review

Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids

Howard Sprecher,[1] Devanand L. Luthria, B. S. Mohammed, and Svetla P. Baykousheva

Department of Medical Biochemistry, The Ohio State University, Columbus, OH 43210

Abstract Recent studies refute the commonly accepted, but untested, hypothesis that 7,10,13,16-22:4 and 7,10,13,16,19-22:5 are desaturated at position 4 by a microsomal acyl-CoA-dependent desaturase. The synthesis of 4,7,10,13,16,19-22:6 occurs via the following reaction sequence: 7,10,13,16,19-22:5→ 9,12,15,18,21-24:5 → 6,9,12,15,18,21-24:6® 4,7,10,13,16,19-22:6. The synthesis of 4,7,10,13,16-22:5 from 7,10,13,16-22:4 takes place via an analogous pathway. According to these pathways the 24-carbon acids that are made in the endoplasmic reticulum move to a site for partial β-oxidation, which is most likely peroxisomes. The products of partial β-oxidation, 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6, then move back to the endoplasmic reticulum where they are used as substrates for membrane lipid biosynthesis. The ability of a fatty acid to serve as a substrate for continued peroxisomal β-oxidation, versus its transfer out of peroxisomes for subsequent endoplasmic reticulum-associated esterification reactions, may be an important control for regulating membrane lipid fatty acid composition. Indeed, the revised pathways of polyunsaturated fatty acid biosynthesis imply that there is considerable intracellular movement and recycling of fatty acids between peroxisomes and the endoplasmic reticulum. In addition, these revised pathways require that two 18-carbon and two 24-carbon acids are substrates for desaturation at position 6. Also, as linoleate and linolenate are metabolized, respectively, to 6,9,12,15,18-24:5 and 6,9,12,15,18,21-24:6, three n–6 acids and three n–3 acids are substrates for malonyl-CoA dependent chain elongation. It remains to be determined how many microsomal enzymes are required to carry out these reactions and whether other ancillary enzymes are expressed in tissues whose membrane lipids accumulate very long-chain polyunsaturated acids with up to 36 carbon atoms.–**Sprecher, H., D. L. Luthria, B. S. Mohammed, and S. P. Baykousheva.** Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J. Lipid Res.* 1995. **36:** 2471–2477.

Supplementary key words peroxisomes • microsomes • acylation • desaturation • chain elongation • polyunsaturated fatty acids

INTRODUCTION

In animals, it is well established that dietary linoleate and linolenate are the respective precursors of long-chain n–6 and n–3 fatty acids. It has generally been assumed that endoplasmic reticulum-associated 6-, 5-, and 4-desaturases are required to metabolize linoleate and linolenate, respectively, to 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6. The fatty acids in these metabolic pathways are then esterified into acceptors to yield neutral lipids and phospholipids. In liver, the synthesis of glycerol-containing lipids is also localized primarily in the endoplasmic reticulum. The biosynthesis of polyunsaturated fatty acids and their subsequent esterification into acceptors may be viewed as two separate, but perhaps coupled, metabolic processes. The primary purpose of this review is to present evidence showing that the endoplasmic reticulum does not contain an acyl-CoA-dependent 4-desaturase. The biosynthesis of both 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6 and their subsequent esterification into acceptors requires intracellular communication between the endoplasmic reticulum and a site for partial β-oxidation which is probably peroxisomes.

DESATURASE ENZYMES

In evaluating the validity of the pathways of polyunsaturated fatty acid biosynthesis, it is appropriate to briefly discuss what is known about the proteins that catalyze position-specific desaturation reactions. It is the reviewer's opinion that much of what we presume to know about these reactions is based primarily on information extrapolated from studies on the 9-desaturase that converts stearoyl-CoA to oleoyl-CoA. In 1965 Schroepfer and Bloch (1) showed that 9-D-proton removal from stearic acid was the rate-limiting step in oleate synthesis. In 1974 Stritmatter et al. (2) purified a 9-desaturase from rat liver and showed that it was a single polypeptide of 53,000 daltons containing one atom of non-heme iron. Desaturation of stearoyl-CoA

[1]To whom correspondence should be addressed.

requires O_2, NADH, and three proteins that are cytochrome b_5, cytochrome b_5 reductase, and the desaturase. The up-regulation in the hepatic synthesis of oleic acid, as induced by feeding a fat-free diet, is due to a 40-fold increase in the level of mRNA for the 9-desaturase which has an in vivo half-life of about 4 h (3). It is now known that two different 9-desaturases are expressed in tissue-specific ways in mice (4) and that the expression of stearoyl-CoA desaturase 1 in mouse liver is regulated by the type of dietary fat (5, 6).

In 1981 Okayasu et al. (7) purified a 6-desaturase from rat liver and showed that cytochrome b_5 and cytochrome b_5 reductase were required to desaturate linoleate to 6,9,12-18:3. The reconstituted system did not desaturate stearate, but it was not determined whether other substrates were desaturated at position 6 or whether this preparation could also introduce double bonds at position 5. Several different cell lines have the ability to desaturate fatty acids at position 5 but not at position 6 (8, 9). It is this type of evidence that supports the hypothesis for position-specific acyl-CoA-dependent 5 and 6 desaturases. However, Pugh and Kates (10) reported that 1-acyl-2-(8,11,14-eicosatrienoyl)-*sn*-glycero-3-phosphocholine was desaturated directly by rat liver microsomes to the arachidonate-containing phospholipid. Stoffel and Ach (11) reported that 11,14-20:2, the chain elongation product of linoleate, was metabolized to 8,11,14-20:3 via a pathway that would require an 8 desaturase. We showed that a number of acids with their first double bond at position 11 were desaturated at position 5, rather than at position 8 (12). Because 6, and particularly the 5-desaturase(s), have not been purified, it is not known *1)* whether cells contain more than one position-specific desaturase; *2)* whether a given desaturase is totally position-specific; or *3)* whether a given protein can introduce double bonds into a variety of substrates as would be required for a 5-desaturase.

There never was any firm evidence for an acyl-CoA-dependent 4-desaturase. In 1960 Klenk and Mohrhauer (13) synthesized a series of carbon-14-labeled n–3 acids and administered them to rats. They isolated various fatty acids, degraded them, and on the basis of their results they proposed a number of biosynthetic pathways, one of which required that 7,10,13,16,19-22:5 was converted to 4,7,10,13,16,19-22:6. However, when [1-^{14}C]7,10,13,16-22:4 was incubated with rat liver and testes microsomes, it was not desaturated (14). When we incubated [1-^{14}C]7,10,13,16,19-22:5 with rat liver microsomes in the presence of ATP, CoASH, and NADH, it also was not desaturated (15). However, when malonyl-CoA and NADPH were included in the incubation, the substrate was converted into two radioactive products that were shown to be 9,12,15,18,21-24:5 and 6,9,12,15,18,21-24:6. The latter compound could serve as a precursor for 4,7,10,13,16,19-22:6 if it was chain-shortened by two carbon atoms. When [1-^{14}C]-7,10,13,16,19-22:5, [3-^{14}C]9,12,15,18,21-24:5, and [3-^{14}C]-6,9,12,15,18,21-24:6 were incubated with rat hepatocytes, all three substrates were metabolized to yield [1-^{14}C]4,7,10,13,16,19-22:6 which was esterified into cellular phospholipids. Studies analogous to these have shown that 7,10,13,16-22:4 is metabolized to 4,7,10,13,16-22:5 via an identical pathway (16).

ROLE OF PEROXISOMES

The above studies show that 7,10,13,16-22:4 and 7,10,13,16,19-22:5 are the respective precursors of 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6 but their synthesis requires intracellular communication between the endoplasmic reticulum and a site for partial β-oxidation which is most likely peroxisomes. It is well documented that peroxisomes function to partially β-oxidize fatty acids and the chain-shortened products may then move to the mitochondria where the process is completed (17, 18). It is also well established that a variety of polyunsaturated fatty acids are partially β-oxidized with the subsequent esterification of chain-shortened metabolites into membrane lipids. We elected to use [3-^{14}C]7,10,13,16-22:4 as a model substrate to study what controls the partial β-oxidation-acylation process. The rationale for using this acid is based on the observation that when it was fed to rats raised on a diet devoid of fat, it was primarily metabolized to yield esterified arachidonate (19). When [3-^{14}C]7,10,13,16-22:4 was incubated with fibroblasts from control patients, it was metabolized to yield esterified arachidonate. Conversely, when it was incubated with fibroblasts from patients with Zellweger's disease, who lack peroxisomes, it was not possible to detect esterified arachidonate (20). When we incubated [1-^{14}C]22:4 (n–6) with peroxisomes, its rate of β-oxidation, as measured by the generation of acid-soluble radioactivity, was independent of microsomes and 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC). With [3-^{14}C]7,10,13,16-22:4 as substrate, the addition of 1-acyl-GPC to peroxisomes depressed the production of acid-soluble radioactivity. When microsomes were also included in the incubation, the rate of production of acid-soluble radioactivity was further depressed. When the phospholipids were isolated from both types of incubation, about 90% of the esterified radioactivity was arachidonate with the remaining 10% being unmetabolized substrate. A puzzling finding was the observation that when peroxisomes were incubated with 1-acyl-GPC, some substrate and arachidonate were esterified into the acceptor. The findings suggested that peroxisomes contained acyl-CoA:1-acyl-GPC acyltrans-

ferase activity. When Nycodenz-purified peroxisomes were assayed for microsomal contamination by measuring NADPH-cytochrome-c reductase activity, the microsomal contamination in peroxisomes correlated exactly with the percentage of contamination found when the specific activity of arachidonoyl-CoA:1-acyl-GPC acyltransferase was measured in peroxisomes and microsomes (21, 22). The esterification activity observed in peroxisomes is thus due to small amounts of microsomal contamination. It has been shown by Das, Horie, and Hajra (23) that peroxisomes are contaminated with small amounts of microsomes even when they are subjected to double Nycodenz gradient centrifugation. Our results thus show that when a microsomal metabolite, such as 22:4(n-6), moves to peroxisomes for partial β-oxidation, the chain-shortened product must move back to the endoplasmic reticulum for esterification. Indeed, the preferred metabolic fate of arachidonate when it was produced via β-oxidation was to move out of peroxisomes for esterification in the endoplasmic reticulum rather than to serve as a substrate for continued β-oxidation (21). Two types of processes may contribute to this type of intracellular regulation. First, rates of acylation of 7,10,13,16-22:4 and 5,8,11,14-20:4 into 1-acyl-GPC by microsomes from rats fed clofibrate were, respectively, 18 and 159 nmols/min per mg of protein (22). Arachidonate is thus a much better substrate for esterification than is 22:4(n-6). Secondly, arachidonate is a relatively poor substrate for peroxisomal β-oxidation (21, 24). Until recently it was generally accepted that 3,6,9,12-18:4 would be produced after one cycle of arachidonate β-oxidation. Tserng and Jin (25) reported that in mitochondria the double bond at position 5 in a number of monounsaturated fatty acids was directly removed via a nucleotide-dependent 5-reductase. This pathway has now been revised to show that it requires NADPH-dependent 2,4-dienoyl reductase and a new enzyme, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase (26, 27) that has been purified from rat liver mitochondria (28, 29). Recently we showed that the removal of both odd-numbered double bonds, during peroxisomal β-oxidation of arachidonate, also requires 2,4-dienoyl-CoA reductase and $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (30). As shown in **Fig. 1,** one complete cycle of arachidonate β-oxidation yields 6,9,12-18:3. The 2-*trans*-8,11,14-20:4 may serve as an intermediate in the β-oxidation cycle. Alternatively, it may be reduced to 8,11,14-20:3 via a nucleotide-dependent 2-*trans*-acyl-CoA reductase which was shown by Dommes et al. (31) to be a different protein than is NADPH-dependent 2,4-dienoyl-CoA reductase. Horie, Suzuki, and Suga (32) have shown that peroxisomes have an acetyl-CoA-dependent chain-elongation system. Reduction of 2-*trans*-8,11,14-20:4 to 8,11,14-20:3 is the last step in chain elongation. The above results show that

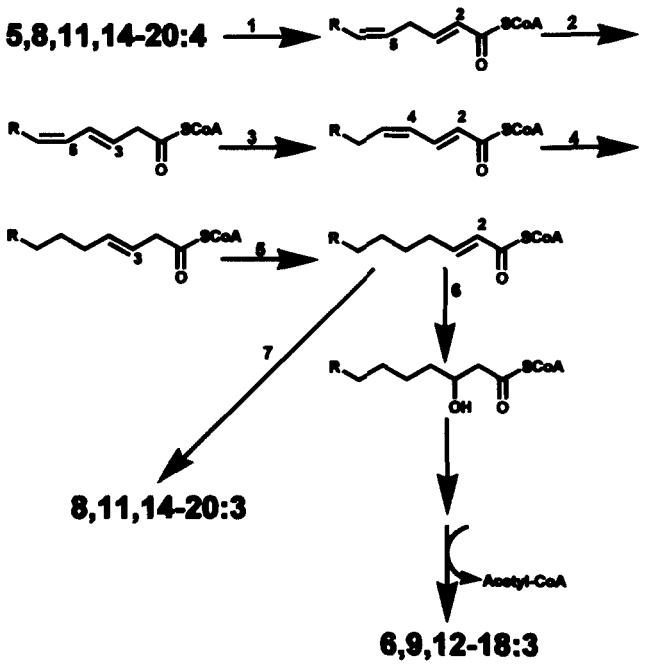

Fig. 1. A possible pathway for the peroxisomal β-oxidation of arachidonic acid as elucidated in reference 30. The enzymes in this proposed pathway are fatty acid oxidase (1), the trifunctional enzyme with Δ^3, Δ^2-enoyl-CoA isomerase activity (2), $\Delta^{3,5}$, $\Delta^{5,4}$-dienoyl-CoA isomerase (3), NADPH-dependent 2,4-dienoyl-CoA reductase (4), the trifunctional enzyme (5 and 6), and an NADPH-dependent 2-*trans*-acyl-CoA reductase (7).

when the microsomal metabolite 22:4(n-6) enters peroxisomes, it is readily β-oxidized but it is a relatively poor substrate for endoplasmic reticulum-associated esterification reactions. As soon as arachidonate is produced, it is preferentially transported out of peroxisomes, via some unknown pathway, for rapid esterification, rather than serving as a substrate for continued β-oxidation.

REGULATION OF PARTIAL β-OXIDATION

The above data show that intracellular communication exists between peroxisomes and the endoplasmic reticulum for a partial β-oxidation-esterification cycle. The revised pathways of polyunsaturated fatty acid biosynthesis, as shown in **Fig. 2,** raise several new questions about the regulation of this partial β-oxidation-acylation process. The pathways imply that linoleate and linolenate are metabolized, respectively, to 24-carbon acids in the endoplasmic reticulum. In theory, any fatty acid can be removed from these pathways for esterification into membrane lipids. If 24-carbon acids are esterified into membrane lipids in the endoplasmic reticulum, it would in essence curtail their movement to peroxisomes for partial β-oxidation. When we incubated the

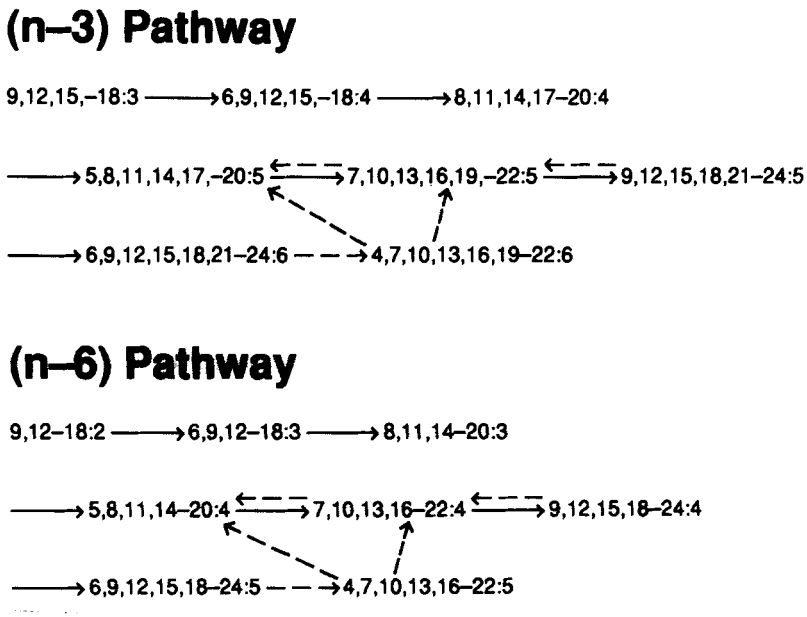

Fig. 2. Revised pathways for the biosynthesis of both 4,7,10,13,16-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid. The solid arrows denote reactions that take place in the endoplasmic reticulum. The dashed arrows show acids that are known substrates for partial β-oxidation followed by esterification of chain-shortened products into membrane lipids.

CoASH derivatives of 20:4(n–6), 22:4(n–6), 24:4(n–6), and 24:5(n–6) with rat liver microsomes, their rates of acylation into 1-acyl-GPC were, respectively, 159, 18, 134, 1, and 1 nmol/min per mg of protein. As 24-carbon acids were, in essence, not esterified, they may preferentially move to peroxisomes for partial β-oxidation. Although neither 24:4(n–6) nor 24:5(n–6) was readily esterified, there are possible important differences as to how their partial β-oxidation products, i.e., 7,10,13,16-22:4 and 4,7,10,13,16-22:5, are processed. The microsomal studies show that 4,7,10,13,16-22:5 is a better substrate for acylation than is 7,10,13,16-22:4. When 7,10,13,16-22:4 is produced from 9,12,15,18-24:4, its continued β-oxidation to 5,8,11,14-20:4 requires only the enzymes for saturated fatty acid degradation. Conversely, when 4,7,10,13,16-22:5 is produced via β-oxidation of 6,9,12,15,18-24:5, its continued β-oxidation to 5,8,11,14-20:4 requires removal of the double bond at position 4. In general, peroxisomal β-oxidation of fatty acids with their first double bond at position 4 proceeds at a slow rate (24, 30), perhaps because both 2,4-dienoyl-CoA reductase and the trifunctional enzyme with Δ^3, Δ^2-enoyl-CoA isomerase activity are required (33). The studies with 22:4(n–6) and 20:4(n–6) as well as with other substrates lead to a working hypothesis suggesting that there is an inverse relationship between rates of peroxisomal β-oxidation and the esterification of fatty acids into acceptors in the endoplasmic reticulum. Competition for endoplasmic reticulum-associated acylation reactions, versus continued peroxisomal β-oxidation, may be an important in vivo type of control in regulating membrane lipid fatty acid composition.

EXTRAHEPATIC PATHWAYS

The preceding discussion focuses on liver metabolism. It is appropriate to ask two other questions. First, do extrahepatic tissues express a microsomal acyl-CoA-dependent 4-desaturase? Wang and Anderson (34) reported that radioactive 24:5(n–3) and 24:6(n–3) were produced when labeled n–3 acids were metabolized to 22:6(n–3) in retinal pigment epithelium. The results suggest that 24-carbon acids are obligatory intermediates in 22:6 (n–3) biosynthesis. In their recent extensive studies on n–3 fatty acid metabolism, Moore et al. (35) reported that normal human fibroblasts metabolized [1-^{14}C]18:3(n–3) to labeled 22:6(n–3), 24:5(n–3), and 24:6(n–3). Conversely, fibroblasts from patients with Zellweger's disease metabolized [1-^{14}C]18:3(n–3) to 24:5(n–3) and 24:6(n–3) but not to 22:6(n–3). In a similar way, [3-^{14}C]-labeled 22:5(n–3), 24:5(n–3), and 24:6(n–3) were all metabolized to 22:6(n–3) in control, but not Zellweger's fibroblasts. These results also document that peroxisomes are required for 22:6(n–3) biosynthesis. When 2 μM [3-^{14}C]22:5(n–3) was incubated with increasing amounts of unlabeled 24:5(n–3) or 24:6(n–3), there was a dose-dependent decrease in the production of labeled 22:6(n–3). The results show that 22:5(n–3) was not desaturated directly to 22:6(n–3) but

rather that the labeled 22:5(n-3) was metabolized to 24-carbon (n-3) acids. As the incubations contained unlabeled 24-carbon acids, the specific activity of 22:6(n-3) precursors was diluted leading to the synthesis of esterified 22:6(n-3) with a lower specific activity. Second, what major differences exist for regulating fatty acid biosynthesis and the esterification of long-chain fatty acids into liver versus extrahepatic membrane lipids? The pathways in Fig. 2 imply that 24:5(n-6) and 24:6(n-3) are in essence end metabolites of microsomal metabolism in liver. These acids then move to peroxisomes where they are chain shortened, respectively, to 22:5(n-6) and 22:6(n-3), followed again by their transfer back to the endoplasmic reticulum for esterification. Membrane lipids from many tissues contain small amounts of very long-chain polyunsaturated fatty acids with 24-36 carbon atoms (36, 37). Clearly, then, in many tissues 24-carbon fatty acids are not end metabolites of microsomal metabolism but rather precursors for families of very long-chain acids which, in turn, are accepted by enzymes that synthesize phospholipids and are not just moved to peroxisomes for partial β-oxidation followed by esterification of chain-shortened metabolites.

CHAIN LENGTH SPECIFIC ENZYMES

Although the absence of a microsomal acyl-CoA-dependent 4-desaturase is of considerable interest, this finding has further ramifications relative to the regulation of fatty acid biosynthesis. First, do microsomes contain chain length position 6-desaturases? As shown in Fig. 2 the biosynthesis of 24:5(n-6) and 24:6(n-3) requires that two substrates in each pathway are desaturated at position 6. In a series of competitive substrate studies, no clear evidence was obtained for chain length-specific acyl-CoA-dependent 6-desaturases (38). It is generally accepted that desaturation of linoleate and linolenate, at position 6, is the rate-limiting step in arachidonate and 20:5(n-3) biosynthesis (39, 40). It would seem unlikely that an enzyme catalyzing a rate-limiting step could be used again to desaturate 24:4(n-6) and 24:5(n-3) at position 6. Second, how many chain-elongating enzymes are present in liver microsomes, and are different forms expressed in extrahepatic tissues that synthesize very long-chain fatty acids? In a recent review Cinti et al. (41) summarize the evidence suggesting that microsomes contain two different malonyl-CoA-dependent condensing enzymes: one of which uses saturated primers while the other accepts unsaturated acyl-CoAs. The respective β-ketoacyl-CoAs are then presumably channeled into a common set of enzymes to complete the chain-elongation processes. According to the pathways depicted in Fig. 2, three fatty acids in each pathway are chain-elongated and two of these reactions take place in sequence. Again, it would seem highly unlikely that a single hepatic condensing enzyme or chain-elongating system could be regulated to accept six different substrates.

PARTIAL β-OXIDATION AS AN ANABOLIC PROCESS

It has long been recognized that long-chain fatty acids are partially β-oxidized with the esterification of chain-shortened metabolites into membrane lipids. The physiological relevance of this process was never apparent. According to the pathways depicted in Fig. 2, the partial β-oxidation of 6,9,12,15,18-24:5 and 6,9,12,15,18,21-24:6, respectively, to 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6 is, in essence, a β-oxidation step that is used in an anabolic mode. Indeed, the partial β-oxidation process may play an important role in regulating how a variety of fatty acids are processed. As shown in Fig. 1,6,9,12-18:3 is produced after one cycle of arachidonate β-oxidation. It remains to be determined whether the preferred metabolic fate of 6,9,12-18:3 is continued β-oxidation or whether, in part, it moves to the endoplasmic reticulum for conversion back to arachidonate. Several studies suggest that chain-shortened products of β-oxidation may have a variety of possible anabolic fates. The α-subunit of transducin, a retinal G protein, contains esterified 14:2(n-6), a finding that implies that this chain-shortened catabolite of n-6 fatty β-oxidation has an anabolic fate (42). Gordon et al. (43) reported that when tritium-labeled 20:4(n-6) was incubated with fibroblasts, 4,7,10-16:3 accumulated in the medium of control cells but not of those obtained from patients with Zellweger's disease. Hansen, Jensen, and von Wettstein-Knowles (44) found that arachidonate was converted to linoleate in rats fed a diet devoid of fat. Three cycles of arachidonate β-oxidation can lead to the production of 14:2(n-6) which, when fed to rats, is metabolized to 18:2(n-6) (45).

n-3 ACIDS VERSUS n-6 ACIDS

Differences in structure between n-3 acids and n-6 acids also play an important role in dictating how fatty acids are processed intracellularly. Membrane lipids generally contain large amounts of 22:6(n-3) but only low levels of 22:5(n-6). This type of compositional difference could be explained if microsomal reaction rates for converting 20:5(n-3) to 24:6(n-3) were more rapid than for the conversion of 20:4(n-6) to 24:5(n-6). How-

ever, individual reaction rates for desaturation and chain elongation of analogous n-3 and n-6 fatty acids were similar (16, 38). Reaction rates for acylation of n-3 versus n-6 acids into 1-acyl-GPC were also similar (46). When [3-^{14}C]22:4(n-6) was injected into the tail vein of rats fed Purina chow, 96% of the esterified radioactivity in liver phospholipids was arachidonate with the remainder being unmetabolized substrate. Conversely, when [3-^{14}C]22:5(n-3) was injected, 20, 56, and 24%, respectively, of the esterified radioactivity was 20:5(n-3), 22:5(n-3), and 22:6(n-3) (47). Clearly, there are differences as to how n-3 versus n-6 fatty acids are processed within the cell that cannot be explained by reaction rates within any single subcellular compartment.

CONCLUSION

Prior to the discovery that microsomes do not contain a 4-desaturase, the synthesis of polyunsaturated fatty acids and their subsequent esterification into membrane lipids could be viewed as a process confined primarily to the endoplasmic reticulum. Why microsomes desaturate fatty acids at positions 9, 6, and 5, but not at position 4, remains to be explained. This review has pointed out that considerable recycling of fatty acids between peroxisomes and microsomes may take place. In theory, any long chain n-3 or n-6 fatty acid could be recycled many times. As long as any n-6 acid retains two double bonds, or an n-3 acid three double bonds, these putative peroxisomal catabolites have the potential of moving back to the endoplasmic reticulum for conversion, via chain elongation and desaturation reactions, back to 20-, 22-, or 24-carbon unsaturated acids. In this regard, rate-limiting reactions in a metabolic sequence, such as desaturation of linoleate by a 6-desaturase, must be reevaluated because even when arachidonate is degraded to 14:2(n-6) or 16:3(n-6) (21), these chain-shortened compounds are converted back to arachidonate without using a 6-desaturase (45, 48).

These studies were supported by NIH grant DK20387.

Manuscript received 10 August 1995 and in revised form 31 August 1995.

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