6 ± 0.2	2.4 ± 0.0
22:6 n-3	32.7 ± 1.7	33.7 ± 1.7	33.4 ± 2.2
24:1	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.3
Σ Saturates	26.3 ± 0.6	25.8 ± 0.2	25.7 ± 0.7
Σ Monoenes	21.0 ± 0.5	20.8 ± 0.8	20.5 ± 0.6
Σ (n-6)	5.8 ± 0.3	5.7 ± 0.0	6.2 ± 0.7
Σ (n-3)	43.1 ± 1.0	44.2 ± 0.9	44.1 ± 1.3
Σ PUFA	48.8 ± 0.9	49.9 ± 1.0	50.3 ± 0.7

(a): trout fed on a commecial diet; (b): trout starved for 30 days; (c): trout starved for 28 days and fed 2 days on a commercial diet prior experiment. Data are means ± SD (n=4 fish). The notation "n.d." indicates not detected

lipids irrespective of feeding regime applied. There were no significant differences between feeding treatments for the incorporation of [1-^{14}C]-18:3n-3 into individual lipid classes. The majority of incorporated radioactivity (72.7% -74.8%) was always recovered in TAG of trout hepatocytes from all the groups. PC was the most radiolabelled phospholipid containing 12.1% of the total radioactivity in fed trout and 11.7% in trout starved for 30 days. The second most labelled phospholipid of hepatocytes from all groups was EGP which contained from 9.1 to 9.5% of the total incorporated radioactivity. The levels of incorporated radioactivity in CE and FFA in hepatocytes of trout from the three groups were not significantly different and ranged from 1.1% in fed trout to 1.7% in starved trout. Approximately 3% of the total radioactivity incorporated in hepatocytes lipids were distributed among other phospholipids, with no significant differences between groups.

3.3.1.3. Effect of starvation and starvation/refeeding regimes on the conversion of [1-^{14}C]-18:3n-3 into 22:6n-3 by trout hepatocytes

The autoradiograms from an $AgNO_3$-TLC plate showing the bands of radioactive PUFA derived from the desaturation and elongation of [1-^{14}C]-18:3n-3 by hepatocytes are presented in Figure 3.3. The FAME were well resolved according to chain length and degree of unsaturation, with the hexaenes having the lowest Rf value followed by the pentaenes, the tetraenes and the trienes. Within a specific unsaturation group, the component with the longest chain had the highest Rf value. The profiles demonstrated that [1-^{14}C]18:3n-3 was converted to longer chain PUFA in hepatocytes of trout from all the three dietary groups. The most intense band corresponded to ^{14}C-18:3n-3 followed by 20:3n-3. Radioactive 22:6n-3 was characterised as a broad band located just above the origin. No bands of radioactivity corresponding to C_{24}-PUFA were detected.

Radiochromatograms of the radiolabelled PUFA formed from the desaturation and elongation of [1-^{14}C]-18:3n-3 by trout hepatocytes are presented in Figure 3.4.

Table 3.5. Effect of starvation and refeeding after starvation on the incorporation of radioactivity into lipid classes of trout hepatocytes incubated with [1-^{14}C]-18:3n-3 for 3 hours.

	Fed (a)	Starved (b)	Starved/fed (c)
Activity *	1.9 ± 0.2	2.0 ± 0.1	1.9 ± 0.3
Lipid class	% distribution of incorporated radioactivity		
SM	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
PC	12.1 ± 1.0	11.7 ± 0.9	11.0 ± 1.1
PS	0.6 ± 0.1	0.6 ± 0.2	0.5 ± 0.2
PI	1.6 ± 0.5	1.5 ± 0.2	1.5 ± 0.2
PG/CL/PA	0.4 ± 0.0	0.7 ± 0.2	0.6 ± 0.1
EGP	9.1 ± 0.7	9.5 ± 0.7	9.3 ± 0.8
CHO	0.5 ± 0.0	0.5 ± 0.2	0.4 ± 0.1
FFA	0.5 ± 0.2	0.6 ± 0.2	0.3 ± 0.2
TAG	74.0 ± 1.4	72.7 ± 2.1	74.8 ± 3.6
CE	1.1 ± 0.6	1.7 ± 0.4	1.3 ± 0.5

(a): trout fed on a commecial diet; (b): trout starved for 30 days; (c): trout starved for 28 days and fed 2 days on a commercial diet prior experiment.

* nmol of ^{14}C-fatty acid incorporated/mg hepatocyte protein. Data are means ± SD (n=4 fish). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters.

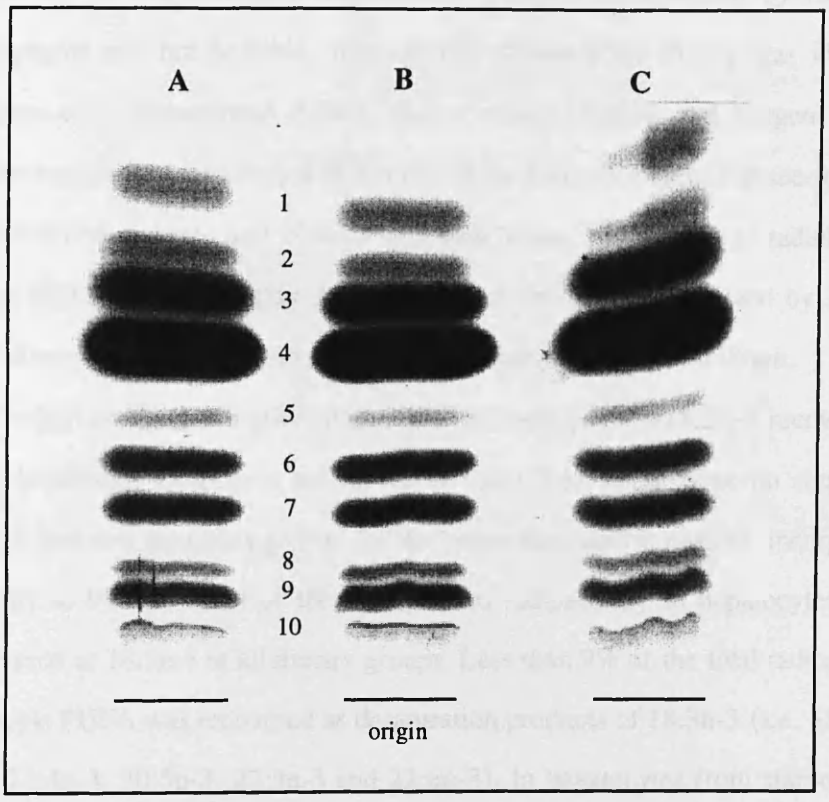

Figure 3.3. Autoradiogram of desaturated and elongated PUFA produced from [1-^{14}C]-18:3n-3 by hepatocytes from rainbow trout fed commercial diet (A), starved for 30 days (B), and starved for 28 days and fed 2 days on a commercial diet (C). 1, 24:3n-3; 2, 22:3n-3; 3, 20:3n-3; 4, 18:3n-3; 5, 22:4n-3; 6, 20:4n-3; 7, 18:4n-3; 8, 22:5n-3; 9, 20:5n-3; 10, 22:6n-3.

Under the chromatographic conditions employed, the radiolabelled 18:3n-3 had a retention time of 19min 47sec. The other trienoic radiolabelled PUFA 20:3n-3 and 22:3n-3 eluted after 24min 46sec and 32min 45sec, respectively. Because of the minute amount of radioactivity incorporated in 24:3n-3 its detection by radio gas chromatography was not possible, although this radiolabelled PUFA was identified by reference to a characterised $AgNO_3$-TLC standard (Wilson and Sargent 1992). Among the tetraenoic radiolabelled PUFA the 18:4n-3 eluted at 20min 28sec followed by 20:4n-3 at 26min 11sec and 22:4n-3 at 35min 06sec. The elution of radiolabelled pentaenoic PUFA proceeded after 35min 13sec for the 20:5n-3 followed by 22:5n-3 at 45min 40sec. Docosahexaenoic acid had a retention time of 47min 05sec.

The percentage distribution of radioactivity from [1-^{14}C]-18:3n-3 recovered in PUFA of hepatocyte total lipids are shown in Table 3.6. There were no significant differences between treatment groups for the percentage distribution of incorporated radioactivity in PUFA. Most of the incorporated radioactivity in hepatocytes lipids was recovered as 18:3n-3 in all dietary groups. Less than 9% of the total radioactivity in hepatocyte PUFA was recovered as desaturation products of 18:3n-3 (*i.e.* 18:4n-3, 20:4n-3, 22;4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). In hepatocytes from starved trout (7.5%) the percentage of total radioactivity recovered as desaturation products was lower than in hepatocytes from fed trout (8.1%). The highest value for incorporated radioactivity in desaturated products was found in hepatocytes from trout subjected to the starvation-refeeding regime, although the value was not significantly different from the other two treatments. The proportion of radioactivity recovered as the "dead end" elongation products of 18:3n-3 (20:3n-3 and 22:3n-3) was not significantly different between hepatocytes from the three groups and ranged from 11.9% in fed trout to 12.8% in starved animals. Only a minor percentage of the total radioactivity incorporated in PUFA was recovered as 22:6n-3 in both hepatocytes from fed trout (0.5%) and in starved trout (0.6%). The chromatographic profiles revealed no radioactive components corresponding to C_{24} carbon PUFA.

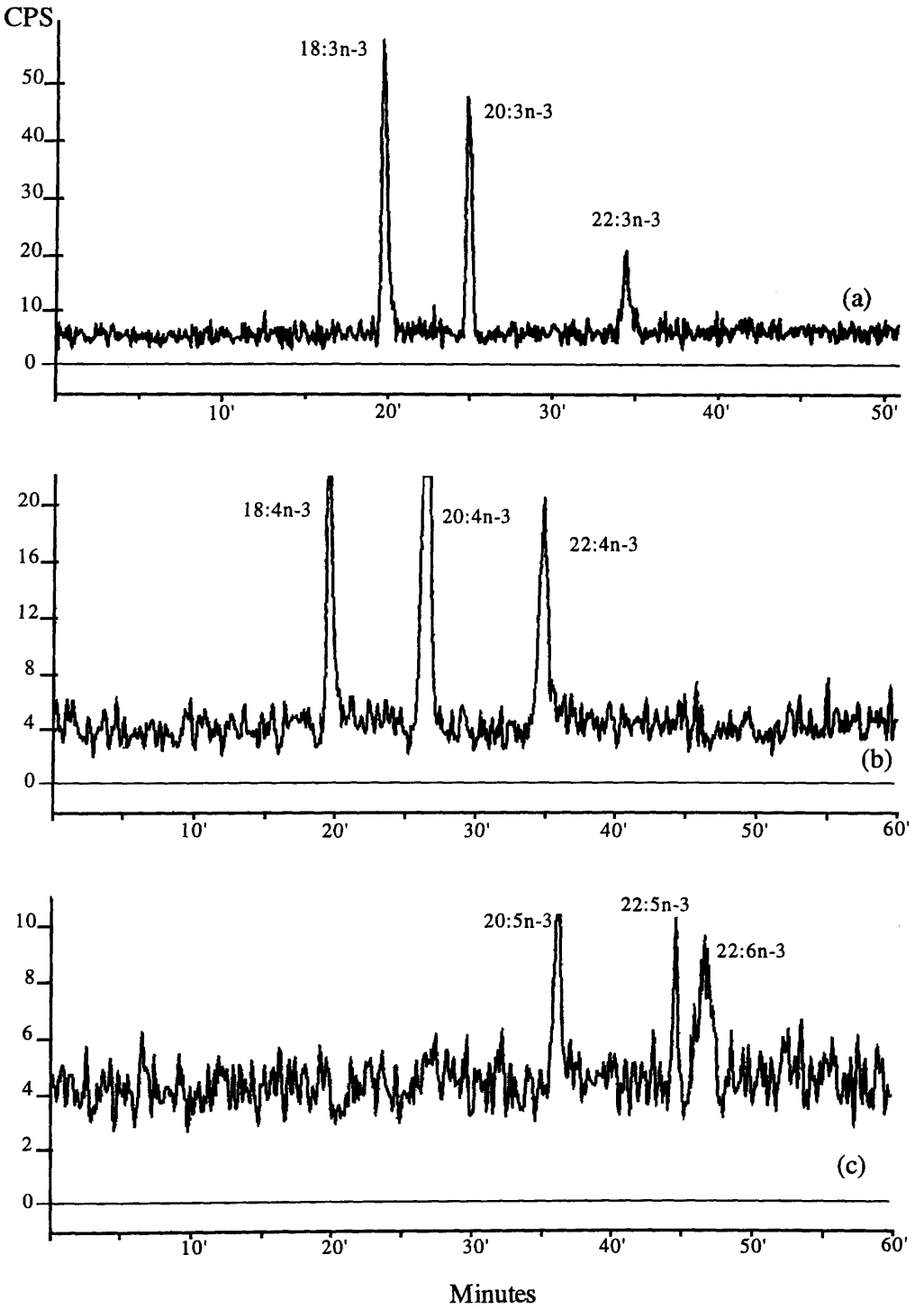

Figure 3.4. Radiochromatograms of radioalabelled PUFA derived from the desaturation and elongation of [1-^{14}C]-18:3n-3 by hepatocytes isolated from rainbow trout. (a), trienes; (b) tetraenes; (c), pentaenes/hexaenes fatty acids.

Table 3.6. Effect of starvation and refeeding after starvation on the distribution of radioactivity in PUFA total lipids of trout hepatocytes incubated with [1-^{14}C]-18:3n-3.

	Fed (a)	Starved (b)	Starved/fed (c)
Activity*	1.9 ± 0.2	2.0 ± 0.1	1.9 ± 0.3
Fatty acid	% distribution of incorporated radioactivity		
18:3n-3	80.0 ± 1.6	80.0 ± 1.2	78.8 ± 1.1
20:3n-3	9.5 ± 1.6	9.5 ± 1.3	9.6 ± 1.1
22:3n-3	2.4 ± 0.9	3.0 ± 0.5	3.2 ± 0.7
24:3n-3	n.d.	n.d.	n.d.
18:4n-3	2.0 ± 0.5	2.1 ± 0.8	2.4 ± 0.6
20:4n-3	2.4 ± 0.5	2.3 ± 0.3	2.6 ± 0.3
22:4n-3	n.d.	n.d.	n.d.
20:5n-3	2.6 ± 0.2	2.0 ± 0.3	2.2 ± 0.4
22:5n-3	0.6 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
24:5n-3	n.d.	n.d.	n.d.
22:6n-3	0.5 ± 0.3	0.6 ± 0.2	0.6 ± 0.3
24:6n-3	n.d.	n.d.	n.d.

(a): trout fed on a commecial diet; (b): trout starved for 30 days; (c): trout starved for 28 days and fed 2 days on a commercial diet prior experiment.
* nmol of ^{14}C-fatty acid incorporated/mg hepatocyte protein/3hours Data are means ± SD (n=4 fish). The notation "n.d." indicates not detected.

3.3.1.4. *Incorporation of radioactivity from [1-^{14}C]-18:3n-3 into lipid class of hepatocytes from juvenile trout: A time course study*

Preliminary results showed that hepatocytes from juvenile rainbow trout were more active in synthesising 22:6n-3 from 18:3n-3 than cells from mature fish. Therefore, it was decided to use cells from the former in the time course experiments. The fish weighed circa 70g (in contrast to the 400g trout used thus far) and were fed on a commercial diet at a daily rate of 2% of the body weight. The viability of the freshly isolated hepatocytes from juvenile trout was an average of 95% as judged by Trypan blue exclusion.

The time course distribution of radioactivity (dpm- disintegration per minute) from [1-^{14}C]-18:3n-3 in hepatocyte lipid classes from juvenile trout is presented in Figure 3.5. Most of the radioactivity from [1-^{14}C]-18:3n-3 was incorporated into the hepatocyte neutral lipids, particularly TAG (20.5 x 10^5 dpm/culture) within one hour of incubation. No significant changes in the proportion of radioactivity recovered in TAG and CHO were observed in the subsequent period from 1 to 24 hours of incubation. In contrast, the proportion of radioactivity recovered in PC and EGP increased significantly (Repeated Measure ANOVA One Factor, $P<0.05$) over the same period. The amount of radioactivity distributed in PC and EGP over the 24 hours increased 2.0 and 2.3 fold, respectively.

3.3.1.5. *Desaturation and elongation of [1-^{14}C]-18:3n-3 by hepatocytes from juvenile trout: A time course study*

The autoradiogram in Figure 3.6 shows the desaturated and elongated PUFA produced from [1-^{14}C]-18:3n-3 by juvenile trout hepatocytes over 24 hours. The profile illustrated clearly that the substrate [1-^{14}C]-18:3n-3 was rapidly desaturated and elongated into longer PUFA by the hepatocytes within the first hour. The broad band for 22:6n-3 (band n° 10) became darker over time which indicated that the proportion of radioactivity recovered as 22:6n-3 increased over the incubation period.

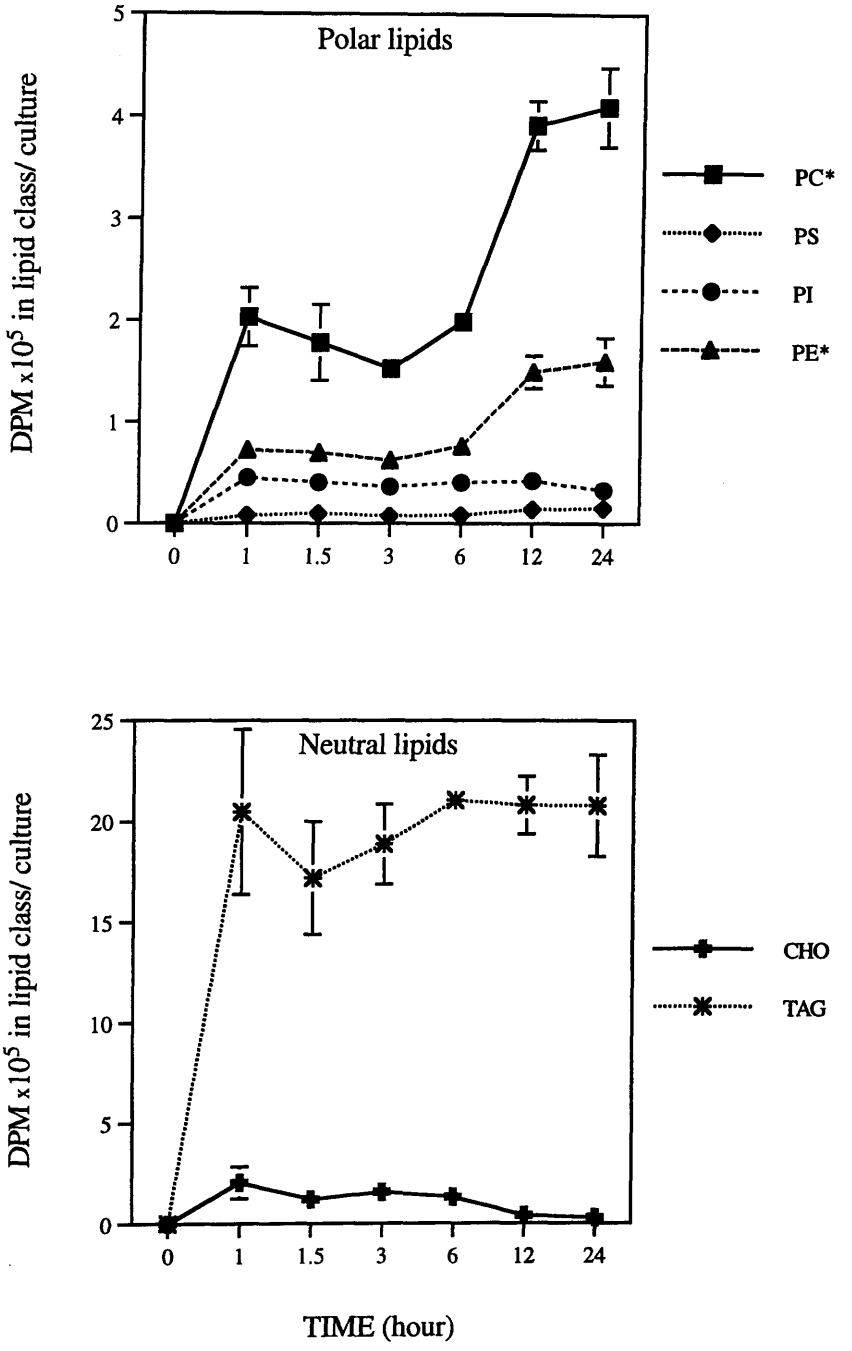

Figure 3.5. Time course distribution of radioactivity (DPM-desintegration per minute) in lipid class in trout hepatocytes incubated with [1-^{14}C]-18:3n-3. The notation "*" indicates significant increase or decrease in percentage over time course (Repeat Measure ANOVA One Factor, $P<0.05$).

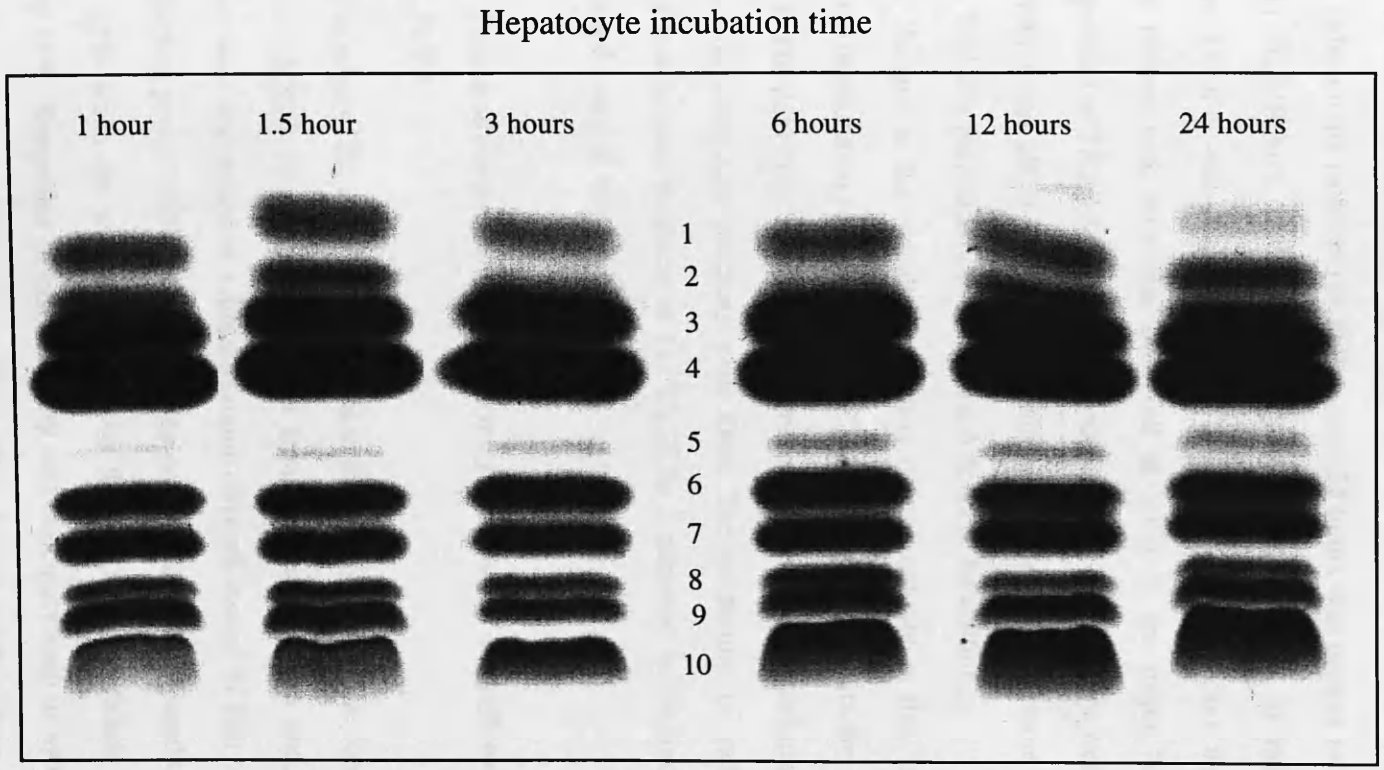

Figure 3.6. Autoradiogram of radiolabelled PUFA in total lipids of hepatocytes isolated from rainbow trout and incubated with [1-^{14}C]-18:3n-3. 1, 24:3n-3; 2, 22:3n-3; 3, 20:3n-3; 4, 18:3n-3; 5, 22:4n-3; 6, 20:4n-3; 7, 18:4n-3; 8, 22:5n-3; 9, 20:5n-3; 10, 22:6n-3.

Figure 3.7 shows that the proportion of radioactivity from [1-^{14}C]-18:3n-3 recovered in 22:6n-3 increased significantly (Repeated Measure ANOVA One Factor, $P<0.05$) from 0.5 x 10^5 DPM/culture after 1 hour to 1.4 x 10^5 DPM/culture after 24 hours. Most of the radioactivity recovered in PUFA over 24 hours was present as the initial substrate, [1-^{14}C]-18:3n-3. No significant change on the amount of radioactivity recovered in 18:3n-3 was observed during 24 hours. A significant increase of radioactivity content over time was observed in 20:3n-3, the major "dead-end" elongation product of 18:3n-3. The levels of incorporated radioactivity recovered as 18:4n-3, 20:4n-3 and 20:5n-3 increased significantly (Repeated Measure ANOVA One Factor, $P<0.05$), particularly over the first 6 hours of incubation.

The changes in the overall distribution of radioactivity in the "dead-end" elongation and desaturation products of [1-^{14}C]-18:3n-3 over 24 hours are presented in Figure 3.8. The data show a significant increase in radioactivity distributed in both desaturation and elongation products with time. The proportion of radioactivity recovered as desaturation products of [1-^{14}C]-18:3n-3 achieved its maximum value between 3 and 6 hours of incubation.

3.3.1.6. Fatty acid composition of hepatocytes from trout fed diet deficient in (n-3) PUFA

At this stage of the study it was decided to attempt to enhance the formation of 22:6n-3 from 18:3n-3 by removing 22:6n-3 from the diet. To this end, juvenile rainbow trout were maintained on a diet containing olive oil instead of fish oil. At the end of the feeding period (120 days) trout fed the control diet had increased their body weight by 23% while the weight of trout fed the (n-3)PUFA-deficient diet had increased by 18%. Symptoms of essential fatty acid deficiency such as swollen pale livers or fin erosion were not observed in either dietary group. There were no significant differences in the numbers of viable hepatocytes prepared from fish in either dietary group with the percentage of viable cells routinely exceeding 97%.

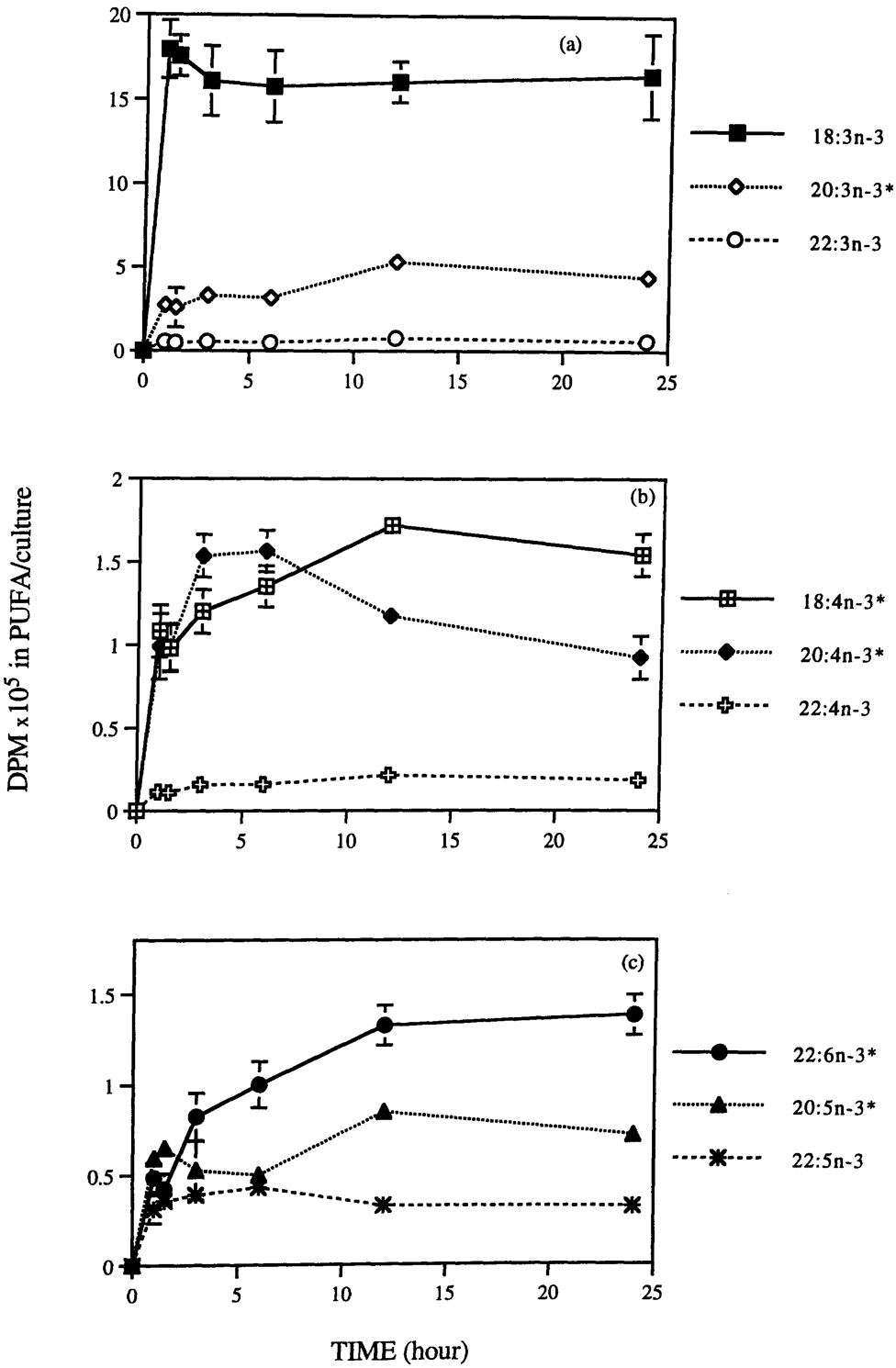

Figure 3.7. Time course of distribution of radioactivity (DPM) in PUFA in total lipids of trout hepatocytes incubated with [1-^{14}C]-18:3n-3. Radioactivity distributed in trienes (a), tetraenes (b) and pentaenes/hexaenes (c).The notation "*" indicates significant increase or decrease in percentage over time course (Repeat Measure ANOVA One Factor, $P<0.05$).Vertical bars represents standard deviation of triplicates.

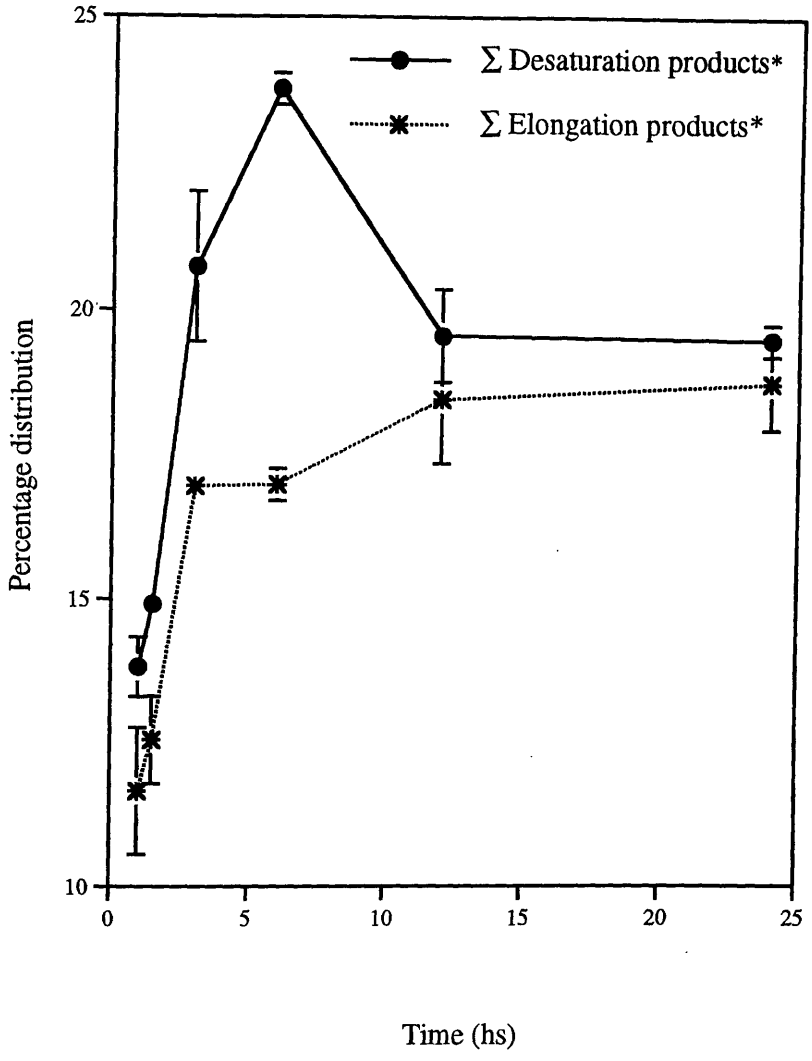

Figure 3.8. Percentage distribution of radioactivity in "dead-end" elongation and desaturation products of [1-^{14}C]-18:3n-3 in hepatocytes isolated from juvenile rainbow trout. The notation "*" indicates significant increase or decrease in percentage over time course (Repeat Measure ANOVA One Factor, $P<0.05$). Vertical bars represent standard deviation of triplicates.

The level of (n-3)PUFA in the experimental diet was negligible compared to the control diet (Table 3.7). Nearly three quarters (71.4%) of the total fatty acids of the experimental diet were oleic acid (18:1n-9) with palmitic (16:0) and linoleic acid (18:2n-6) comprising 11.8% and 9.6%, respectively, of the total.

The percentage fatty acid composition of hepatocytes from trout fed the two diets are presented in Table 3.7. The proportion of (n-3) PUFA in the total lipid of hepatocytes from trout fed the (n-3) PUFA deficient diet was approximately half from those of trout fed the control diet. The levels of 20:5n-3 and 22:6n-3 in hepatocyte total lipid from deficient trout were 40% and 53% respectively, compared to the levels in cells from control fish. At the same time a 62% increase in the levels of monoenoic fatty acids was observed in hepatocyte total lipid from deficient trout compared to the levels in hepatocytes from control fish. In particular, the proportion of oleic acid (18:1n-9) increased from 14.5% of the total fatty acids in control hepatocytes to 30.4% in cells from trout fed deficient diet. The content of (n-6) PUFA in hepatocyte lipid from deficient fish was approximately 2-fold higher than that from control trout.

Levels of 20:5n-3 and 22:6n-3 in the polar lipids of hepatocytes from trout fed the diet deficient in (n-3) PUFA were less than half those from trout fed the control diet. The depletion of 20:5n-3 and 22:6n-3 from the neutral lipid fraction of hepatocytes from fish fed the experimental diet was even more pronounced, so that the levels were 20 and 10-fold respectively less than observed in cells from control fish. The level of total (n-6) PUFA in the polar fraction of the (n-3)PUFA-deficient cells was significantly higher than in control cells, due to higher proportions of 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6. The levels of oleic acid in both polar and neutral lipid fractions of hepatocytes isolated from control fish were approximately half those observed in hepatocytes from fish fed the (n-3)PUFA deficient diet. The fatty acids 18:2n-9 and 20:2n-9 were absent from control hepatocytes but were present in small amounts in the total, polar and particularly in the neutral lipid fraction in hepatocytes from trout fed the diet deficient in (n-3)PUFA.

Table 3.7. Fatty acid compositions (wt%) of dietary lipids and the polar and neutral lipid fractions isolated from the hepatocytes of trout fed the two diets.

Fatty acids	Dietary lipids		Total lipids		Polar lipids		Neutral lipids	
	Control	(n-3) deficient	Control	(n-3) deficient	Control	(n-3) deficient	Control	(n-3) deficient
14:0	5.4 ± 0.2	n.d.	1.9 ± 0.3	1.3 ± 0.5	1.4 ± 0.3	0.6 ± 0.2	1.9 ± 0.4	2.4 ± 0.6
15:0	n.d.	n.d.	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	n.d.	2.5 ± 0.9	0.2 ± 0.0
16:0	14.1 ± 0.3	11.8 ± 0.3	17.3 ± 0.8	15.0 ± 1.3	20.5 ± 1.8	17.4 ± 1.3	11.6 ± 1.6	12.6 ± 1.8
16:1n-7	5.3 ± 0.4	1.4 ± 0.1	4.6 ± 0.4	5.7 ± 0.7	3.3 ± 1.0	3.0 ± 0.5	3.2 ± 1.0	7.4 ± 0.9
18:0	n.d.	2.9 ± 0.1	3.9 ± 0.3	5.1 ± 0.7	5.2 ± 0.4	5.6 ± 0.6	3.9 ± 0.8	3.6 ± 0.5
18:1n-9	19.2 ± 1.4	71.4 ± 1.6	14.5 ± 1.4	30.4 ± 1.1	13.7 ± 1.3	25.5 ± 1.8	23.7 ± 2.4	53.1 ± 4.3
18:2n-9	n.d.	n.d.	n.d.	0.3 ± 0.0	n.d.	0.6 ± 0.1	n.d.	1.1 ± 0.1
18:2n-6	1.8 ± 0.2	9.6 ± 0.3	2.7 ± 0.6	3.7 ± 0.5	0.9 ± 0.2	4.2 ± 1.1	2.2 ± 0.8	1.9 ± 0.9
18:3n-6	n.d.	n.d.	0.3 ± 0.0	0.6 ± 0.1	0.1 ± 0.0	0.4 ± 0.3	0.7 ± 0.1	0.1 ± 0.0
18:3n-3	2.2 ± 0.1	0.6 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.2	0.8 ± 0.1	0.2 ± 0.0
20:1n-9	10.1 ± 0.3	0.4 ± 0.0	4.7 ± 0.8	5.0 ± 0.4	2.9 ± 0.9	4.0 ± 0.9	6.5 ± 1.7	6.7 ± 1.4
20:2n-9	n.d.	n.d.	n.d.	0.6 ± 0.1	n.d.	0.9 ± 0.2	n.d.	2.7 ± 1.1
20:2n-6	n.d.	n.d.	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	2.5 ± 0.3	0.7 ± 0.2	1.6 ± 0.6
20:3n-6	n.d.	n.d.	0.2 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	3.9 ± 0.8	0.5 ± 0.1	0.5 ± 0.0
20:4n-6	0.9 ± 0.0	0.2 ± 0.0	2.6 ± 0.4	5.9 ± 0.4	2.3 ± 0.5	7.8 ± 1.5	1.1 ± 0.4	1.0 ± 0.3
20:4n-3	0.9 ± 0.1	n.d.	0.7 ± 0.2	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	2.7 ± 0.8	0.1 ± 0.0
20:5n-3	6.2 ± 0.1	n.d.	6.0 ± 0.7	2.4 ± 0.6	3.7 ± 0.8	1.4 ± 0.5	3.8 ± 0.9	0.2 ± 0.0
22:1n-11	14.3 ± 0.2	0.4 ± 0.0	2.4 ± 0.5	1.3 ± 0.8	0.3 ± 0.0	0.2 ± 0.0	2.4 ± 0.8	1.9 ± 0.9
22:5n-6	0.3 ± 0.0	n.d.	0.2 ± 0.0	1.1 ± 0.4	0.8 ± 0.1	2.7 ± 0.4	0.3 ± 0.0	0.2 ± 0.0
22:5n-3	2.3 ± 0.1	n.d.	1.6 ± 0.5	0.7 ± 0.2	1.5 ± 0.6	0.7 ± 0.0	1.6 ± 0.5	0.4 ± 0.0
22:6n-3	10.4 ± 0.3	0.3 ± 0.0	35.4 ± 2.1	18.8 ± 1.2	40.9 ± 2.5	18.2 ± 1.7	20.4 ± 2.3	2.1 ± 0.9
Σ Sats	21.9 ± 1.5	14.8 ± 1.3	23.4 ± 2.4	21.6 ± 3.1	27.3 ± 2.3	23.6 ± 2.5	19.9 ± 1.1	18.8 ± 1.5
Σ Monos	50.1 ± 3.6	74.3 ± 4.6	26.2 ± 2.3	42.4 ± 2.1	20.2 ± 1.4	32.7 ± 2.9	35.8 ± 2.4	69.1 ± 4.3
Σ PUFA	28.0 ± 2.7	11.0 ± 1.2	50.4 ± 3.6	35.0 ± 3.2	51.6 ± 3.2	43.9 ± 3.0	34.8 ± 2.7	12.1 ± 1.3
Σ n-6	3.0 ± 0.7	9.8 ± 0.5	6.4 ± 1.2	12.4 ± 1.4	5.0 ± 0.6	21.5 ± 2.2	5.5 ± 0.5	5.3 ± 0.7
Σ n-3	25.0 ± 2.1	0.9 ± 0.0	44.0 ± 2.4	22.6 ± 1.2	46.6 ± 2.8	20.9 ± 1.4	29.3 ± 2.8	3.0 ± 0.6
n-3/n-6	8.3 ± 0.9	0.1 ± 0.0	6.9 ± 0.3	1.8 ± 0.5	9.3 ± 0.8	1.0 ± 0.1	5.3 ± 0.7	0.6 ± 0.0

Data are means ± S.D. (n=3 fish). The notation 'n.d.' indicates not detected.

3.3.1.7. Distribution of radioactivity from [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 in lipid classes of hepatocytes from trout fed diet deficient in (n-3) PUFA

The distribution of radioactivity incorporated from [1-^{14}C]-18:3n-3 and [1-^{14}C]20:5n-3 into individual lipids of trout hepatocytes is presented in Table 3.8. More radioactivity (2.5 times) was incorporated into total lipids from [1-^{14}C]-18:3n-3 than from [1-^{14}C]-20:5n-3 but there was no difference between dietary groups in the amount of either carbon-14 labelled substrate incorporated. More than 70% of the radioactivity from [1-^{14}C]-18:3n-3 was incorporated into neutral lipid by hepatocytes from both dietary groups. A significantly higher amount (One Way ANOVA, $P<0.05$) of radioactivity from [1-^{14}C]-18:3n-3 was recovered in the cholesterol ester fraction of the cells from the experimental group than the control group (4.5% Vs 0.9%). No significant differences between dietary groups were found in the proportion of radioactivity from [1-^{14}C]-18:3n-3 recovered in free fatty acids, which was always less than 2%. Phosphatidylcholine (PC) was the most radiolabelled phospholipid containing 14.1% of the total radioactivity incorporated from [1-^{14}C]-18:3n-3 in control fish and 17.5% in fish fed the (n-3) PUFA-deficient diet. The second most labelled phospholipid from [^{14}C]-18:3n-3 was PE, accounting for approximately 5% of the total recovered radioactivity in both dietary groups. Significantly less radioactivity from [1-^{14}C]-18:3n-3 was incorporated into PI from fish fed the deficient diet than the control diet.

There were no significant differences between dietary groups for the incorporation of [1-^{14}C]-20:5n-3 into hepatocyte neutral lipids with about 50% of the incorporated radioactivity recovered in triacylglycerols from both groups of fish. A significantly higher proportion (One Way ANOVA, $P<0.05$) of radioactivity from [1-^{14}C]-20:5n-3 was recovered in PC from the (n-3) PUFA-deficient group (33.6%) compared to the control group (25.7%). No significant differences were found between the two groups in the distribution of radioactivity from [1-^{14}C]-20:5n-3 in

Table 3.8. Incorporation of radioactivity into lipid classes of hepatocytes isolated from trout fed the two diets and incubated with [1-^{14}C]18:3n-3 or [1-^{14}C] 20:5n-3.

^{14}C substrate	[1-^{14} C]-18:3(n-3)		[1-^{14} C]-20:5(n-3)	
Diets	Control	(n-3) Deficient	Control	(n-3) Deficient
Activity†	2.2 ± 0.1	2.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
% distribution of incorporated radioactivity				
Class				
SM	0.8 ± 0.3	0.6 ± 0.1	0.9 ± 0.7	0.7 ± 0.1
PC	14.1 ± 3.2	17.5 ± 2.2	25.7 ± 2.5	33.6 ± 0.5*
PS	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.7	1.6 ± 0.9
PI	2.9 ± 0.7	1.6 ± 0.4*	7.9 ± 0.6	2.4 ± 0.2*
PA/PG/CL	1.0 ± 0.3	0.8 ± 0.2	1.0 ± 0.3	1.0 ± 0.5
EGP	5.1 ± 0.8	5.5 ± 0.7	8.0 ± 0.8	8.4 ± 2.6
CHO	3.7 ± 1.2	3.9 ± 1.2	2.5 ± 0.6	2.3 ± 0.4
FFA	1.7 ± 1.1	1.7 ± 0.3	0.9 ± 0.2	1.2 ± 0.4
TAG	68.8 ± 7.5	66.4 ± 5.1	51.0 ± 4.0	47.9 ± 3.5
CE	0.9 ± 0.3	4.5 ± 0.2*	0.7 ± 0.1	1.0 ± 0.2
Total polar	24.9 ± 2.3	23.5 ± 1.8	44.9 ± 3.4	47.6 ± 3.1
Total neutral	75.1 ± 3.6	76.5 ± 3.5	55.1 ± 3.2	52.4 ± 1.2

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours Data are means ± SD (n=6 fish). The notation "*" indicates significant different from control (One Way ANOVA, $P<0.05$). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters

PE, PS and SM. However, the proportion of label recovered in PI fraction was three-fold higher in the control than the experimental group.

3.3.1.8. Desaturation and elongation of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 by hepatocytes from trout fed diet deficient in (n-3) PUFA

Figure 3.9 is a typical autoradiogram from a $AgNO_3$-TLC plate showing the bands of radioactive fatty acids resulting from the elongation and desaturation products of [1-^{14}C]-18:3n-3 present in the total, polar and neutral lipid fractions. Figure 3.9 demonstrates that [1-^{14}C]-18:3n-3 was converted to longer chain PUFA in hepatocytes from both dietary groups and also shows that the C_{20} and C_{22} fatty acids resulting from the desaturation and elongation of [1-^{14}C]-18:3n-3, *i.e.* 20:5n-3, 22:5n-3 and 22:6n-3, were present mainly in the polar lipid fraction. In contrast, the "dead end" elongation products of 18:3n-3, *i.e.* 20:3n-3, 22:3n-3, 24:3n-3, were mainly present in the neutral lipid fraction. Radioactive 22:6n-3 present in the total lipid and polar lipid fraction was always characterised as a broad band located just above the origin. Radiolabelled 24:6n-3 was found predominantly in neutral fractions and was located above the 22:6n-3 band.

The autoradiogram from a $AgNO_3$ plate in Figure 3.10 shows the radiolabelled PUFA products derived from the desaturation and elongation of [1-^{14}C]-20:5n-3. The profiles indicate that radiolabelled 22:6n-3 was substantially distributed in the polar lipids whereas radioactive 24:6n-3 was predominantly detected in the neutral lipid fraction. Radiolabelled 24:5n-3 was present in all lipid fractions, especially in the neutral lipids and was located on the TLC plate at nearest to the solvent front and above the 22:5n-3 band. Although FAME were well resolved in most cases, it was occasionally difficult to separate 24:6n-3 and 22:6n-3, especially when the mass of 22:6n-3 present on the TLC plates was high (polar fractions). Therefore, the fraction containing both these fatty acids was routinely taken for their subsequent individual quantitation by radio gas-liquid chromatography.

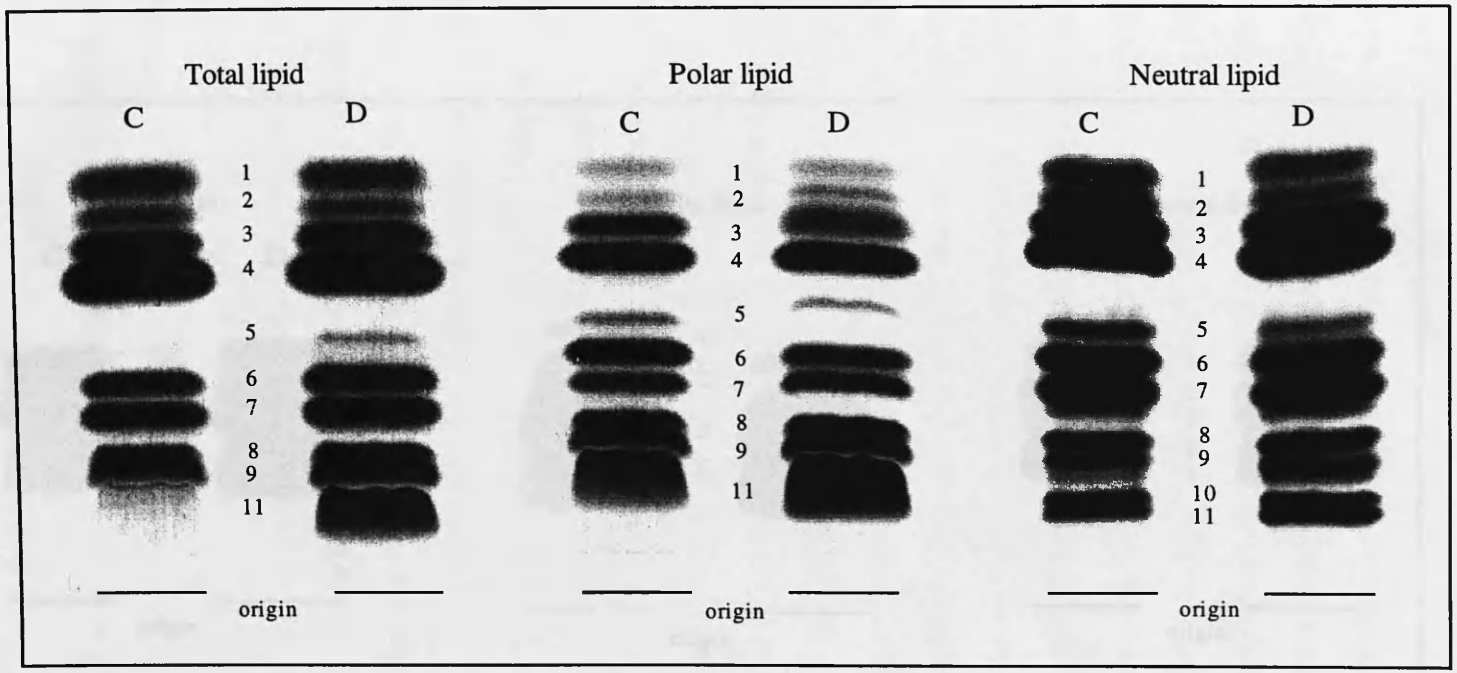

Figure 3.9. Autoradiograms of elongated and desaturated PUFA products in total, neutral and polar fractions of hepatocytes isolated from trout fed the control (C) and diet deficient in (n-3) PUFA (D) and incubated with [1-^{14}C]-18:3n-3. 1, 24:3n-3; 2, 22:3n-3; 3, 20:3n-3; 4, 18:3n-3; 5, 22:4n-3; 6, 20:4n-3; 7, 18:4n-3; 8, 22:5n-3; 9, 20:5n-3; 10, 24:6n-3; 11, 22:6n-3.

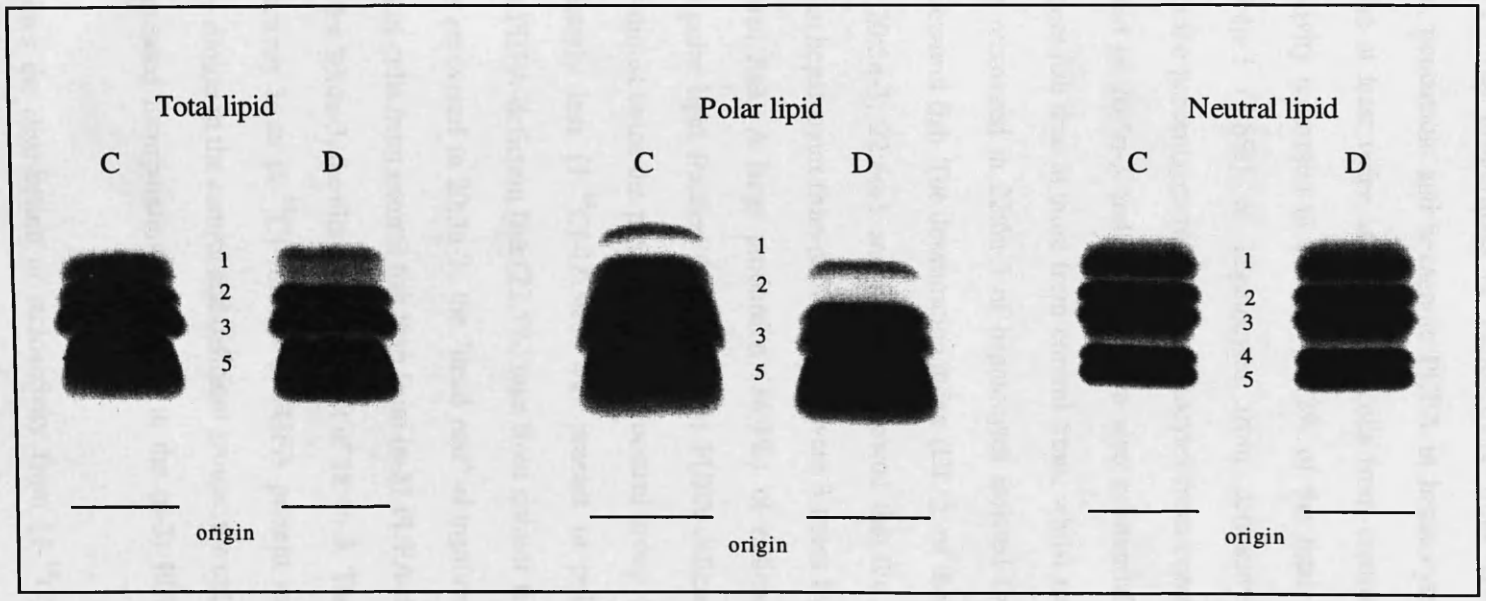

Figure 3.10. Autoradiograms of elongated and desaturated PUFA prducts in total, neutral and polar fractions of hepatocytes isolated from trout fed the control (C) and diet deficient in (n-3) PUFA (D) and incubated with [1-^{14}C]-20:5n-3. 1, 24:5n-3; 2, 22:5n-3; 3, 20:5n-3; 4, 24:6n-3; 5, 22:6n-3.

The percentage distributions of radioactivity from [1-^{14}C]-18:3n-3 recovered in PUFA of the total, the polar and neutral lipid fractions of trout hepatocytes are presented in Table 3.9. The proportions of radioactivity from [1-^{14}C]-18:3n-3 recovered in tetraenoic, pentaenoic and hexaenoic PUFA in hepatocytes total lipid from deficient fish were at least twice as high as in cells from control trout. The percentages of radioactivity recovered in 18:4n-3 (11.2% of the total incorporated radioactivity) and 20:4n-3 (5.8%) of hepatocytes from deficient trout were significantly higher than the percentages found in hepatocytes from control fish. The radioactivity levels found in 20:5n-3 and 22:5n-3 were also substantially higher in hepatocytes from deficient fish than in those from control trout, whilst nearly 6 times more radioactivity was recovered in 22:6n-3 of hepatocytes isolated from deficient trout than in cells from control fish. The desaturation index (DI, Σ of the proportions recovered in 18:4n-3, 20:5n-3, 22:6n-3 and 24:6n-3) showed that the desaturation and elongation activity in hepatocytes from deficient trout were 3 times higher than in hepatocytes from control fish. A large proportion (36.4%) of radioactivity from [1-^{14}C]-18:3n-3 in the polar lipid fraction from the (n-3) PUFA-deficient fish was recovered as 22:6n-3, almost twice the proportion in the control group (18.4%). At the same time significantly less [1-^{14}C]-18:3n-3 was present in polar lipids in hepatocytes from (n-3) PUFA-deficient fish (22.5%) than from control fish (31.6%). More radioactivity was recovered in 20:3n-3, the "dead end" elongation product of 18:3n-3, in polar lipids of cells from control fish than from (n-3) PUFA-deficient fish and the same was true for 20:4n-3, the elongation product of 18:4n-3. The percentage distributions of radioactivity from [1-^{14}C]-18:3n-3 in PUFA present in the neutral lipid fraction were very similar in the control and deficient groups, the only significant difference being an increased incorporation in 18:4n-3 in the (n-3) PUFA-deficient trout (Table 3.9).

Table 3.10 shows the distribution of radioactivity from [1-^{14}C]-20:5n-3 in PUFA present in the total, the polar and neutral lipid fractions. The distribution of radioactivity from [1-^{14}C]-20:5n-3 in hepatocyte total lipid showed that hepatocytes

Table 3.9. Percentage distribution of radioactivity in PUFA total, polar and neutral lipids of hepatocytes isolated from trout fed the two diets and incubated with [1-^{14}C]-18:3n-3

	Total lipid		Polar lipid		Neutral lipid	
Diet	Control	(n-3) deficient	Control	(n-3) deficient	Control	(n-3) deficient
Activity†	2.1 ± 0.3	2.0 ± 0.6	0.5 ± 0.3	0.5 ± 0.3	1.6 ± 0.6	1.6 ± 0.5
Fatty acids						
18:3n-3	81.9 ± 1.4	63.1 ± 1.3 *	31.6 ± 5.3	22.5 ± 0.8 *	45.4 ± 5.2	45.9 ± 4.6
20:3n-3	3.3 ± 0.4	3.2 ± 0.7	6.1 ± 0.4	3.7 ± 0.5 *	9.5 ± 2.8	9.3 ± 2.7
22:3n-3	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.6 ± 0.3	1.9 ± 0.5
24:3n-3	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.2	1.2 ± 0.1	2.1 ± 0.2	2.3 ± 0.4
18:4n-3	5.3 ± 0.5	11.2 ± 0.6 *	7.5 ± 0.7	8.9 ± 0.7	8.9 ± 0.8	12.2 ± 1.0 *
20:4n-3	2.5 ± 0.1	5.8 ± 0.8 *	10.5 ± 2.6	4.6 ± 1.7 *	9.3 ± 4.8	7.0 ± 0.5
22:4n-3	0.2 ± 0.0	0.5 ± 0.3	1.5 ± 0.5	1.5 ± 0.2	1.6 ± 0.7	1.9 ± 0.8
20:5n-3	2.1 ± 0.3	5.4 ± 0.5 *	17.4 ± 1.3	15.3 ± 3.6	5.9 ± 4.7	5.3 ± 2.8
22:5n-3	1.0 ± 0.4	2.5 ± 0.3 *	4.6 ± 0.9	4.8 ± 1.0	3.9 ± 1.6	4.1 ± 1.0
24:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6n-3	1.1 ± 0.1	5.9 ± 0.1 *	18.4 ± 2.2	36.4 ± 7.5 *	6.5 ± 3.2	6.3 ± 0.7
24:6n-3	n.d.	n.d.	n.d.	n.d.	5.3 ± 2.9	3.8 ± 0.7
DI	0.1 ± 0.0	0.4 ± 0.1 *	1.4 ± 0.3	2.7 ± 0.5 *	0.6 ± 0.1	0.6 ± 0.2

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=6 fish). The notation "*" indicates significant different from control (One Way ANOVA, $P<0.05$). The abreviation "n.d." indicates not detected. ᵃ The desaturation index "DI" is given as (18:4+20:5+22:6+24:6)/18:3n-3.

from deficient trout desaturated and elongated 72% more radiolabelled substrate than control hepatocytes. The proportion of radioactivity recovered in 22:6n-3 of hepatocyte total lipids was 3.7 times higher in cells from deficient fish than those from control trout. A significantly higher amount of radioactivity was found in 24:5n-3 from hepatocytes isolated from deficient fish than from controls. The proportion of radioactivity recovered in 22:6n-3 of polar lipids of hepatocytes from deficient trout was 1.6-fold higher than that observed in hepatocytes from control fish. Substantial levels of radioactivity were present in 24:6n-3 in both the control and deficient groups, but only in the neutral lipid fraction Radioactivity was present in 24:5n-3, particularly in the neutral lipid fraction. Significantly less radioactivity was present in 24:5n-3 in the total lipid and polar lipid from trout fed the (n-3)PUFA-deficient diet. The DI establishes that the overall desaturation/elongation activity had increased 4.5 times in total lipid and 2.4 times in the polar lipids of the hepatocytes from (n-3) PUFA-deficient group relative to the control group. For the neutral lipid fraction the increase was much smaller (18%).

3.3.2. Desaturation and elongation activity in liver microsomes from stimulated trout

Having induced the desaturation and elongation activity for both 18:3n-3 and 20:5n-3 in hepatocytes by removing 22:6n-3 from the trout diet, it was decided to investigate the desaturation and elongation metabolism in the liver microsomes from stimulated juvenile rainbow trout.

3.3.2.1. Fatty acid composition of liver microsomes from stimulated trout

Tests to assess the purity of the microsomal preparation demonstrated that catalase specific activity was 0.042 units/mg protein in the microsomal fraction compared with 0.749 units/mg protein in the peroxisomal-rich fraction. Similarly,

Table 3.10. Percentage distribution of radioactivity in PUFA total, polar and neutral lipids of hepatocytes isolated from trout fed the two diets and incubated with [1-^{14}C]-20:5n-3.

	Total lipid		Polar lipid		Neutral lipid	
Diet	Control	(n-3) deficient	Control	(n-3) deficient	Control	(n-3) deficient
Activity†	1.9 ± 0.4	2.0 ± 0.7	0.5 ± 0.2	0.4 ± 0.3	1.6 ± 0.7	1.6 ± 0.1
Fatty acids						
20:5n-3	75.6 ± 2.5	43.9 ± 1.7 *	55.5 ± 0.5	39.7 ± 0.7 *	30.2 ± 1.3	28.6 ± 0.9
22:5n-3	13.3 ± 0.8	14.2 ± 1.5	9.5 ± 1.5	10.1 ± 0.5	25.7 ± 1.0	22.8 ± 1.2
24:5n-3	1.0 ± 0.3	4.9 ± 0.1 *	6.1 ± 0.5	4.3 ± 0.4 *	10.7 ± 0.8	11.2 ± 0.7
22:6n-3	10.1 ± 1.2	37.2 ± 0.6 *	28.9 ± 1.2	45.9 ± 0.7 *	18.4 ± 0.5	19.3 ± 2.2
24:6n-3	n.d	n.d	n.d.	n.d.	15.0 ± 0.4	18.1 ± 2.2
DI [a]	0.2 ± 0.1	0.9 ± 0.3 *	0.5 ± 0.1	1.2 ± 0.2 *	1.1 ± 0.6	1.3 ± 0.5

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=6 fish). The notation "*" indicates significant different from control (One Way ANOVA, $P<0.05$). The abreviation "n.d." indicates not detected. [a] The desaturation index "DI" is given as (22:6+24:6)/20:5n-3.

succinate dehydrogenase activity was 10-fold lower in microsomal preparation than in the mitochondrial fraction (Figure 3.1).

The percentage fatty acid composition of liver microsomes from trout fed the two diets are presented in Table 3.11. The fatty acid composition of liver microsomes from trout fed the (n-3) PUFA deficient diet followed a similar pattern of that observed with intact hepatocytes from fish in the same dietary group and shared a relative lower proportion of (n-3)PUFA and higher proportions of (n-6)PUFA and monoenoic fatty acids compared to controls. The proportion of (n-3) PUFA in total lipid of liver microsomes isolated from trout fed deficient diet was approximately 3-fold higher than in microsomes from trout fed control diet. The levels of 20:5n-3 and 22:6n-3 in the total lipid of liver microsomes from trout fed the deficient diet were respectively 7.6-fold and 2.6-fold lower than in lipid of microsomes from control fish. The contents of (n-6)PUFA and monoenoic fatty acids in microsomal lipid from deficient trout were twice as high as those of control fish whilst the levels of 20:4n-6 and 18:1n-9/7 were approximately 3-fold higher.

3.3.2.2. Distribution of radioactivity from [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 into lipid class of liver microsomes isolated from stimulated trout

The distribution of radioactivity incorporated from [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 into individual lipid class of microsomal fraction from trout liver is presented in Table 3.12. There were no significant differences between dietary groups for the incorporation of either [1-^{14}C]-18:3n-3 or [1-^{14}C]-20:5n-3 into individual lipid class of trout liver microsomes. Slightly more radioactivity incorporated from [1-^{14}C]-18:3n-3 was recovered in neutral than in polar lipids whereas the opposite was true for the distribution of radioactivity from [1-^{14}C]-20:5n-3. The majority of radioactivity from [1-^{14}C]-18:3n-3 was recovered in TAG (47.8 - 48.3%) from both dietary groups. PC (36.1 - 37.1%) was the second most labelled lipid class, followed by cholesterol ester (5.6 - 5.7%) and EGP (3.6-4.1%). Conversely, PC

Table 3.11. Fatty acid compositions (wt%) of liver microsomes total lipid isolated from trout fed the two diets.

	Trout liver microsomes	
Fatty acid	Control	(n-3) PUFA deficient
14:0	2.2 ± 0.0	0.7 ± 0.0
15:0	0.4 ± 0.0	0.1 ± 0.0
16:0	18.0 ± 1.1	15.3 ± 0.4
16:1n-7	2.4 ± 0.4	3.2 ± 0.4
16:2n-3	0.3 ± 0.0	0.1 ± 0.0
17:0	0.3 ± 0.0	0.1 ± 0.1
16:3n-3	n.d.	0.1 ± 0.1
16:4n-3	n.d.	n.d.
18:0	4.0 ± 0.3	6.0 ± 0.6
18:1n-9/7	11.7 ± 0.8	33.6 ± 1.1
18:2n-9	n.d.	0.7 ± 0.1
18:2n-6	3.1 ± 0.2	1.0 ± 1.7
18:3n-6	n.d.	n.d.
18:3n-3	0.4 ± 0.2	n.d.
18:4n-3	0.3 ± 0.3	0.2 ± 0.1
20:1n-9	3.8 ± 1.4	1.5 ± 0.0
20:2n-9	n.d.	1.5 ± 0.2
20:2n-6	0.6 ± 0.1	n.d.
20:3n-9	n.d.	2.8 ± 0.4
20:3n-6	0.8 ± 0.5	3.4 ± 0.4
20:4n-6	3.6 ± 0.5	10.0 ± 0.9
20:3n-3	n.d.	n.d.
20:4n-3	0.6 ± 0.0	n.d.
20:5n-3	6.9 ± 1.1	0.9 ± 0.2
22:1n-11	1.0 ± 0.2	0.1 ± 0.0
22:4n-6	0.1 ± 0.1	0.8 ± 0.1
22:5n-6	1.2 ± 0.6	3.3 ± 0.7
22:5n-3	1.4 ± 0.3	0.5 ± 0.0
22:6n-3	36.9 ± 0.7	14.0 ± 2.7
Σ Sats	24.5 ± 1.5	22.2 ± 1.0
Σ Monos	18.8 ± 2.0	38.5 ± 1.5
Σ (n-6)	8.0 ± 1.4	14.4 ± 3.1
Σ (n-3)	46.9 ± 2.6	15.8 ± 3.1
Σ PUFA	56.7 ± 1.2	39.3 ± 6.2

Data are means ± S.D. (n=3 fish). The notation 'n.d.' indicates not detected

Table 3.12. Incorporation of radioactivity into lipid classes of liver microsomes isolated from trout fed the two diets and incubated with [1-^{14}C]18:3n-3 or [1-^{14}C] 20:5n-3.

^{14}C substrate	[1-^{14}C]-18:3n-3		[1-^{14}C]-20:5n-3	
Activity†	1.9 ± 0.5	1.8 ± 0.6	2.2 ± 0.7	2.1 ± 0.7
Class	Control	(n-3)PUFA deficient	Control	(n-3)PUFA deficient
SM	0.4 ± 0.3	0.5 ± 0.3	1.0 ± 0.1	0.8 ± 0.1
PC	37.1 ± 0.7	36.1 ± 0.9	39.9 ± 0.1	37.7 ± 1.7
PS	0.7 ± 0.2	0.8 ± 0.1	1.1 ± 0.2	2.0 ± 1.1
PI	2.5 ± 0.1	2.4 ± 0.1	9.0 ± 0.6	9.3 ± 1.4
PG/CL/PA	0.7 ± 0.3	0.4 ± 0.2	0.7 ± 0.0	0.7 ± 0.1
EGP	3.6 ± 0.4	4.1 ± 0.4	5.9 ± 0.1	7.7 ± 0.5
CHO	1.2 ± 0.4	1.4 ± 0.1	3.4 ± 0.2	3.8 ± 0.2
FFA	0.4 ± 0.1	0.4 ± 0.0	4.3 ± 1.4	5.8 ± 0.9
TAG	47.8 ± 0.7	48.3 ± 0.4	30.6 ± 0.4	28.6 ± 1.2
CE	5.7 ± 0.7	5.6 ± 0.5	4.1 ± 0.1	3.4 ± 0.0
Total polar	49.9 ± 2.1	44.3 ± 2.5	57.6 ± 1.8	58.4 ± 2.8
Total neutral	55.1 ± 1.3	55.7± 2.3	42.4 ± 2.4	41.6 ± 2.2

† nmoles of ^{14}C-fatty acid incorporated/mg protein/3 hours. Data are means ± S.D. (n=3 fish). For each labelled substrate the symbol "*" denotes significantly different ($P<0.05$) from the control. If no symbol appears, values are not significantly different. SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; EGP, ethanolamine phosphoglycerides; CHO, cholesterol; FFA, free fatty acid; TAG, triacylglycerols; CE, cholesterol esters.

(37.7-39.9%) was the major labelled lipid class in liver microsomes from both dietary groups incubated with [1-^{14}C]-20:5n-3. At least 10 times more radioactivity from [1-^{14}C]-20:5n-3 was recovered as FFA than from [1-^{14}C]-18:3n-3. Similarly, more radioactivity from [1-^{14}C]-20:5n-3 was recovered in EGP than from [1-^{14}C]-18:3n-3.

3.3.2.3. Desaturation and elongation of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 by liver microsomes from stimulated trout

The autoradiograms from a $AgNO_3$-TLC plate in Figure 3.11 show the radiolabelled PUFA resulting from the desaturation and elongation of [1-^{14}C]-18:3n-3 by microsomes isolated from deficient and control trout. The profiles indicate that although [1-^{14}C]-18:3n-3 was extensively converted to highly unsaturated fatty acids, nevertheless no radioactive band for 22:6n-3 was identified among the products. The "dead-end" elongation products of [1-^{14}C]-18:3n-3 were found in both polar and neutral fractions, but were more abundant in the latter. The bands of C_{24} (n-3)PUFA (24:5n-3 and 24:6n-3) were found in both polar and neutral lipid fractions of trout microsomes incubated with [1-^{14}C]-18:3n-3. Radio-gas chromatography revealed that the radioactive band of 24:5n-3 and 18:4n-3 in the total lipid fraction were only partially resolved in most cases. The combined band was subjected to a second run with toluene/acetonitrile (95:5 by vol.) for total separation and subsequent quantitation.

Figure 3.12 shows an autoradiogram of the radioactive PUFA products derived from the desaturation and elongation of [1-^{14}C]-20:5n-3 by liver microsomes from the two groups of trout. The profiles indicate that only 22:5n-3, 24:5n-3 and 24:6n-3 were labelled and well resolved. No radioactive band of 22:6n-3 was detected among the labelled PUFA.

The quantitative incorporation of radioactivity from [1-^{14}C]18:3n-3 into PUFA of trout liver microsomes lipids is shown in Table 3.13. The majority of radioactivity remained as 18:3n-3 itself, while large amounts of radioactivity (24-29%) were

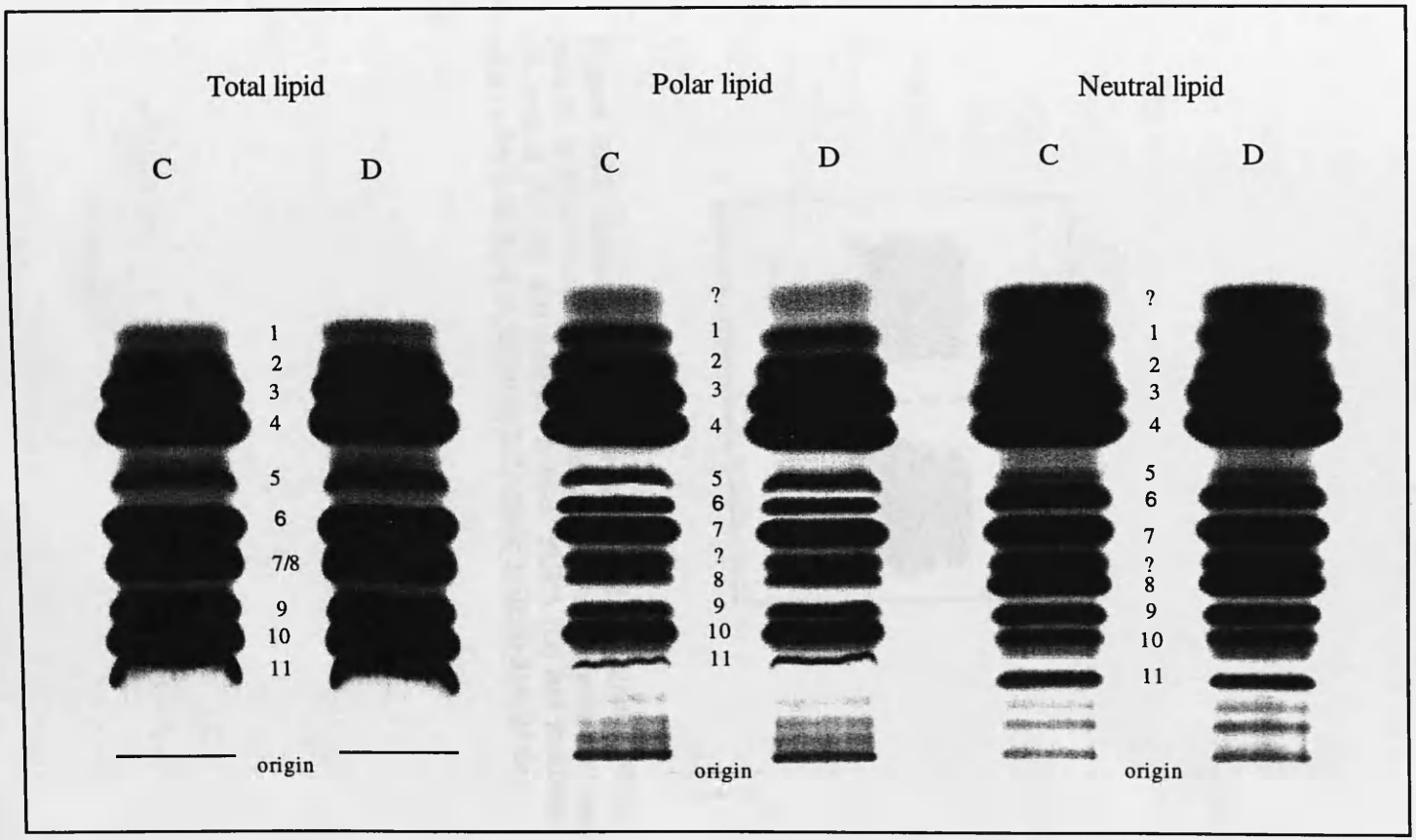

Figure 3.11. Autoradiograms of elongated and desaturated PUFA products in total, neutral and polar fractions of liver microsomes isolated from trout fed the control (C) and diet deficient in (n-3) PUFA (D) and incubated with [1-^{14}C]-18:3n-3. 1, 24:3n-3; 2, 22:3n-3; 3, 20:3n-3; 4, 18:3n-3; 5, 22:4n-3; 6, 20:4n-3; 7, 18:4n-3; 8, 24:5n-3; 9, 22:5n-3; 10, 20:5n-3; 11, 24:6n-3; ? not identified.

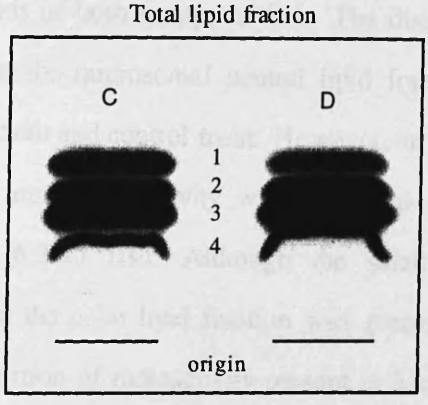

Figure 3.12. Autoradiogram of elongated and desaturated PUFA prducts in total fraction of liver microsomes isolated from trout fed the control (C) and diet deficient in (n-3) PUFA (D) and incubated with [1-^{14}C]-20:5n-3. 1, 24:5n-3; 2, 22:5n-3; 3, 20:5n-3; 4, 24:6n-3.

recovered as "dead end" elongation products of [1-^{14}C]18:3n-3, particularly 20:3n-3, in microsomes from both group of fish. A significantly higher proportion of radioactivity was recovered in 18:4n-3 of liver microsomes from deficient fish (5.9%) than from control trout (4.2%). The level of radiolabelled 24:6n-3 was significantly higher (One Way ANOVA, $P<0.05$) in microsomal lipid from deficient trout than from controls. The majority of the radioactivity (53-57%) remained as [1-^{14}C]18:3n-3 in the polar and neutral lipid fractions of microsomal lipid from both (n-3) PUFA-deficient and control trout. More radioactivity was recovered in the 20:5n-3 of polar lipids than neutral lipids of both groups of fish. The distribution of radioactivity in PUFA recovered from the microsomal neutral lipid fraction was not significantly different between deficient and control trout. However, in the microsomal polar lipid fraction, significantly more radioactivity was recovered as 18:4n-3 from deficient (9.0%) than control (6.3%) fish. Although the small amount of radioactivity recovered in 24:5n-3 in the polar lipid fraction was greater for the control than the deficient fish, the proportion of radioactivity present in 24:6n-3 was 4-fold higher in deficient than in control fish.

No radioactivity was detected in 22:6n-3 in total lipid recovered from microsomes incubated with [1-^{14}C]20:5n-3 from either group of fish (Table 3.14). Significantly more radioactivity was incorporated into the elongation products of [1-^{14}C]20:5n-3, *i.e.* 22:5n-3 and 24:5n-3, by microsomes from the livers of control than from deficient fish. Notably, the proportion of radioactivity recovered in 24:6n-3 from liver microsomes of deficient fish was very substantial and 2.5-fold higher than observed with control fish.

3.3.3. Biosynthesis of 22:6n-3 from C_{24} (n-3) PUFA in rainbow trout hepatocytes

At this stage of the study it was decided to investigate the possible further metabolism of C_{24} PUFA, formed in microsomes from 18:3n-3, in trout hepatocytes. As a prelude to this, the metabolism of 20:5n-3 was first studied in the hepatocytes. A

Table 3.13. Percentage distribution of radioactivity in PUFA in total, polar and neutral lipids of liver microsomes isolated from trout fed the two diets and incubated with [1-^{14}C] 18:3n-3.

	Total lipid		Polar lipid		Neutral lipid	
Diet	Control	(n-3) deficient	Control	(n-3) deficient	Control	(n-3) deficient
Activity†	2.2 ± 0.4	2.1 ± 0.5	1.7 ± 0.2	1.6 ± 0.2	1.8 ± 0.1	1.8 ± 0.1
Fatty acids						
18:3n-3	60.3 ± 2.4	58.0 ± 2.3	54.3 ± 3.8	52.7 ± 4.5	56.7 ± 4.8	55.9 ± 5.2
20:3n-3	20.0 ± 1.4	20.1 ± 1.7	21.3 ± 3.2	19.0 ± 4.9	22.5 ± 3.7	23.4 ± 4.5
22:3n-3	2.3 ± 0.7	2.1 ± 0.9	3.0 ± 0.5	3.0 ± 1.0	3.2 ± 0.5	3.0 ± 0.6
24:3n-3	2.0 ± 0.8	1.0 ± 0.5	1.5 ± 0.3	1.9 ± 0.2	2.1 ± 0.3	2.2 ± 0.2
18:4n-3	4.2 ± 0.4	5.9 ± 0.6 *	6.3 ± 0.5	9.0 ± 0.3 *	6.0 ± 0.5	5.8 ± 1.0
20:4n-3	3.5 ± 0.7	3.3 ± 0.8	0.7 ± 0.0	0.3 ± 0.0	1.9 ± 0.7	2.1 ± 0.3
22:4n-3	0.7 ± 0.4	0.8 ± 0.3	1.4 ± 0.3	1.6 ± 0.2	0.4 ± 0.0	0.4 ± 0.1
20:5n-3	2.2 ± 1.3	2.9 ± 0.5	6.2 ± 0.4	6.0 ± 1.1	1.4 ± 0.6	1.3 ± 0.5
22:5n-3	1.4 ± 0.4	1.3 ± 0.3	1.2 ± 0.1	1.5 ± 0.1	1.5 ± 0.8	1.2 ± 0.3
24:5n-3	2.5 ± 0.3	2.3 ± 0.6	3.7 ± 0.3	2.7 ± 0.4 *	3.2 ± 0.4	3.1 ± 0.6
22:6n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24:6n-3	0.9 ± 0.2	2.2 ± 0.6 *	0.6 ± 0.0	2.4 ± 0.6 *	1.0 ± 0.3	1.4 ± 0.7

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=6 fish). The notation "*" indicates significantly different from control (One Way ANOVA, $P<0.05$). The abreviation "n.d." indicates not detected.

Table 3.14. Percentage distribution of radioactivity in PUFA of total lipids of liver microsomes isolated from trout fed the two diets and incubated with [1-^{14}C] 20:5n-3.

Diets	Control	(n-3) PUFA deficient
Activity†	2.2 ± 0.2	2.2 ± 0.0
Fatty acid		
20:5n-3	52.1 ± 1.0	57.8 ± 1.0*
22:5n-3	23.4 ± 0.7	13.8 ± 2.1*
24:5n-3	15.2 ± 0.4	4.7 ± 0.1*
22:6n-3	n.d.	n.d.
24:6n-3	9.3 ± 0.1	23.6 ± 1.2*

† nmoles of ^{14}C fatty acid incorporated/mg protein/3 hours. Data are means ± S.D. (n=3). For each labelled substrate the superscript symbol '*' denotes significantly different ($P<0.05$) from the control. The notation "n.d." represents not detected

typical radiochromatogram of the radioactive PUFA formed by the desaturation and elongation of [1-^{14}C]20:5n-3 by (n-3) deficient trout hepatocytes over the period of 3 hours is presented in Figure 3.13a. The profiles show clearly that [1-^{14}C]-20:5n-3 was desaturated and elongated by trout hepatocytes to form four radiolabelled PUFA products which were identified as 22:5n-3, 24:5n-3, 24:6n-3 and 22:6n-3. Under the chromatographic conditions employed, the ^{14}C-labelled 22:6n-3 product had a retention time of 47 minutes. In sharp contrast to this findings, when the [1-^{14}C]20:5n-3 was incubated with microsomes isolated from trout liver, the 22:6n-3 radioactive peak (Rt = 47 min.) was never present in the chromatographic profile (Figure 3.13b).

When ^{14}C-24:5n-3 was recovered from microsomal incubations and presented to hepatocytes a large proportion (41.9%) of radioactivity was recovered as hexaenoic fatty acids (22:6n-3, 22.9% and 24:6n-3, 19.0%), whereas only 1.6% was present in 22:5n-3 and 20:5n-3 (Table 3.15). Most (56.5%) of the radioactivity remained as 24:5n-3. The autoradiogram in Figure 3.14a confirms that ^{14}C-24:5n-3 was converted to 24:6n-3 and 22:6n-3 and also illustrates that retroconversion to 22:5n-3 and 20:5n-3 occurred, since significant amounts of radioactivity were present in these PUFA.

To investigate the last step of the formation of 22:6n-3 from C24 PUFA, radiolabelled 24:6n-3 isolated from microsomes was incubated with hepatocytes from deficient trout (Table 3.15). The autoradiogram in Figure 3.14b shows the $AgNO_3$-TLC profiles resulted from the time-course incubation of trout hepatocytes in the presence of ^{14}C-24:6n-3. It was notable that the density of a band corresponding to 22:6n-3 increased with time. The time-course of changes in percentage distribution of radioactivity in hepatocyte PUFA over a period of 6 hours is shown in Figure 3.15. The proportion of total radioactivity recovered in 22:6n-3 increased gradually and significantly (Repeated Measure ANOVA One Factor, $P<0.05$) as the proportion recovered in the substrate 24:6n-3 decreased. At the end of the incubation period, 34.6% of the total radioactivity recovered was present as 22:6n-3. The proportion of radioactivity recovered as 24:6n-3 decreased significantly (Repeated Measure

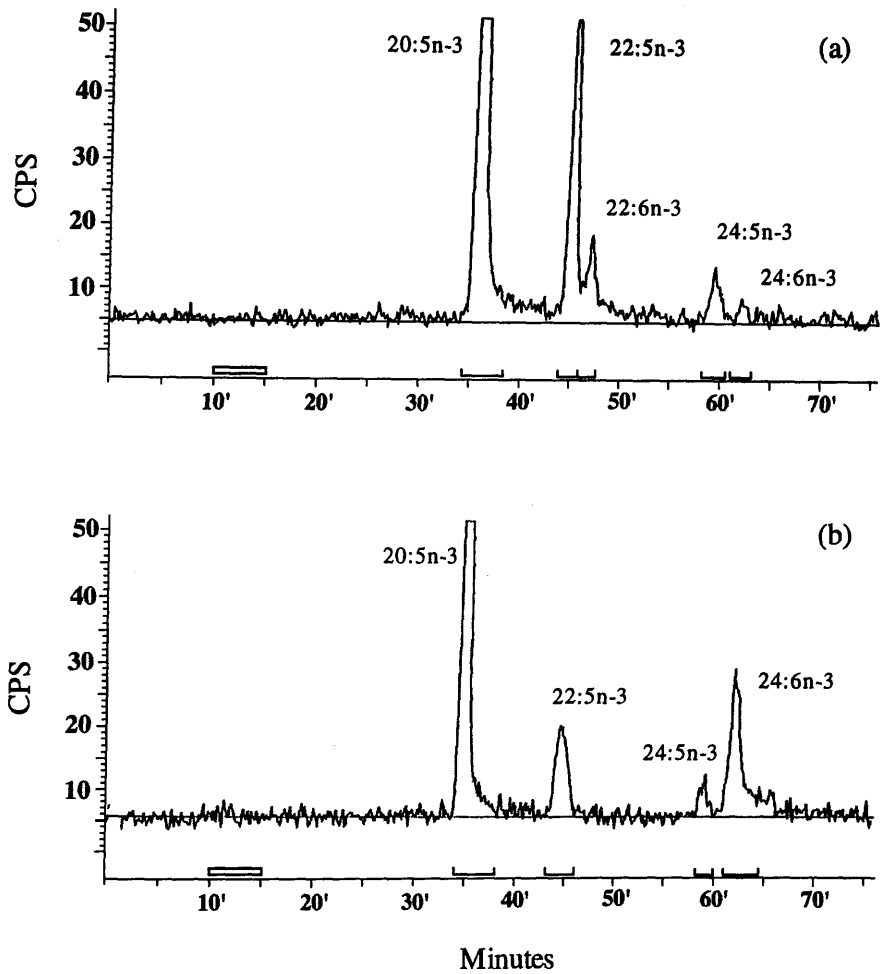

Figure 3.13. Radiochromatograms of the desaturation and elongation [1-^{14}C]-20:5n-3 by hepatocytes (a) and liver microsomes (b) from stimulated rainbow trout. Hepatocytes and microsomes were incubated for 3 hours at 12°C.

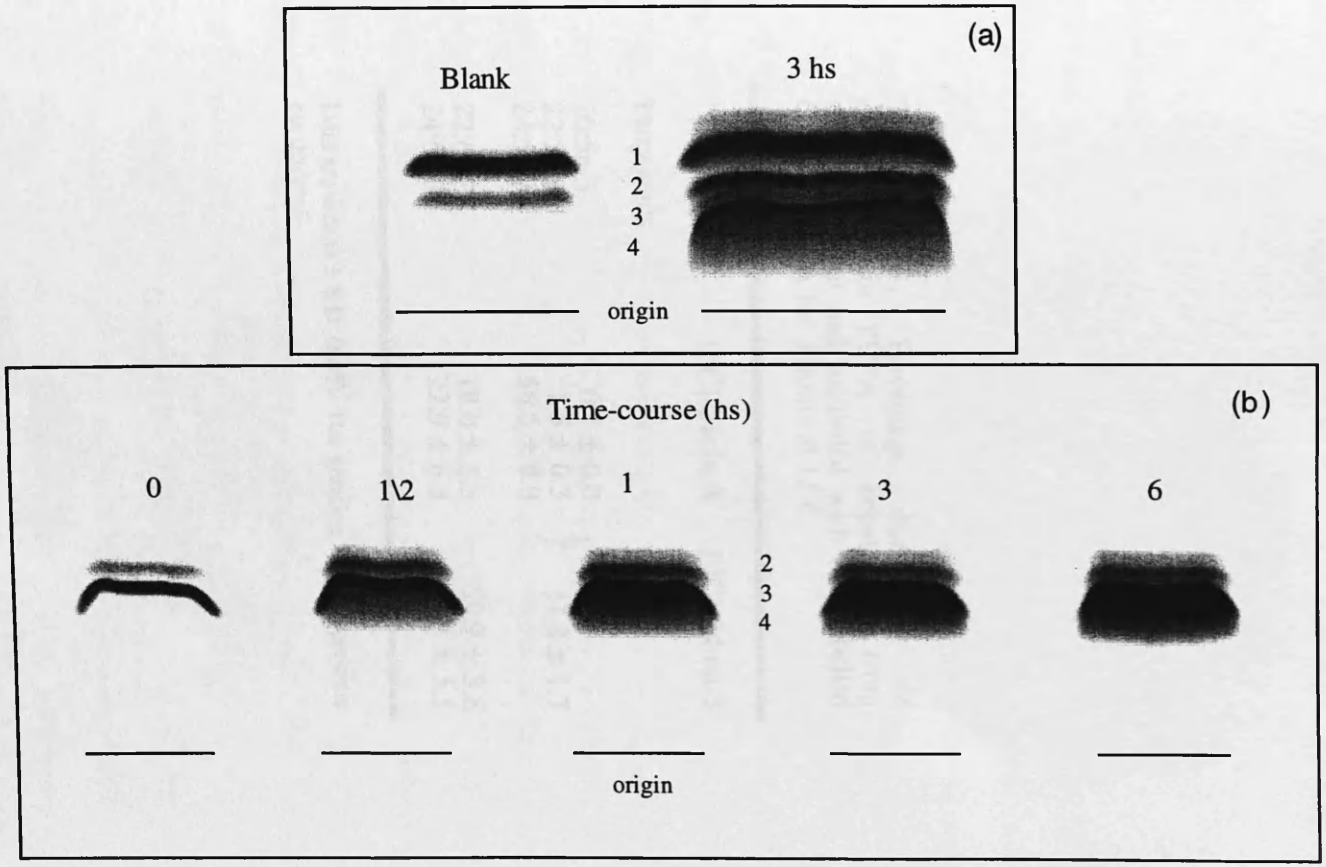

Figure 3.14. Autoradiograms from AgNO3-TLC plates showing the conversion of C24-(n-3)PUFA to 22:6n-3. (a) trout hepatocytes incubated with ^{14}C-24:5n-3 for 3 hours; (b) time-course incubation of trout hepatocytes in the presence of ^{14}C-24:6n-3. 1, 24:5n-3; 2, 22:5n-3/20:5n-3; 3, 24:6n-3; 4, 22:6n-3.

Table 3.15. Percentage distribution of radioactivity in PUFA of hepatocytes from stimulated trout and incubated with radiolabelled C_{24}-(n-3)PUFA for 3 hours at 12°C

^{14}C-substrate	[^{14}C]-24:5n-3	[^{14}C]-24:6n-3
Fatty acids		
20:5n-3	0.1 ± 0.0	
22:5n-3	1.5 ± 0.3	11.8 ± 1.7
24:5n-3	56.5 ± 9.9	
22:6n-3	19.0 ± 5.3	59.9 ± 3.8
24:6n-3	22.9 ± 6.3	28.2 ± 5.1

Data are means ± S.D. (n=3). The notation "n.d." represents not detected

ANOVA One Factor, $P<0.05$) from 95.2% to 54.2% *i.e.* by 41% over the time-course period. Table 3.15 shows that 11.8% of the total radioactivity from ^{14}C-24:5n-3 was recovered in retroconversion products (Σ 24:5n-3, 22:5n-3, 20:5n-3) after 3 hours incubation.

3.4. Discussion

The lipid class composition of hepatocytes from trout starved for 30 days (treatment B) was not significantly different from that of either hepatocytes from fed (treatment A) or from trout that were fed for 2 days following the 28 days of starvation (treatment C). The results also demonstrated that no significant changes in the hepatocyte fatty acid composition occurred when fish were subjected to either starvation or starvation-refeeding regimes. Similarly, a recent study on the induction of Δ9-desaturation in rainbow trout has demonstrated that fatty acid composition of trout liver lipids is unaffected by a 2-week starvation period (Tocher, *et al.,* 1996).

In the present study, the [1-^{14}C]-18:3n-3 incorporated by hepatocytes was predominantly recovered in the triacylglycerol fraction. Neither the total radioactive incorporation rate nor its distribution in different lipid classes were affected by starvation and starvation-refeeding regimes. Previous work using European eel has also demonstrated that the distribution of radioactivity from [1-^{14}C]-acetate into liver and gills lipid class is not significantly different between fed and 7 week-starved eel (Hansen and Abraham, 1983; Abraham *et al.*, 1984).

Freshly isolated hepatocytes from rainbow trout used in the present experiments were capable of converting radiolabelled 18:3n-3 to longer PUFA, including the biologically important 22:6n-3. The profiles demonstrated that [1-^{14}C]18:3n-3 was converted to longer chain PUFA in hepatocytes of trout from all three dietary groups. The patterns observed on the autoradiograms clearly demonstrated that [1-^{14}C]-18:3n-3 was desaturated to 18:4n-3 which was subsequently elongated to 20:4n-3 and 22:4n-3. The profiles showed in addition that [1-^{14}C]-18:3n-3 was subjected to successive elongation reactions to form "dead-end"

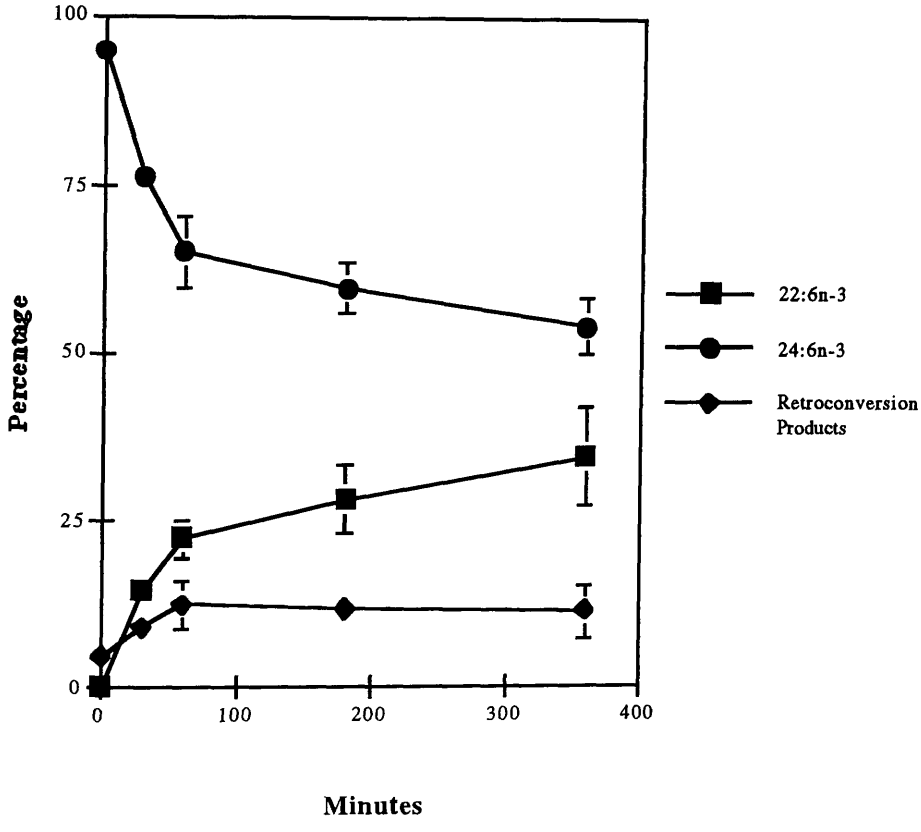

Figure 3.15. Percentage distribution of radioactivity in isolated trout hepatocytes incubated with [^{14}C]-24:6n-3. Cells were incubated for 6 hours at 12°C. Each point represents the mean ± standard deviation of triplicates.

products such as 20:3n-3, 22:3n-3 and 24:3n-3, whose bands appeared on the $AgNO_3$-TLC plates positioned above the [1-^{14}C]-18:3n-3. The presence of radioactive 20:5n-3 band indicated that 20:4n-3 was desaturated by the rainbow trout hepatocytes. The radiolabelled 20:5n-3 was elongated in turn to form 22:5n-3. The detection of nine radiolabelled products derived from the desaturation and elongation of [1-^{14}C]-18:3n-3 in total lipids confirms that these hepatocytes possessed functional elongases and desaturases, particularly Δ6 and Δ5-desaturases.

It is known that starvation suppresses lipogenic activity in mammals (Brenner 1981) and fish. Studying the effects of starvation on hepatic lipogenic enzymes, Lin *et al.* (1977) observed that two days of withholding food was sufficient to decrease the hepatic fatty acid synthesis in liver slices by 50%. Tocher, *et al.* (1996) have recently reported that the Δ9 desaturation activity in hepatocytes from trout starved for 3 weeks was low in comparison with fed trout, since only 2% of [1-^{14}C]-18:0 was converted to longer monoenoic fatty acids. The re-introduction of a commercial diet following starvation resulted in a substantial increase of desaturation activity with 50% of [1-^{14}C]-18:0 being converted to longer Δ9-desaturase products. The present results demonstrate that the desaturation and elongation activity in trout hepatocytes is not significantly affected by either starvation or starvation-refeeding regimes used here. The conversion of [1-^{14}C]-18:3n-3 to longer (n-3) PUFA in hepatocytes was less than 9% with no appreciable differences between groups. Nevertheless there was a trend for desaturation activity to increase in hepatocytes if the starvation period was prolonged. A longer starvation period may have elicited significant differences in desaturation activity.

Hepatocytes isolated from juvenile rainbow trout showed that the radioactivity from [1-^{14}C]-18:3n-3 were more rapidly incorporated into hepatocyte neutral lipid, particularly the triacylglycerol fraction, that with mature fish. Although no significant changes in the proportion of radioactivity recovered in TAG was observed during 24 hours, the levels recovered in polar lipids, particularly in PC and EGP exhibited a considerable increase with time. These results are consistent with the initial

incorporation of 18:3n-3 into TAG and a turnover of TAG to release 18:3n-3 for desaturation and elongation to PUFA which are then esterified into phospholipids. A previous study of the incorporation of radiolabelled C_{18}-PUFA by rainbow trout hepatocytes has reported that at least 5 times more radioactivity from [1-^{14}C]-18:3n-3 was incorporated into neutral than in polar lipids, and that among the neutrals, over 90% of the isotope was found in TAG (Sellner and Hazel 1982ab). Later, Hazel (1983) found that the radioactivity from [1-^{14}C]-18:3n-3 incorporated into trout hepatocyte phospholipids was preferentially located in PC and PE, which is in accordance with the results of the present study. Moreover, the same authors reported that the products of Δ6 and Δ5 desaturation (*i.e.*: 18:4n-3, 20:5n-3) and C_{18} and C_{20} elongation products (*i.e.*: 20:4n-3, 22:5n-3) were mainly found in the phospholipid fraction, and they suggested that trout hepatocytes tended to retain the polyunsaturated substrate and their derivatives in the FFA pool before storing them as TAG which are subsequently available for providing substrates for desaturation and elongation and incorporation into membrane phospholipids *via* acyltransferases (Sellner and Hazel 1982ab; Hazel 1983). This may explain in the present study, the substantial decrease of radiolabel recovered in TAG over time concomitant with the increase of label in PC and EGP.

Hepatocytes from juvenile rainbow trout were capable of desaturating and elongating substantially more [1-^{14}C]-18:3n-3 to longer chain PUFA than hepatocytes isolated from adult fish (weighing 400-450g). Within 3 hours of incubation hepatocytes form juvenile trout converted 38% of the [1-^{14}C]-18:3n-3 to longer chain PUFA compared to cells from adult trout which converted only 20% of the radioactive substrate. Radioactivity recovered in 22:6n-3 was at least 7 times higher in hepatocytes from juvenile trout than in those from adult fish. Since both juvenile and adult trout were fed the same commercial diet, the differences in desaturation activity reflects the age of the animals. Although the effect of age on the desaturation and elongation in fish hepatocytes has not been yet investigated, Bourre *et al.*, (1990) established that Δ6-desaturation in mice liver, measured by the conversion of

[1-^{14}C]-18:3n-3 into longer chain PUFA, decreased significantly with the age of the animals.

Several authors have reported previously that rainbow trout are capable of converting linolenic acid to highly unsaturated fatty acids such as 20:5n-3 and 22:6n-3 both *in vivo* and *in vitro* (Owen, *et al.*, 1975; Kanazawa, *et al.*, 1979; Sellner and Hazel 1982ab; Hagve, *et al.*, 1986). Previous *in vivo* studies using rainbow trout and turbot demonstrated that approximately 70% of the total radioactivity from[1-^{14}C]-18:3n-3 injected in trout was recovered in 22:6n-3, whereas in turbot the proportion of radioactivity recovered as 22:6n-3 was negligible (Owen, *et al.*, 1975). Studying the conversion of 18:3n-3 into highly unsaturated fatty acids in various fish species, Kanazawa *et al.* (1979) observed that in the trout, 10% of the radioactivity from injected [1-^{14}C]-18:3n-3 present in the polar fraction was recovered as 22:6n-3, which was at least twice as much as the proportions found in the other fish species. *In vitro* studies using isolated hepatocytes from thermally acclimated rainbow trout demonstrated that cold-acclimated fish converted up to 2.3% of the total radioactivity from [1-^{14}C]-18:3n-3 into 22:6n-3 (Sellner and Hazel 1982ab). Similarly, the patterns of radioactivity distribution showed that hepatocytes from juvenile trout used in the current study readily converted [1-^{14}C]-18:3n-3 highly unsaturated fatty acids. The increase of radioactivity recovered in products such as 18:4n-3, 20:5n-3 and particularly 22:6n-3 within a few hours of incubation indicates that the desaturation and elongation reactions to form 22:6n-3 in trout proceeded rapidly. Comparing the polyunsaturated fatty acid metabolism between hepatocytes from rat and trout, Hagve *et al.* (1986) found that the rate of formation of 22:6n-3 form 18:3n-3 in trout, which was 2.1% of the total radioactivity from [1-^{14}C]-18:3n-3, was 3 fold higher than in rats.

The results from time course incubations revealed that the distribution of radioactivity in desaturated/elongated products (*i.e.*: 18:4n-3, 20:4n-3, 22:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3) and "dead-end" elongation products (*i.e.*: 20:3n-3, 22:3n-3 and 24:3n-3) increased over time. The decreased proportion of radioactivity

recovered in desaturated/elongated products after 6 hours, particularly in 20:4n-3 and 22:5n-3 was unexpected. Because there was no appreciable reduction of the proportion of radioactivity recovered in 18:3n-3 after 6 hours, it is possible that the decrease of 20:4n-3 and 22:5n-3 may be attributed to the formation of 20:5n-3 and 22:6n-3 as the proportion of both these PUFA increased over time.

Thus, the experiments described here demonstrated that the incorporation rate of [1-^{14}C]-18:3n-3, its distribution into lipid class in trout hepatocytes, as well as the desaturation and elongation activity were unaffected by the starvation and fast-refeeding regimes used here. Although the desaturation and elongation activities in hepatocytes from adult trout were low compared to these hepatocytes from juvenile trout, docosahexaenoic acid was still formed in detectable amounts.

The use of olive oil in the experimental diet effectively deprived the trout of a dietary supply of (n-3)PUFA while maintaining the proportion of dietary energy supplied as lipid and at the same time providing a sufficiency of (n-6)PUFA. The lower growth rate observed for trout fed olive oil compared to trout fed fish oil confirms a previous nutritional study which demonstrated that trout fed a diet containing 80% of the total fatty acids as 18:1n-9 grew more slowly than those with a high intake of (n-3)PUFA (Castell *et al.*, 1972b). The absence in this study of visible symptoms of essential fatty acid deficiency in fish maintained on olive oil for three months is in keeping with a longer time being required for the manifestation of such symptoms (Castell *et al.*, 1972b).

The fatty acid composition of the isolated hepatocytes established that the dietary regime using olive oil was very effective in reducing the endogenous level of (n-3)PUFA. The ratios of (n-3)/(n-6) PUFA in hepatocytes from trout fed deficient diet were always substantially lower in relation to the ratios in control fish. The accumulation of high levels of 18:1n-9 in hepatocyte lipids from the experimental fish is clear evidence that the fatty acid composition of liver tissue directly responds to changes in the fatty acid composition of dietary lipid. The increased content of hepatocyte (n-9) PUFA, *i.e.* 18:2n-9 and 20:2n-9, observed in the present study

confirms previous reports that a deficiency of (n-3)PUFA results in an increase in the level of (n-9)PUFA in trout (Castell *et al.*, 1972a; Watanabe *et al.*, 1974) and other fish species (Cowey *et al.*, 1976; Takeuchi *et al.*, 1980; Olsen *et al.*, 1990). No 20:3n-9 was observed in the hepatocytes from fish fed the experimental diet. The experimental diet used here contained less than the minimum amount of (n-3)PUFA required to satisfy the requirements of rainbow trout for these PUFA (Henderson and Tocher 1987), but it contained almost 10% 18:2n-6 PUFA. Utilisation of 18:2n-6 as a substrate for desaturation and elongation enzymes in preference to 18:1n-9 can account for the relative small amount of 18:2n-9 and 20:2n-9 formed and absence of 20:3n-9, this inference being supported by the presence of elevated and quite substantial levels of 20:2n-6, 20:3n-6, 20:4n-6 and 22:5n-6 in the hepatocytes from the experimental fish.

Hepatocytes from both dietary groups incorporated over 75% of the total radioactivity from [1-^{14}C]18:3n-3 into fatty acids in the neutral lipid fraction which consisted of FFA, TAG and CE. In contrast, of the lesser amounts of radioactivity incorporated from [1-^{14}C]20:5n-3, almost half was recovered in the polar lipid fraction. This demonstrates that the hepatocyte esterification enzymes have substrate preferences resulting in the general situation in trout, that the phospholipids contain high levels of C_{20} and C_{22} PUFA whereas the triacylglycerols are richer in C_{18} PUFA (Henderson and Tocher 1987).

It is well established that rainbow trout can efficiently desaturate and elongate 18:3n-3 to 20:5n-3 and 22:6n-3 (Castell *et al.*, 1972a; Owen *et al.*, 1975; Hazel 1979; Kanazawa *et al.*, 1979; Leger *et al.*, 1980; Sellner and Hazel 1982ab; Hazel 1983; Hagve *et al.*, 1986; Tocher and Sargent, 1990ab), and it has been reported that trout hepatocytes form 22:6n-3 from either 18:3n-3 or 20:5n-3 at a higher rate than rat hepatocytes (Hagve *et al.*, 1986). The rates of formation of 22:6n-3 observed in the present study in hepatocytes from trout fed olive oil are considerably higher than those reported previously for cells prepared from trout maintained on a commercial fish diet (Hagve *et al.*, 1986).

The first step in the conversion of 18:3n-3 to 22:6n-3 in animals is Δ6 desaturation to form 18:4n-3. In rats the rate of Δ6 desaturation is influenced by nutritional factors including the essential fatty acid status and levels of dietary energy and protein (Brenner 1981). Increased Δ6 desaturation activity elicited by essential fatty acid deficiency can be attributed to the absence of dietary, preformed long chain PUFA, necessitating their formation from shorter chain, less unsaturated fatty acids. In line with this, the present results indicate that 18:3n-3 supplied to trout hepatocytes with a low level of endogenous long chain (n-3)PUFA is converted more extensively to 22:6n-3 than the same substrate presented to hepatocytes containing high levels of 20:5n-3 and 22:6n-3. This confirms analytical evidence from studies in which rainbow trout fed diets rich in lard and poor in (n-3)PUFA desaturated and chain elongated more 18:3n-3 and 18:2n-6 than trout fed fish oil diet (Leger *et al.*, 1980). In rats this inhibition is also evident *in vitro* since the addition of 22:6n-3 to liver microsomes significantly depressed the low rate of desaturation of 18:3n-3 to 22:6n-3 (Brenner and Peluffo 1966). Likewise, liver microsomes from rats fed diets based on fish oil had lower Δ6 and Δ5 desaturation activities than microsomes from rats fed either sunflower or linseed oil (Christiansen *et al.*, 1991). Although the formation of 22:6n-3 from 20:5n-3 has already been studied in fish (Hagve *et al.*, 1986; Tocher and Sargent 1990ab; Linares and Henderson 1991), none of the studies have investigated the effect of dietary (n-3)PUFA on the elongation / desaturation activity. The results of the present study demonstrate that the rate of conversion of 20:5n-3 to 22:6n-3 is also subject to control by the level of long chain (n-3)PUFA in the diet.

It was notable that hepatocytes incorporated radioactivity from [1-^{14}C]18:3n-3 into 24:6n-3, whereas both 24:5n-3 and 24:6n-3 contained radiolabel in hepatocytes incubated with [1-^{14}C]20:5n-3. Recent studies on the metabolism of radiolabelled 20:5n-3 in juvenile seabream *in vivo* have also reported radioactive 24:5n-3 and 24:6n-3 among the desaturated and elongated metabolites (Mourente and Tocher 1993ab; Mourente and Tocher 1994). These two C_{24} PUFA are known to occur in small amounts in the TAG of pike (Henderson *et al.*, 1995) and some marine fish

(Linko and Karinkanta 1970; Ota, Kawabata *et al.*, 1994; Ota, Chihara *et al.*, 1994), but they have not been reported to occur in trout lipids.

The present study demonstrates that the ability of trout hepatocytes to synthesise 22:6n-3 from both 18:3n-3 and 20:5n-3 can be markedly stimulated by eliminating long chain (n-3)PUFA from the fish's diet, particularly 22:6n-3.

The endoplasmic reticulum in fish liver cells possesses enzyme systems capable of desaturating and chain-elongating unsaturated fatty acids. The results in the present study proved that liver microsomes isolated from trout fed a diet deficient in (n-3) PUFA were in fact depleted in this PUFA series, and as with intact hepatocytes, they exhibited a significantly higher desaturation and elongation activity compared to microsomes from control trout. Previous studies using fish liver have demonstrated that isolated microsomes exhibit *in vitro* a high desaturation and chain elongation activity for various PUFA substrates without forming significant amounts of 22:6n-3 (Ninno *et al.*, 1974; De Torrengo and Brenner 1976; Muci *et al.*, 1992). Although the effects of dietary lipids on microsomal desaturation and elongation activity has never been reported in fish, it is known that liver microsomes from rats fed a diet rich in (n-3) PUFA, particularly 22:6n-3 and 20:5n-3, showed lower desaturation and elongation activity relative to animals fed a diet with a low content of (n-3) PUFA (Garg *et al.*, 1988; Christiansen *et al.*, 1991). Likewise, Brenner and Peluffo (1966) demonstrated that rat microsomal desaturation and elongation activities were suppressed by the addition of long chain PUFA, such as 22:6n-3 to the medium.

Although the rate of formation of 22:6n-3 by trout hepatocytes was substantially enhanced by feeding the fish a diet deficient in (n-3) PUFA, the results here clearly demonstrate that crude microsomes prepared from the livers of fish fed either the control or (n-3)PUFA-deficient diets were unable to form 22:6n-3 in detectable amounts. The trout microsomes, however, readily elongated 20:5n-3 to 22:5n-3 and thence to 24:5n-3, and also generated substantial amounts of 24:6n-3, the $\Delta 6$ desaturation product of 24:5n-3. Moreover, the distribution of radioactivity from both radiolabelled 18:3n-3 and especially from 20:5n-3 showed that significantly

more radioactivity was recovered as 24:6n-3, and less as 24:5n-3, in microsomes from (n-3)PUFA - deficient fish than from control fish. This reinforces the evidence for a higher rate of Δ6-desaturation activity in (n-3)PUFA - deficient trout.

The distribution of radioactivity in liver microsome lipid classes indicated that radioactivity from [1-^{14}C]-20:5n-3 was preferentially esterified into phospholipids compared to neutral lipids, whereas radioactivity from [1-^{14}C]-18:3n-3 was found predominantly in neutral lipids, especially in TAG. This distribution pattern found in microsomes is further evidence that the trout liver esterification enzymes have a substrate preference in line with the general composition in trout tissue, whereby phospholipids contain high levels of C_{20} and C_{22} PUFA and the triacylglycerols are richer in C_{18} PUFA.

The present work demonstrates that freshwater fish such as trout can substantially desaturate and elongate both 18:3n-3 and 20:5n-3 to 22:6n-3. However, to date, there is no experimental evidence to support the direct formation of 22:6n-3 from 22:5n-3 by Δ4-desaturation in fish tissues. Contrary to the known situation in isolated hepatocytes, the microsomal fraction from trout liver was unable to produce 22:6n-3 from either 18:3n-3 or 20:5n-3. This strongly indicates that, as is the case in rats (Voss *et al.*, 1991), the formation of 22:6n-3 in fish requires other cellular organelles in addition to endoplasmic reticulum. The commonly accepted hypothesis that the desaturation of 22:5n-3 to 22:6n-3 is catalysed by a Δ4-desaturase in rat liver was recently refuted by (Voss *et al.*, 1991). In these studies, it was demonstrated that 22:5n-3 was the precursor of 22:6n-3 through a pathway which involves firstly the Δ6-desaturation of 24:5n-3 to 24:6n-3 in the microsomes followed by a partial peroxisomal ß-oxidation whereby 24:6n-3 is chain-shortened to 22:6n-3. The accumulation of substantial amounts of radioactivity from [1-^{14}C]20:5n-3 in 24:6n-3 and 24:5n-3 in microsomal lipids provides evidence that the formation of 22:6n-3 *via* C_{24} intermediates with subsequent peroxisomal chain shortening may operate in trout. The possibility that the radioactive 24:6n-3 formed in isolated microsomes in the present study was formed by direct elongation of 22:6n-3, itself formed by

conventional direct Δ4 desaturation of 22:5n-3, seems remote, since the elongation of any 22:6n-3 formed by direct Δ4-desaturation would have to be so fast to preclude even traces of radioactivity being detected in 22:6n-3. This is not compatible with the ready detection of chain elongation products from a wide range of PUFA substrates and intermediates in this study.

The observation that, in hepatocytes, the Δ6-desaturation of added 24:5n-3 to 24:6n-3 and conversion to 22:6n-3 predominated over retroconversion to 22:5n-3 and 20:5n-3 indicates that under the conditions employed here the major metabolic fate of 24:5n-3 formed in hepatocytes from (n-3) PUFA-deficient trout is the biosynthesis of 22:6n-3. This is keeping with the results of an earlier study with hepatocytes from rats using [3-^{14}C]-22:5n-3 which showed that 22:5n-3 was a poor substrate for retroconversion and was mainly designated for 22:6n-3 formation (Schlenk *et al.*, 1969; Voss *et al.*, 1992).

Hepatocytes from rainbow trout directly converted radiolabelled 24:6n-3 to 22:6n-3. The results show that the concentration of radiolabelled 24:6n-3 decreased proportionally to the increase of radiolabelled 22:6n-3. Retroconversion of 24:6n-3 to 24:5n-3 was negligible, *i.e.* the primary fate of 24:6n-3 is conversion to 22:6n-3. This clearly demonstrated that the last metabolic step for the 22:6n-3 formation in trout hepatocytes is a partial ß-oxidation of 24:6n-3, which presumably takes place in peroxisomes. Although 24:6n-3 can be substantially ß-oxidised in the peroxisomes to form 22:6n-3, further ß-oxidation of 22:6n-3 is unlikely to proceed at the same rate. The 22:6n-3 molecule contains the maximum number of methylene interrupted *cis* double bonds along the carbon chain making it conformationally an awkward substrate for removing 2 carbons by ß-oxidation. The peroxisomal ß-oxidation of 22:6n-3 in mammalian livers has been generally found to occur at a low rate (Hiltunen *et al.*, 1986; Hovik and Osmundsen 1987; Mannerts and Van Veldhoven 1993). The reason for this is that the 22:6n-3 molecule has Δ4 double bond, which unlike all the (n-3) PUFA in animal tissues, requires a NADPH dependent enzyme, 2,4-dienoyl-CoA reductase, during the first cycle of its ß-oxidation. Although in theory 22:6n-3

can be ß-oxidised by the 2,4-dienoyl-CoA reductase pathway, in practice this enzymatic step is probably heavily regulated and occurs at a limited rate comparing to the main ß-oxidation pathways which do not require NADPH. This generally results in the accumulation of 22:6n-3 in tissues.

The importance of retroconversion in the biosynthesis of PUFA was recently emphasised by *in vivo* study using deutered eicosatrienoic acid (20:3n-3) fed to mice (Schenck *et al.*, 1996). These authors found that 51.5% of the total deuterated metabolites from 11,14,17-20:3n-3-3,3,4,4,8,8,9,9-d_8 arose from initial retroconversion (*i.e.*: 11,14,17-20:3n-3 ➡ 9,12,15-18:3n-3). In addition to retroconversion, 42.1% of the metabolites were derived from the classical pathway involving Δ5-desaturase (*i.e.*: 11,14,17-20:3n-3 ➡ 5,11,14,17-20:4n-3) and 6.4% from a pathway which utilises Δ8-desaturation (*i.e.*: 11,14,17-20:3n-3 ➡ 8,11,14,17-20:4n-3) (Schenck *et al.*, 1996). Recent studies with marine teleost fish demonstrated that hepatic peroxisomes contribute to the ß-oxidation of the major proportion of long chain (n-3) PUFA, and indicate that a high oxidative capacity of peroxisomes from fish liver could be a catabolic pathway that sustains anabolic processes (Crockett and Sidel 1993ab).

CHAPTER 4

STUDIES ON THE ELONGASES, Δ6 AND Δ5-DESATURASE ACTIVITIES IN TROUT LIVER MICROSOMES

4.1. Introduction

The work described in the previous section established that the biosynthesis of docosahexaenoic acid in rainbow trout hepatocytes proceeds *via* C_{24} PUFA intermediates (*i.e.* 24:5n-3 and 24:6n-3) and involves a Δ6-desaturase rather than, as previously assumed, a Δ4-desaturase. However, it is not known whether the Δ6-desaturase required for converting 18:3n-3 to 18:4n-3 is the same enzyme that also converts 24:5n-3 to 24:6n-3. If there are two different enzymes engaged in this process, how are they regulated? Alternatively, if there is only one Δ6-desaturase, what are the factors that define its affinity and activity for different chain-length PUFA? A recent study, using both C_{18} and C_{24} substrates, demonstrated that C_{18} PUFA inhibited the desaturation and elongation of the C_{24} substrates to a great degree than *vice versa*, indicating that there was always a preferential desaturation of 18:3n-3 rather than 24:5n-3 (Geiger *et al.*, 1993). Although the authors suggested that these results were consistent with a single Δ6-desaturase, additional studies including the isolation of Δ6-desaturase are required to understand more about the mechanism involved in the regulation of 22:6n-3 biosynthesis.

Several studies have reported that cyclopropene fatty acids (CPFA) such as sterculic and malvalic acids, usually present in Baobab seed oil, are effectively able to inhibit desaturation activity in microsomes isolated from rat liver (Andrianaivo-Rafehivola *et al.*, 1993; Cao *et al.*, 1993; Andrianaivo-Rafehivola *et al.*, 1994) and yeast (Rolph *et al.*, 1990). Although there have been no studies regarding the effects of cyclopropenoid acids on the desaturation activity in trout, several lines of evidence have indicated that CPFA affect protein and lipid metabolism in trout (Malevski *et al.*,

1974; Struthers *et al.*, 1975; Selivonchick *et al.*, 1981). Cao *et al.*, (1993) found that the presence of cyclopropenoic acids in a fresh Baobab oil diet markedly depressed Δ6 and Δ5-desaturation in liver microsomes from rat, while Rolph *et al.*, (1990) observed that approximately 90% of [1-^{14}C]-18:0 desaturation by the oleaginous yeast *Rhodotorula gracilis* was inhibited in the presence of sterculic acid. The mechanism by which the cyclopropene fatty acids inhibit desaturation is not yet fully understood, but it was suggested that inhibition possibly resulted from the formation of a carbon-sulphur bond between a carbon atom of the cyclopropene ring and a thiol group at the active site of the desaturase (Andrianaivo-Rafehivola *et al.*, 1993).

Curcumin, a yellow pigment in turmeric (dry powdered rhizome of the plant *Curcuma longa* Linn) and widely used as spice and colorant in the food industry is a potent inhibitor of mammalian lipoxygenase, cyclooxygenase and lipid peroxidases (Huang *et al.*, 1991; Reddy and Lokesh 1994; Reddy and Lokesh 1994), and Δ6 and Δ5-desaturases (Fujiyama-Fujiwara *et al.*, 1992; Shimizu *et al.*, 1992; Kawashima *et al.*, 1996). Its inhibitory effect on fatty acid desaturation and elongation was also reported in the yeast *Mortierella alpina* (Shimizu *et al.*, 1992). Kawashima *et al.*, (1996) recently reported that the aromatic ring conjugated with the double bond positioned between carbons 1 and 2, the 4 hydroxy and the 3 methoxy groups are all structural features of the curcumin molecule which play important roles in the inhibition of rat liver microsomal fatty acid desaturation.

The use of specific inhibitors of desaturation metabolism is a potentially useful tool for examining the mechanism of Δ5 and Δ6-desaturation in the process of 22:6n-3 biosynthesis. The aim of the study described below was to investigate the inhibitory effects of curcumin and cyclopropene fatty acids on the desaturation and elongation of (n-3) fatty acids in trout hepatocytes and liver microsomes. In addition, the affinities of elongases, Δ6 and Δ5-desaturases for (n-3) and (n-6) substrates, were investigated in trout liver microsomes.

4.2. Materials and Methods

4.2.1. Materials

Cyclopropene fatty acids (>82% sterculic acid, 5% malvalic acid) were donated by Prof. J. Bézard (Unité de Recherche de Nutrition Cellulaire et Metabolique, Université de Bourgogne, Dijon, France). [1-^{14}C]Linolenic acid (54mCi/mmol) and [1-^{14}C]eicosapentaenoic acid (specific activity 58mCi/mmol) were purchased from Amersham International plc (Bucks., UK). [1-^{14}C]-Arachidonic acid (57.0 mCi/mmol), [1-^{14}C]-linoleic acid (53.0mCi/mmol), and [1-^{14}C]-eicosatrienoic acid (47mCi/mmol) were purchased from DuPont de Nemours (Dreiech, Germany). Curcumin and all the reagents used in the hepatocyte and microsomal incubations were purchased from SIGMA Chemicals Ltd.(Poole, Dorset, UK).

4.2.2. Experimental fish.

The holding, diet and feeding regime of juvenile rainbow trout (approx.80g) used in the followed experiments were described previously in section 2.2.2.

4.2.3. Preparation of trout hepatocytes and liver microsomes

Hepatocytes and liver microsomes from rainbow trout were isolated by the methods described previously for in sections 2.2.3 and 3.2.3, respectively.

4.2.4. Incubations with [^{14}C]-fatty acids

Freshly isolated hepatocytes and liver microsomes from rainbow trout were incubated with 9.3 nmoles (0.5μCi) of [1-^{14}C]-linolenic acid or 8.6nmoles (0.5μCi) [1-^{14}C]-eicosapentaenoic acid in the presence of 100μmolar of curcumin or 100μmolar cyclopropene fatty acids. Hepatocytes were incubated in 5 ml Medium 199 at a concentration of 2.5×10^6 cells ml^{-1} (section 2.2.5). The standard microsomal desaturation and elongation system (section 3.2.7) contained 3.0mg of microsomal protein in a total volume of 1.3ml. Curcumin was added to the incubations in ethanol

85%. Cyclopropane fatty acids and radiolabelled substrates were added to the incubation medium as their potassium salts bound to BSA.

To determine Δ6-desaturase activity for (n-3) and (n-6) PUFA substrates, the microsomes were incubated in the standard desaturation and elongation system (section 3.2.7) with 9.5 nmoles (0.5μCi) [1-^{14}C]-linoleic acid or 9.3 nmoles (0.5μCi) [1-^{14}C]-linolenic acid. For Δ5-desaturase activity the incubations were performed with 2.1nmoles (0.1μCi) [1-^{14}C]-20:3n-6 or 2.0 nmoles (0.1μCi) [3-^{14}C]-20:4n-3 (prepared according to the method in section 3.2.8 and containing 0.1μCi of [3-^{14}C]-20:4n-3 in 0.609μg of the fatty acid). In an experiment to determine elongation activity for (n-3) and (n-6) PUFA substrates, trout liver microsomes were incubated with either 9.5 nmoles (0.5μCi) [1-^{14}C]-linoleic acid or 9.3 nmoles (0.5μCi) [1-^{14}C]-linolenic acid, or 9.0 nmoles (0.5μCi) [1-^{14}C]-arachidonic acid or 8.6 nmoles (0.5μCi) [1-^{14}C]-eicosapentaenoic acid in the presence of 2mM KCN. All incubations contained 3.0mg of microsomal protein in 1.3ml of the standard desaturation system (section 3.2.7) and were carried out for 3 hours at 12°C.

4.2.5. Incorporation of radioactivity into PUFA from hepatocytes and liver microsomes.

The determination of the incorporated radioactivity from radiolabelled fatty acids in PUFA of hepatocytes and liver microsomes of trout, as well as the identification or radiolabelled PUFA products, were assessed by the methods described in sections 3.2.11 and 3.2.12.

4.2.6. Statistical analysis

The percentage distribution of incorporated radioactivity from radiolabelled fatty acids in PUFA of hepatocytes and liver microsomes are means of separate determinations of individual fish. The percentages were normalised by arcsin transformation and the significant differences between incubations were evaluated by

One Way Factorial ANOVA. The data were subjected to post hoc Scheffé's F test and significant differences between incubations were reported in Tables if $P<0.05$.

4.3. Results

4.3.1. Viability of trout hepatocytes in the presence of inibitors

The viability of trout hepatocytes incubated for 3 hours with curcumin or cyclopropene fatty acids (CPFA) is presented in Figure 4.1. No significant differences in percentage viability (assessed by Trypan Blue exclusion) were observed in hepatocytes incubated with inhibitors compared to controls.

4.3.2. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-18:3n-3 by trout hepatocytes

The effects of curcumin and CPFA on the desaturation and elongation of 18:3n-3 by hepatocytes isolated from trout are presented in Table 4.1. The rate of incorporation of radiolabelled 18:3n-3 into total lipids of hepatocytes from trout species was unaffected by the presence of either curcumin or CPFA in the culture medium. No significant differences were observed in the absolute amount of incorporated [1-^{14}C]-18:3n-3 into total lipid of hepatocytes from trout.

Trout hepatocytes incubated with CPFA retained a significantly higher proportion of recovered radioactivity as [1-^{14}C]-18:3n-3 (One Way ANOVA, $P<0.05$, Scheffé's F test) than hepatocytes incubated with curcumin or controls. No significant differences were observed on the proportion of radioactivity recovered in the individual "dead-end" elongation product of [1-^{14}C]-18:3n-3 between trout hepatocytes incubated with either inhibitors or control. Nevertheless, the proportion of radioactivity found among the total "dead-end" elongation products of 18:3n-3 in hepatocytes incubated with CPFA was significantly lower (One Way ANOVA, $P<0.05$, Scheffé's F test) than in the controls and in hepatocytes incubated with curcumin.

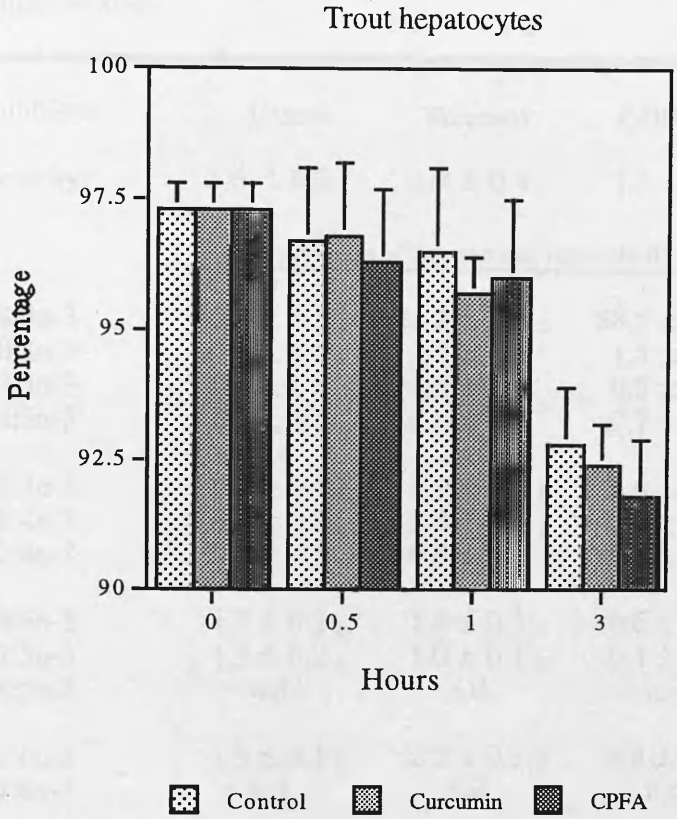

Figure 4.1. Viability (%) of hepatocytes isolated from rainbow trout and incubated for 3 hours in the presence of inhibitors. CPFA is the abreviation for cyclopropene fatty acids. Vertical bars indicated standard deviation of triplicates.

Table 4.1. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-18:3n-3 by hepatocytes isolated from rainbow trout.

Inhibitors	Control	Curcumin	CPFA
Activity†	1.6 ± 0.3	1.7 ± 0.4	1.5 ± 0.2
	% distribution of incorporated radioactivity		
18:3n-3	82.2 ± 0.5 a	83.2 ± 1.4 a	88.5 ± 0.5 b
20:3n-3	1.9 ± 0.0	2.1 ± 0.3	1.5 ± 0.2
22:3n-3	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
24:3n-3	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
18:4n-3	6.6 ± 0.4 a	5.1 ± 0.5 b	4.6 ± 0.2 b
20:4n-3	3.1 ± 0.1 a	3.2 ± 0.2 a	2.0 ± 0.0 b
22:4n-3	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.1
20:5n-3	1.7 ± 0.2 a	1.4 ± 0.3 a	0.6 ± 0.1 b
22:5n-3	1.3 ± 0.2 a	1.0 ± 0.1 a	0.4 ± 0.0 b
24:5n-3	n.d.	n.d.	n.d.
22:6n-3	1.5 ± 0.1 a	2.2 ± 0.1 b	0.8 ± 0.1 c
24:6n-3	n.d.	n.d.	n.d.
"Dead-end"	3.1 ± 0.2 a	3.4 ± 0.3 a	2.6 ± 0.4 b
Tetraenes	10.1 ± 0.5 a	8.8 ± 0.8 a	7.1 ± 0.2 b
Pentaenes	3.0 ± 0.2 a	2.4 ± 0.8 a	1.0 ± 0.1 b
Hexaenes	1.5 ± 0.1 a	2.2 ± 0.1 b	0.8 ± 0.1 c

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significant different (One Way ANOVA, $P<0.05$). Absence of a subscript indicates not significantly different. Trout hepatocytes were incubated for 3 hours at 12°C. CPFA, cyclopropene fatty acids.

The overall proportion, as well as the pattern of distribution of radioactivity from [1-^{14}C]-18:3n-3 in PUFA of trout hepatocytes incubated with curcumin was not significantly different to that in the control cells. Nevertheless, a significantly lower proportion of radioactivity was present in PUFA from curcumin than CPFA incubations. The levels of radioactivity from [1-^{14}C]-18:3n-3 in tetraenoic and pentaenoic PUFA found in hepatocytes incubated with CPFA were significantly lower (One Way ANOVA, $P<0.05$, Scheffé's F test) relative to control cells. The amount of radioactivity present in 20:5n-3 and 22:5n-3 in hepatocytes exposed to CPFA were respectively 2.5 and 3.4 times lower than in controls. Almost half as much radioactivity was found in 22:6n-3 of hepatocytes incubated with cyclopropenoic acids as in control cells.

4.3.3. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-20:5n-3 by trout hepatocytes

Table 4.2 shows the effects of curcumin and CPFA on the desaturation and elongation of [1-^{14}C]-20:5n-3 by hepatocytes from trout. No significant differences were observed in the incorporation rate of [1-^{14}C]-20:5n-3 between hepatocytes incubated with inhibitors and controls. Radiolabelled 20:5n-3 was significantly less (One Way ANOVA, $P<0.05$, Scheffé's F test) desaturated and elongated by trout hepatocytes incubated with CPFA compared to those incubated with curcumin or controls. The levels of radioactivity recovered in the elongation products of 20:5n-3 (22:5n-3 and 24:5n-3) were lowest in hepatocytes subjected to CPFA followed by those incubated with curcumin and were highest in controls. Similarly, significantly less radioactivity from [1-^{14}C]-20:5n-3 was recovered in the hexaenoic PUFA in hepatocytes incubated with CPFA than in the other two group of cells. The proportion of radioactivity recovered in 22:6n-3 of hepatocytes incubated with CPFA was 3 times lower than controls.

Table 4.2. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-20:5n-3 by hepatocytes isolated from rainbow trout.

Inhibitors	Control	Curcumin	CPFA
Activity†	2.1 ± 0.5	2.0 ± 0.7	1.9 ± 0.5
Fatty acid	% distribution of incorporated radioactivity		
20:5n-3	74.5 ± 0.6 $_a$	78.2 ± 0.7 $_b$	89.1 ± 1.3 $_c$
22:5n-3	15.8 ± 0.4 $_a$	14.8 ± 0.3 $_b$	7.3 ± 0.8 $_c$
24:5n-3	3.1 ± 0.2 $_a$	2.2 ± 0.3 $_b$	1.8 ± 0.2 $_b$
22:6n-3	4.4 ± 0.2 $_a$	3.2 ± 0.5 $_b$	1.4 ± 0.3 $_c$
24:6n-3	2.1 ± 0.4 $_a$	1.7 ± 0.2 $_b$	0.6 ± 0.2 $_c$
Pentaenes	93.5 ± 0.5 $_a$	95.1 ± 0.6 $_b$	98.1 ± 0.6 $_c$
Hexaenes	6.5 ± 0.5 $_a$	4.9 ± 0.6 $_b$	2.0 ± 0.4 $_c$

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significant different (One Way ANOVA, $P<0.05$). Absence of a subscript indicates not significantly different. Trout hepatocytes were incubated for 3 hours at 12°C. CPFA, cyclopropene fatty acids.

4.3.4. Effect of inhibitors on the desaturation and elongation of [1-^{14}C]-18:3n-3 by microsomes isolated from trout liver

Table 4.3 shows the effects of inhibitors on the conversion of [1-^{14}C]-18:3n-3 to other PUFA by liver microsomes from trout. Microsomes from trout exposed to curcumin converted significantly less (One Way ANOVA, $P<0.05$, Scheffé's F test) [1-^{14}C]-18:3n-3 (20.6% of the total incorporated radioactivity) than those incubated with either CPFA (31.9%) or controls (61.6%). The proportion of radioactivity distributed among the "dead-end" products of [1-^{14}C]-18:3n-3 in liver microsomes incubated with CPFA was significantly higher (One Way ANOVA, $P<0.05$, Scheffé's F test) than in the other two groups, particularly in 20:3n-3 which was nearly four times higher than in controls. Substantially less radioactivity from [1-^{14}C]-18:3n-3 was recovered in tetraenoic and pentaenoic PUFA in liver microsomes incubated with inhibitors. The proportion of label detected in 18:4n-3 and 20:4n-3 from trout liver microsomes incubated with CPFA was respectively 18 and 5 times lower than in controls. Among the pentaenoic PUFA, 20:5n-3 was the most labelled PUFA in all the three groups, with the lowest proportion of radioactivity found in microsomes incubated with CPFA. The proportion of label recovered in 20:5n-3 in control microsomes was 33.5 and 9 times higher than in those treated with CPFA and curcumin, respectively. The level of radioactivity present in the hexaenoic PUFA 24:6n-3 of trout liver microsomes was highest in the control group (16.7%) followed by those incubated with curcumin (0.9%) and CPFA (0.6%).

4.3.5. Effect of inhibitors on the desaturation and elongation of [1-^{14}C]-20:5n-3 by microsomes isolated from trout liver

The effects of curcumin and cyclopropenoic acids on the conversion of [1-^{14}C]-20:5n-3 to PUFA by liver microsomes from trout are shown in Table 4.4. The data show that significantly less (One Way ANOVA, $P<0.05$, Scheffé's F test)

Table 4.3. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-18:3n-3 by liver microsomes isolated from rainbow trout.

Inhibitors	Control	Curcumin	CPFA
Activity†	2.1 ± 0.2	1.9 ± 0.9	2.0 ± 0.2
Fatty acid	% distribution of incorporated radioactivity		
18:3n-3	38.4 ± 4.5 a	79.4 ± 6.5 b	68.1 ± 7.9 c
20:3n-3	6.3 ± 1.2 a	9.8 ± 2.4 a	23.5 ± 5.5 b
22:3n-3	1.6 ± 0.4	1.4 ± 0.8	2.0 ± 0.3
24:3n-3	1.9 ± 0.8	2.2 ± 0.5	2.2 ± 0.0
18:4n-3	11.0 ± 1.6 a	2.4 ± 1.2 b	0.6 ± 0.4 c
20:4n-3	8.6 ± 1.1 a	1.7 ± 0.5 b	1.8 ± 1.7 b
22:4n-3	1.5 ± 0.7 a	0.3 ± 0.1 b	0.5 ± 0.2 b
20:5n-3	13.4 ± 1.3 a	1.5 ± 0.5 b	0.4 ± 0.2 b
22:5n-3	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.1
24:5n-3	n.d.	n.d.	n.d.
22:6n-3	n.d.	n.d	n.d.
24:6n-3	16.7 ± 2.7 a	0.9 ± 0.4 b	0.6 ± 0.1 b
"Dead-end"	9.9 ± 2.5 a	13.4 ± 2.3 a	27.7 ± 4.6 b
Tetraenes	21.2 ± 3.4 a	4.8 ± 2.2 b	3.0 ± 1.1 b
Pentaenes	13.9 ± 2.8 a	1.5 ± 0.9 b	0.6 ± 0.3 c
Hexaenes	16.7 ± 2.7 a	0.9 ± 0.4 b	0.6 ± 0.1 b

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significant different (One Way ANOVA, $P<0.05$). Absence of a subscript indicates not significantly different. Trout hepatocytes were incubated for 3 hours at 12°C. CPFA, cyclopropene fatty acids.

[1-^{14}C]-20:5n-3 was converted to longer chain pentaenoic and hexaenoic PUFA by trout microsomes incubated with CPFA or curcumin compared to controls. The amount of radioactivity present in 22:5n-3 was highest in microsomes incubated with CPFA (50.4%), than in those with curcumin (36.7%) and lowest in the controls (14.1%). Conversely, the proportion of radioactivity found in 24:5n-3 was highest in controls (29.8%), followed by those incubated with curcumin (7.9%) and lowest in microsomes exposed to CPFA (3.8%). The radioactivity levels found in the hexaenoic PUFA 24:6n-3 of control microsomes was significantly (One Way ANOVA, $P<0.05$, Scheffé's F test) and at least 12 times higher than in those incubated with inhibitors.

4.3.6. Desaturase substrate specificity for (n-3) and (n-6) PUFA in trout liver microsomes

Table 4.5 compares the rates of conversion of [1-^{14}C]-18:2n-6 and [1-^{14}C]-18:3n-3 to PUFA by liver microsomes from rainbow trout. There were no significant differences between (n-3) and (n-6) C_{18}-substrates the total radioactivity incorporated into microsomal lipids. Nevertheless, significantly more [1-^{14}C]-18:3n-3 than [1-^{14}C]-18:2n-6 (29.4% and 7.2%, respectively of the total recovered radioactivity) (One Way ANOVA, $P<0.05$, Scheffé's F test) was desaturated by the microsomes to longer chain PUFA. No significant differences were observed in the proportion of radioactivity distributed among the C_{20} "dead-end" elongation products from either of the two C_{18}-substrates. However, the proportions of radioactivity recovered in C_{22} "dead-end" elongation products were substantially higher for (n-6) than for (n-3) PUFA. Approximately 5 times more radioactivity from [1-^{14}C]-18:3n-3 was recovered as the Δ6-desaturase product (18:4n-3) than in the product from [1-^{14}C]-18:2n-6 (18:3n-6). The amount of radioactivity from [1-^{14}C]-18:3n-3 present in the C_{20} elongation product (20:4n-3) of microsomal lipids was significantly higher than in the product from [1-^{14}C]-18:2n-6 (20:3n-6). The PUFA

Table 4.4. Effects of inhibitors on the desaturation and elongation of [1-^{14}C]-20:5n-3 by liver microsomes isolated from rainbow trout.

Inhibitors	Control	Curcumin	CPFA
Activity†	2.0 ± 0.3	2.0 ± 0.6	2.1 ± 0.5
Fatty acid	% distribution of incorporated radioactivity		
20:5n-3	28.3 ± 5.3 $_a$	53.1 ± 3.7 $_b$	44.8 ± 3.9 $_c$
22:5n-3	14.1 ± 3.5 $_a$	36.7 ± 3.3 $_b$	50.4 ± 6.5 $_c$
24:5n-3	29.8 ± 4.3 $_a$	7.9 ± 1.6 $_b$	3.8 ± 1.3 $_c$
22:6n-3	n.d.	n.d.	n.d.
24:6n-3	27.8 ± 2.8 $_a$	2.3 ± 1.2 $_b$	1.0 ± 0.8 $_b$
Pentaenes	72.2 ± 6.4 $_a$	97.7 ± 6.6 $_b$	99.0 ± 6.8 $_b$
Hexaenes	27.8 ± 2.4 $_a$	2.3 ± 1.1 $_b$	1.0 ± 0.5 $_c$

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significant different (One Way ANOVA, $P<0.05$). Absence of a subscript indicates not significantly different. Trout hepatocytes were incubated for 3 hours at 12°C. CPFA, cyclopropene fatty acids.

Table 4.5. Distribution of radioactivity from radiolabelled (n-3) and (n-6) C_{18} fatty acids in PUFA of liver microsomes isolated from rainbow trout.

C_{18}-Substrates	[1-^{14}C]-18:3n-3	[1-^{14}C]-18:2n-6
Activity†	1.9 ± 0.4	1.9 ± 0.7
PUFA products	% of incorporated radioactivity	
Substrate	57.5 ± 3.4 a	79.7 ± 2.1 b
C_{20} "dead-end"	12.8 ± 0.7	11.5 ± 0.8
C_{22} "dead-end"	0.3 ± 0.3 a	1.5 ± 0.3 b
C_{24} "dead-end"	0.1 ± 0.1 a	n.d. b
C_{18} Δ6-desaturation	6.6 ± 0.5 a	1.4 ± 0.7 b
C_{20} elongation	8.7 ± 3.1 a	4.1 ± 0.7 b
C_{22} elongation	n.d.	n.d.
C_{20} Δ5-desaturation	8.9 ± 1.4 a	0.5 ± 0.1 b
C_{22} elongation	3.1 ± 1.1 a	0.4 ± 0.1 b
C_{24} elongation	0.6 ± 0.1	0.5 ± 0.1
C_{24} Δ6-desaturation	1.5 ± 0.6 a	0.3 ± 0.1 b

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significantly different (One Way ANOVA, $P<0.05$). Absence of a subscription indicates no significant difference. The notation "CxΔy desaturation" indicate that the fatty acid contained "x" carbons in the acyl chain and was subjected to a desturation reaction at position Δ"y". The notation "Cx elongation" or "Cx dead-end" indicate that the fatty acid was elongated to "x" number of carbons in the acyl chain.

products of Δ5-desaturation and C_{22} elongation from [1-^{14}C]-18:3n-3 were also found to contain significantly more radioactivity than those derived from [1-^{14}C]-18:2n-6.

When liver microsomes from trout were also incubated with [3-^{14}C]-20:4n-3 or [1-^{14}C]-20:3n-6 to determine Δ5-desaturase activity between the two series of PUFA, more [3-^{14}C]-20:4n-3 was significantly (One Way ANOVA, $P<0.05$, Scheffé's F test) converted to longer chain PUFA (57.7%) by trout liver microsomes than [1-^{14}C]-20:3n-6 (22.3%) (Table 4.6). The level of radioactivity recovered as the Δ6-desaturase products (*i.e.*: 24:5n-6 and 24:6n-3) was substantially higher in microsomes incubated with [3-^{14}C]-20:4n-3 than with [1-^{14}C]-20:3n-6.

4.3.7. Elongation activity with (n-3) and (n-6) PUFA in trout liver microsomes

The elongation activity in trout liver microsomes for (n-3) and (n-6) PUFA was determined by incubating the liver microsomes with radiolabelled fatty acids in the presence of KCN. Figure 4.2 shows autoradiograms from $AgNO_3$-TLC plates indicating that the desaturation of radiolabelled fatty acids was completely inhibited by the presence of KCN. The bands of radioactive elongation products were identified (section 4.2.9) and are shown on the profiles. The data in Table 4.7 show in general that (n-3) PUFA substrates were significantly (One Way ANOVA, $P<0.05$, Scheffé's F test) more elongated by liver microsomes than (n-6) PUFA substrates. Nearly twice as much radioactivity was recovered among the total elongation products of [1-^{14}C]-18:3n-3 compared to [1-^{14}C]-18:2n-6, whereas within the C_{20}-substrates 2.5 times more radiolable was found among the total elongation products of [1-^{14}C]-20:5n-3 than in those derived from [1-^{14}C]-20:4n-6.

4.4. Discussion

The present results establish that cell viability as well as the esterification activity of all radiolabelled fatty acids into trout hepatocyte lipid *in vitro* is unaffected

Table 4.6. Distribution of radioactivity from radiolabelled (n-3) and (n-6) C_{20} fatty acids in PUFA of liver microsomes isolated from rainbow trout.

C_{20}-Substrates	^{14}C-20:4n-3	^{14}C-20:3n-6
Activity†	0.9 ± 0.3	1.0 ± 0.4
PUFA products	% of incorporated radioactivity	
Substrate	38.2 ± 1.0 a	72.2 ± 2.1 b
C_{22} elongation	4.3 ± 0.6	5.5 ± 0.6
C_{20} Δ5-desaturation	24.6 ± 0.7 a	15.5 ± 2.9 b
C_{22} elongation	6.6 ± 1.3	5.8 ± 1.0
C_{24} elongation	3.4 ± 1.0 a	1.0 ± 0.2 b
C_{24} Δ6-desaturation	22.8 ± 1.0 a	n.d. b

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significantly different (One Way ANOVA, $P<0.05$). Absence of a subscription indicates no significant difference. The notation "CxΔy desaturation" indicate that the fatty acid contained "x" carbons in the acyl chain and was subjected to a desturation reaction at position Δ"y". The notation "Cx elongation" or "Cx dead-end" indicate that the fatty acid was elongated to "x" number of carbons in the acyl chain.

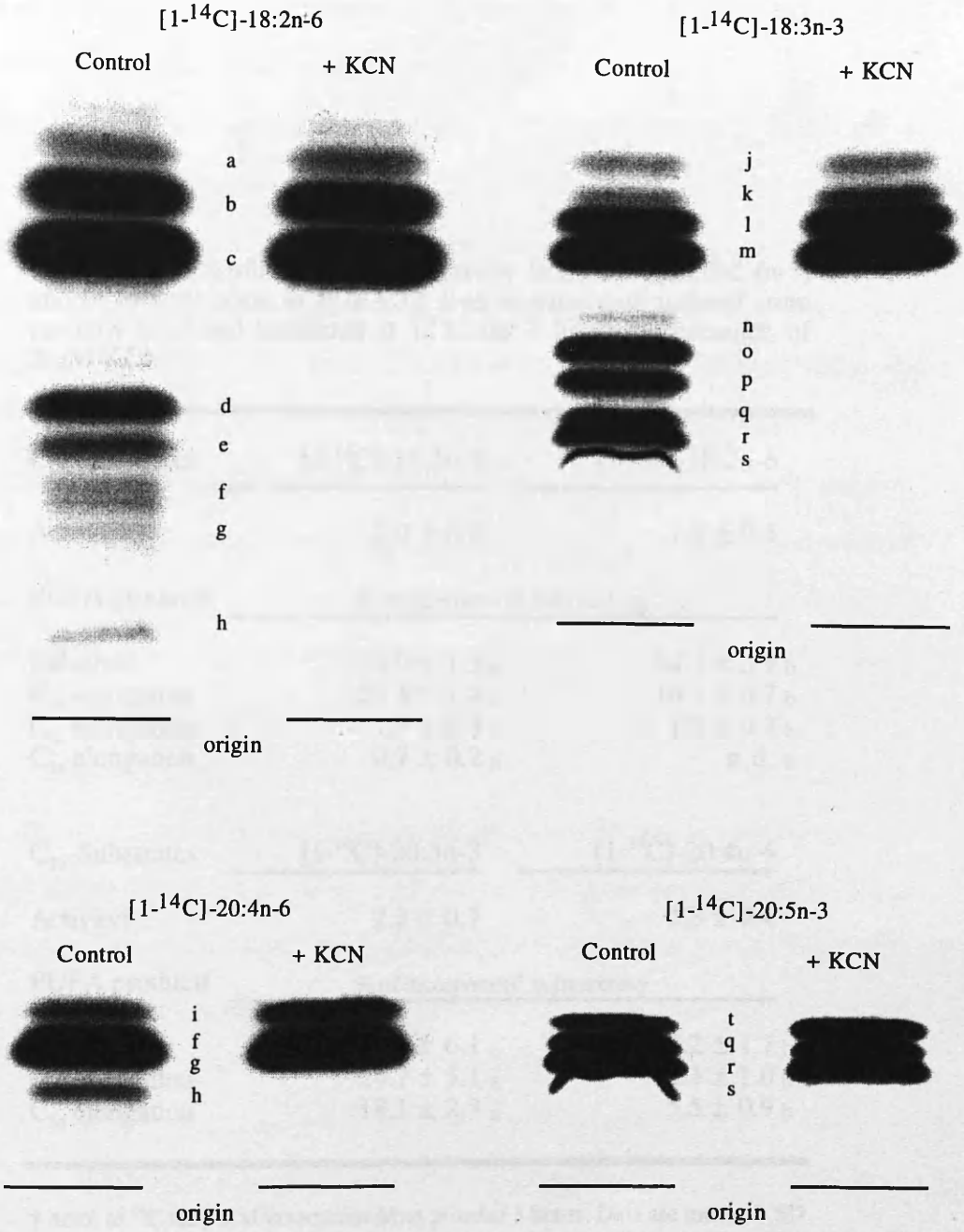

Figure 4.2. Autoradiograms of radiolabelled PUFA generated by liver microsomes from trout incubated with C_{18} and C_{20} radioactive substrates in the presence of KCN. a, 22:2n-6; b, 20:2n-6; c, 18:2n-6; d, 20:3n-6; e, 18:3n-6; f, 22:4n-6; g, 20:4n-6; h, 24:5n-6; i, 24:4n-6; j, 24:3n-3; k, 22:3n-3; l, 20:3n-3; m, 18:3n-3; n, 22:4n-3; o, 20;4n-3; p, 18:4n-3; q, 22:5n-3; r, 20:5n-3; s, 24:6n-3; t, 24:5n-3.

Table 4.7. Distribution of radioactivity from radiolabelled (n-3) and (n-6) fatty acids in PUFA of liver microsomes isolated from rainbow trout and incubated at 12°C for 3 hs in the presence of 2mM KCN.

C_{18}-Substrates	[1-^{14}C]-18:3n-3	[1-^{14}C]-18:2n-6
Activity†	2.0 ± 0.3	1.9 ± 0.5
PUFA products	% of incorporated radioactivity	
Substrate	74.9 ± 1.3 $_a$	84.4 ± 0.9 $_b$
C_{20} elongation	22.4 ± 1.8 $_a$	14.3 ± 0.7 $_b$
C_{22} elongation	1.9 ± 0.3 $_a$	1.3 ± 0.2 $_b$
C_{24} elongation	0.7 ± 0.2 $_a$	n.d. $_b$
C_{20}-Substrates	[1-^{14}C]-20:5n-3	[1-^{14}C]-20:4n-6
Activity†	2.2 ± 0.7	2.3 ± 0.4
PUFA products	% of incorporated radioactivity	
Substrate	57.2 ± 6.1 $_a$	82.2 ± 1.7 $_b$
C_{22} elongation	24.7 ± 5.1 $_a$	12.3 ± 1.0 $_b$
C_{24} elongation	18.1 ± 2.3 $_a$	5.5 ± 0.9 $_b$

† nmol of ^{14}C fatty acid incorporated/mg protein/ 3 hours. Data are means ± SD (n=3 fish). Different subscript letters denote significantly different (One Way ANOVA, $P<0.05$). Absence of a subscript indicates no significant difference. The notation "Cx elongation" indicate that the fatty acid was elongated to "x" number of carbons in the acyl chain.

by the presence of either curcumin or cyclopropene fatty acids (CPFA) at the concentrations employed here.

The results also demonstrate that the overall desaturation and elongation activities in hepatocytes and liver microsomes from rainbow trout were markedly depressed in the presence of either inhibitor. Several studies have reported that rainbow trout is extremely sensitive to the effects of cyclopropenoid fatty acids (Malevski *et al.*, 1974; Struthers *et al.*, 1975; Loveland *et al.*, 1979; Hendricks *et al.*, 1980; Selivonchick *et al.*, 1981; Chikwem 1987). For example, when feeding rainbow trout a diet containing CPFA, Selivonchick *et al.* (1981) observed that liver cells contained grossly altered membranes, particularly confined to a degranulation of the rough-surfaced endoplasmic reticulum. Protein metabolism is also affected by CPFA as Malevski *et al.* (1974) demonstrated that a lower liver protein concentration as well as a decrease in the incorporation of ^{14}C-labelled amino acids into liver proteins occurred in trout fed CPFA.

The Δ6-desaturation activity (using 18:3n-3 as substrate) in trout hepatocytes was inhibited by 39% in the presence of CPFA and 8% in the presence of curcumin. Furthermore, the conversion of [1-^{14}C]-20:5n-3 to 22:6n-3 *via* C_{24}-PUFA intermediates by trout hepatocytes was inhibited by 69% in the presence of CPFA and 25% when exposed to curcumin. These results indicate that CPFA are a more effective inhibitor than curcumin for both Δ6-desaturase reactions in trout hepatocytes. Moreover, these results indicated that the formation of 22:6n-3 from 20:5n-3 *via* Δ6-desaturation of 24:5n-3 in hepatocytes was more susceptible to inhibition by both inhibitors than the Δ6-desaturase reaction using 18:3n-3 as substrate. The relatively high reduction of 22:6n-3 formation from 20:5n-3 by the inhibitors in trout cells, particularly by CPFA, might be partially associated with the depletion of 24:5n-3, the Δ6-desaturase substrate, resulting from the inhibition of the preceding elongation steps. However, the results showed that elongation reactions, especially the formation of "dead-end" products in trout hepatocytes was unaffected by either of the two inhibitors. A previous study performed with rat hepatocytes

demonstrated that Δ5-desaturation was significantly inhibited by curcumin and suggested that the inhibitory action of curcumin on hepatocytes was not entirely specific to desaturation but also to acylation and elongation reactions (Fujiyama-Fujiwara *et al.*, 1992). The lower levels of radioactivity from [1-^{14}C]-18:3n-3 found in pentaenoic PUFA of hepatocytes incubated with inhibitors suggests that Δ5-desaturation activity was inhibited in this study.

The inhibitory effect of curcumin and CPFA on the conversion of [1-^{14}C]-18:3n-3 or [1-^{14}C]-20:5n-3 to longer-chain PUFA in liver microsomes isolated from trout was substantially more accentuated than in the case of intact hepatocytes. Around 90% inhibition of the Δ6-desaturation activity (18:3n-3 ➡ longer chain PUFA) occurred in the presence of either curcumin or CPFA in trout microsomes, whilst the microsomal Δ5-desaturation activity was reduced by at least 90% with curcumin and by 93% with CPFA. The formation of tetracosahexaenoic acid (24:6n-3) from [1-^{14}C]-20:5n-3 in liver microsomes of trout was inhibited by at least 92% when exposed to either of the two inhibitors. These observations are in agreement with the results of previous studies which have shown that Δ9, Δ6 and Δ5-desaturation activities in liver microsomes from rat were all highly inhibited in the presence of CPFA (Raju and Reiser 1967; Andrianaivo-Rafehivola *et al.*, 1993; Cao *et al.*, 1993) or curcumin (Shimizu *et al.*, 1992; Kawashima *et al.*, 1996). Shimizu *et al.*, (1992) observed that curcumin added to microsomal incubations at a concentration of 100μmolar was effective in inhibiting Δ5-desaturase by 50% and Δ6-desaturase by 20%.

The lower inhibitory effect of CPFA and curcumin in intact hepatocytes from trout compared to the inhibition in microsomal incubations might be related to the non-availability of the inhibitors in the intact cells. It is possible that curcumin and CPFA could have been metabolised by microsomal or/and cytoplasmic detoxification enzymes, such as glutathione S-transferase (GST), glutathione peroxidases and cytochrome P_{450}. A recent study has demonstrated that curcumin among other natural anticarcinogens is effective in inducing GST activity in liver and intestine of rats

(Nijhoff *et al.*, 1993). Other investigators (Reddy and Lokesh 1994) have demonstrated that the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione transferase were all higher in liver homogenates of rats fed curcumin than in controls. In trout, CPFA are known to reduce the concentration of liver microsomal cytochrome P_{450} as well as the activity of NADPH cytochrome c reductase (Loveland *et al.*, 1979).

The degrees and patterns of inhibition of CPFA and curcumin on Δ6-desaturation activity using either [1-^{14}C]-18:3n-3 or [1-^{14}C]-20:5n-3 were very similar. This provides evidence that the Δ6-desaturation mechanism in both reactions (18:3n-3 ➡ 18:4n-3 and 24:5n-3 ➡ 24:6n-3) is similar, at least in terms of susceptibility to inhibitors. Since the inhibitory effects of CPFA and curcumin are not entirely specific to Δ6-desaturase, it is difficult to characterise in terms of inhibitory response the metabolic similarities and features of the two Δ6-desaturation reactions. It was observed in microsomal incubations from trout liver that CPFA were more effective in inhibiting the reaction 24:5n-3 ➡ 24:6n-3 than the reaction 18:3n-3 ➡ 18:4n-3, which indicated that fatty acid chain length is an important structural factor of the acyl molecules competing for the active site(s) of the desaturase(s). Nevertheless, the endogenous concentration of radiolabelled substrates 18:3n-3 and 24:5n-3 available for these reactions were unlikely to be equal at proportions which precludes conclusions being drawn regarding the specificity of Δ6-desaturase in the two pathways. Studying the chain length specificity for Δ6-desaturase in rat liver microsomes, Geiger *et al.* (1993) found that, when an enzyme-saturating level of [3-^{14}C]-24:5n-3 was incubated with 120nmol of [1-^{14}C]-18:3n-3, the production of 24:6n-3 was inhibited by 81%, whereas when [1-^{14}C]-18:3n-3 was incubated with 120nmol [3-^{14}C]-24:5n-3, the formation of 18:4n-3 was inhibited by only 27%, showing preferential Δ6-desaturation of 18:3n-3 rather than 24:5n-3.

The results described above showed that the Δ6-desaturase activity in trout liver microsomes was 5-fold higher with 18:3n-3 than with 18:2n-6 as substrate, whereas the Δ5-desaturase the activity with 20:4n-3 was nearly twice as high as with

20:3n-6. A problem in estimating Δ5-desaturase activity using radiolabelled C_{18}-substrates arises from the availability of the Δ5 substrates (*i.e.:* 20:4n-3 and 20:3n-6) generated *in situ* from [1-^{14}C]-18:3n-3 and [1-^{14}C]-18:2n-6 are dependent essentially on the preceding C_{18} Δ6-desaturase and C_{20} elongation activities. For instance, the Δ5-desaturase activity for (n-3)PUFA estimated indirectly in the present study from 18:3n-3 was 18-fold higher than for 18:2n-6, whereas when radiolabelled 20:3n-6 and 20:4n-3 were used as direct substrates the difference in activity between the two series was only 2-fold. Consequently, false estimations of Δ5-desaturase activity may have been obtained in the past, particularly for the (n-3) series, since radiolabelled 20:4n-3 is not available commercially.

Studies performed with isolated hepatocytes from thermally acclimated rainbow trout have demonstrated that, irrespective of the temperature to which the fish were acclimated, the desaturation and elongation activities were always higher for (n-3) PUFA than for (n-6) substrates (Sellner and Hazel, 1982ab). Comparing the desaturation and chain elongation of essential fatty acids in hepatocytes of rainbow trout and rat, Hagve *et al.* (1986) observed that a larger fraction of the substrate was Δ6-desaturated with 18:3n-3 than with 18:2n-6. Similarly, this Δ6-desaturase substrate preference has been found in fish cell lines (Tocher and Dick 1990; Tocher and Sargent 1990; Tocher 1993) and in human CaCo-2 cell lines (Chen and Nilsson, 1993). Several injection studies using C_{18} and C_{20} (n-3) and (n-6) radiolabelled substrates performed in marine (Mourente and Tocher 1993ab; Linares and Henderson 1991) and freshwater fish (Olsen *et al.*, 1990; Henderson *et al.*, 1995) have also reported that both Δ6 and Δ5-desaturase activities were higher with (n-3) than (n-6) PUFA substrates.

The differences observed in this study regarding the activity of the desaturation enzymes in microsomes with different PUFA substrates are in agreement with observations reported previously by others. Ninno *et al.*, (1974) observed that the order of microsomal Δ6-desaturation activity in freshwater fish *(Pimelodus maculatus)* and rat was 18:3n-3>18:2n-6>18:1n-9. The presence of radiolabelled

(n-6) C_{24}-PUFA among the desaturation and elongation products of 20:3n-6 is evidence that the formation of 22:5n-6 in trout proceeds *via* Δ6-desaturation of 24:4n-6 to 24:5n-6, in a similar manner to (n-3) PUFA. In fact, Mohammed *et al.*, (1995) have recently established that the pathway for the metabolism of 22:5n-6 in rat liver proceeds in a similar way to that proposed for 22:6n-3 by Voss *et al.* (1991).

The use of KCN in microsomal incubations allowed the evaluation of elongation activity without the interference of the desaturation system. It is well known that microsomal stearoyl coenzyme A desaturation is an aerobic, cytochrome-dependent process and, therefore, very sensitive to cyanide (Holloway and Katz 1972; Hiwatashi *et al.*, 1975). The desaturation of all the radiolabelled substrates used in the experiments described above was completely inhibited by KCN, which is consistent with desaturation system in trout liver involving the complex of NADH-cytochrome b_5 oxidoreductase and cytochrome b_5, and utilising molecular oxygen (Holloway and Katz 1972). The elongation activities found here for all C_{18} and C_{20} PUFA substrates examined were significantly higher with (n-3) than with (n-6) PUFA substrates. Higher elongation activities for (n-3) PUFA substrates and particularly the formation of "dead-end" products of 18:3n-3 have also been reported previously for isolated trout hepatocytes (Sellner and Hazel 1982b). Likewise, in a study of the PUFA metabolism in primary astroglial cells prepared from turbot, Tocher (1993) found that elongation activity predominated over desaturation, particularly with [1-^{14}C]-18:3n-3 as a substrate which led to the production of significant amounts of labelled 20:3n-3, 22:3n-3 and 24:3n-3 ("dead-end" products). Several lines of evidences have indicated that the microsomal fatty acid elongation system in mammals is a multiple condensing enzyme system with different chain-length specificity and connected to a single or multiple pathways (Cinti *et al.*, 1992). The mechanism and regulation of microsomal fatty acid elongation system are not yet understood.

CONCLUDING REMARKS

Although rainbow trout, like all vertebrates are not able to synthesise linoleic (18:2n-6) and linolenic acid (18:3n-3) from oleic acid (18:1n-9), their ability to desaturate and elongate these C_{18}-PUFA has been confirmed in the current study. At the same time, details of the biosynthetic pathway leading to the formation of 22:6n-3 have been established.

The present study demonstrated that primary culture of hepatocytes from rainbow trout is a suitable *in vitro* system to investigate the biosynthesis of PUFA in fish. Trout hepatocytes incubated at 12°C in an appropriate balanced salt medium containing a low concentration of serum were viable and metabolically active for several days. Radiolabelled 18:3n-3 to these cells could be efficiently incorporated into cellular lipid and rapidly desaturated and elongated into longer-chain and more unsaturated fatty acids, including 20:5n-3 and 22:6n-3. The technique of argentation thin-layer chromatography combined with radio-gas chromatography was found to be appropriate for this study, revealing clearly all the radiolabelled metabolites derived from the conversion of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 and involved in the synthesis of 22:6n-3.

The diagram in Figure 5.1 shows all the products generated from the desaturation and elongation of 18:3n-3 by trout hepatocytes. The high proportion of radioactivity recovered as "dead-end" products of 18:3n-3 (i.e.: 20:3n-3, 22:3n-3 and 24:3n-3), found predominantly in the hepatocyte neutral lipid fraction, indicates that trout hepatocytes contain active elongases responsible for modifying dietary C_{18}-PUFA into longer-chain fatty acids, especially when these substrates are provided in excess. It is possible that the "dead-end" products stored in the neutral lipid fraction can be further retroconverted for subsequent utilisation by desaturating and elongating enzymes. As illustrated in Figure 5.1 linolenic acid (18:3n-3) is desaturated at position 6 by a Δ6-desaturase to form 18:4n-3 which is rapidly elongated to 20:4n-3

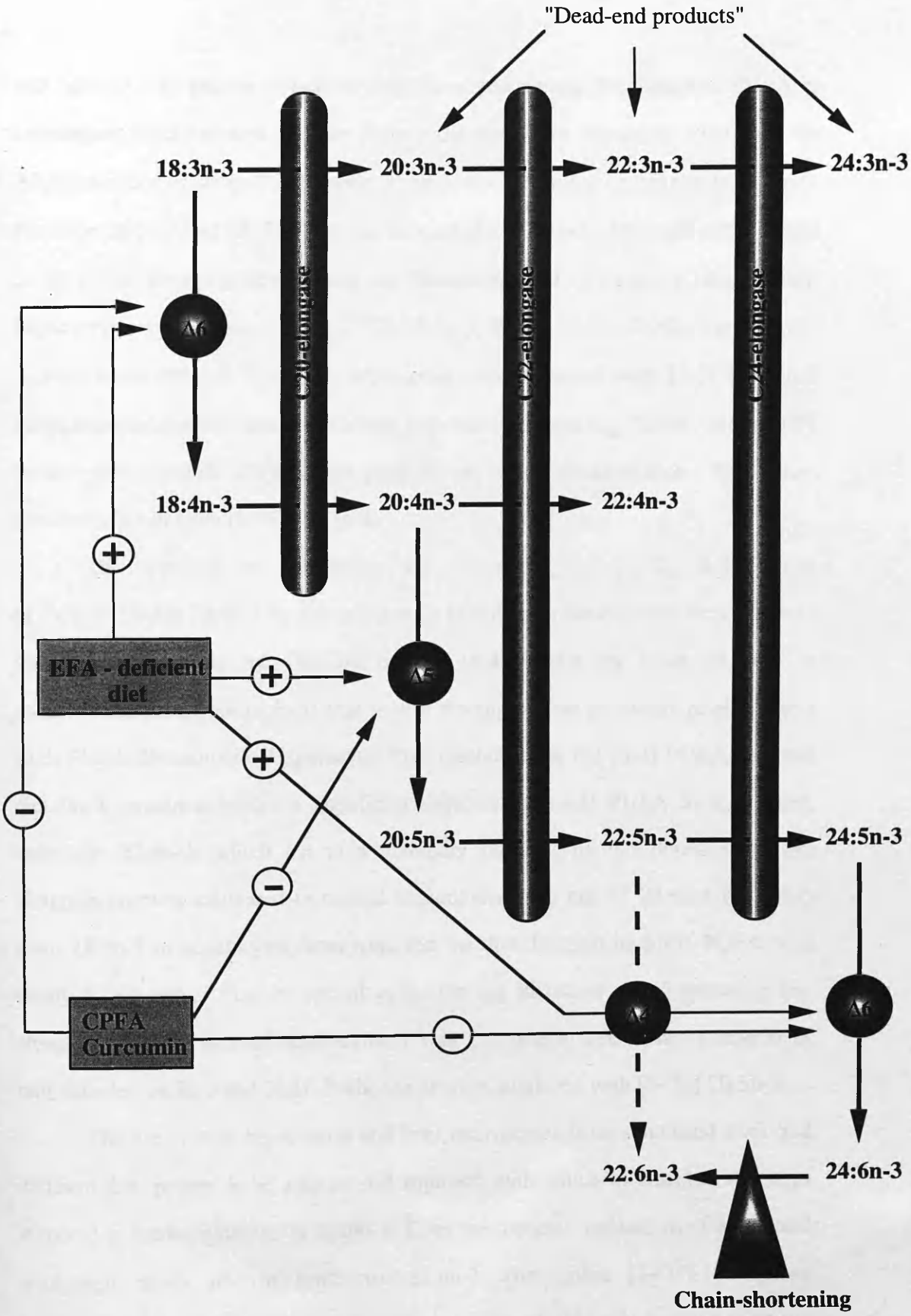

Figure 5.1. Metabolic pathway for the biosynthesis of 22:6n-3 from 18:3n-3 in rainbow trout hepatocytes. CPFA, cyclopropene fatty acids; EFA, essential fatty acids.

and 22:4n-3. The pattern of radioactivity recovered among the tetraenoic PUFA in hepatocytes lipid revealed that the main route for these tetraenoic PUFA is the Δ5-desaturation of 20:4n-3 to generate 20:5n-3, rather than the elongation to 22:4n-3. Eicosapentaenoic acid (20:5n-3) is then elongated to 22:5n-3. Although radiolabelled 22:6n-3 was always present among the desaturated and elongated products when hepatocytes were incubated with [1-^{14}C]-18:3n-3, the C_{24}-PUFA, 24:5n-3 and 24:6n-3, were never detected. However, when cells were incubated with [1-^{14}C]-20:5n-3 substantial amounts of radioactivity were recovered as these C_{24}-PUFA, presumably because the substrate 20:5n-3 was provided at higher concentrations than when generated *in situ* from [1-^{14}C]-18:3n-3.

The approach of stimulating the conversion of [1-^{14}C]-18:3n-3 and [1-^{14}C]-20:5n-3 to 22:6n-3 by removing (n-3) PUFA from the rainbow trout diet was successful. Replacing the fish oil rich in (n-3) PUFA by olive oil rich in monosaturates and poor in (n-3) PUFA was the appropriate procedure to elaborate a (n-3) PUFA-deficient diet. Hepatocytes from rainbow trout fed (n-3) PUFA deficient diet for 3 months exhibited a significant depletion of (n-3) PUFA in their lipid, especially 22:6n-3, which led to a markedly increase of the desaturation and elongation activity compared to control hepatocytes. The rate of 22:6n-3 formation from 18:3n-3 in hepatocytes from trout fed the diet deficient in (n-3) PUFA was nearly 6-fold higher than in control cells. Having increased the desaturation and elongation activity in trout hepatocytes it was possible to detect the formation of radiolabelled 24:5n-3 and 24:6n-3 when cells were incubated with [1-^{14}C]-20:5n-3.

The use of both hepatocytes and liver microsomes from stimulated trout (fed deficient diet) proved to be a successful approach with which to elucidate the steps involved in the biosynthesis of 22:6n-3. Liver microsomes isolated from stimulated trout were never able to synthesise 22:6n-3 from either [1-^{14}C]-18:3n-3 or [1-^{14}C]-20:5n-3, which indicated that the process of 22:6n-3 formation require another cellular organelle or organelles to addition of endoplasmic reticulum. Instead, microsomes incubated with radiolabelled PUFA substrates, particularly

[1-^{14}C]-20:5n-3, were capable of accumulating considerably amounts of radiolabelled 24:5n-3 and 24:6n-3 in their lipids under the experimental conditions employed. To establish that the biosynthesis of 22:6n-3 proceeded *via* these C_{24}-PUFA intermediates, the radiolabelled 24:5n-3 and 24:6n-3 were isolated from microsomal incubations by argentation chromatography, purified and used as substrates in incubations with intact hepatocytes from stimulated trout. The recovery of radioactivity in 22:6n-3 from both radiolabelled 24:5n-3 and 24:6n-3 was conclusive evidence that the biosynthesis of 22:6n-3 in fish can proceed by the same pathway as proposed for mammals (Voss *et al.*, 1991), involving a Δ6-desaturation of 24:5n-3 to 24:6n-3 followed by the chain shortening of the latter to generate 22:6n-3.

The study of the regulation of the desaturation and elongation activity is crucial in understanding the mechanism involved in the biosynthesis of PUFA. It was demonstrated here that Δ6 and Δ5-desaturases and elongases from trout liver microsomes exhibited a higher affinity for (n-3) PUFA substrates than for (n-6) PUFA. Moreover, it was demonstrated that the activities of Δ6 and Δ5-desaturases in trout liver are highly sensitive to the effects of plant inhibitors such as cyclopropene fatty acids and curcumin. Because of the lack of specificity of these inhibitors for Δ6-desaturase, it was not possible to draw clear conclusions in relation to the Δ6-desaturases involved in the reactions 18:3n-3 ➡ 18:4n-3 and 24:5n-3 ➡ 24:6n-3. However, it was found that the desaturation of 24:5n-3 in trout microsomes was substantially more affected than the desaturation of 18:3n-3, which suggests that fatty acid substrate chain length might play an important role in determining the enzyme activity.

Finally, further studies are required on the role of peroxisomes in the biosynthesis of 22:6n-3, particularly in relation to how the transport of PUFA between microsomes and peroxisomes proceeds and which cytosolic or membrane factors are involves in this mechanism. Further investigation of the relative affinities of the Δ6-desaturase for C_{18} and C_{24} substrates and its inhibition by long chain PUFA is also required to understand the regulatory mechanism involved in the biosynthesis

of 22:6n-3. The establishment of whether this pathway for the biosynthesis of 22:6n-3 is ubiquitous in nature is also a relevant subject for further study. This study extends the evidence suggesting that it may be ubiquitous in vertebrates.

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