Chapter 2

Pathways for the Biosynthesis of Polyunsaturated Fatty Acids

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Arachidonic Acid Biosynthesis

It is generally accepted that linoleic acid is metabolized to arachidonic acid in the endoplasmic reticulum as follows: 9,12-18:2 → 6,9,12-18:3 → 8,11,14-20:3 → 5,8,11,14-20:4. This pathway requires the participation of position-specific acyl-CoA-dependent 6- and 5-desaturases with an intervening malonyl-CoA-dependent chain-elongation step. When the pathways of unsaturated fatty acid biosynthesis were being elucidated, Stoffel and Ach (1) presented evidence that linoleate was chain-elongated to 11,14-20:2, which was then desaturated at position 8 to yield 8,11,14-20:3. We synthesized [1-^{14}C]11,14-20:2 and [1-^{14}C]11,14,17-20:3. When they were incubated with rat liver microsomes, they were desaturated at position 5 to yield 5,11,14-20:3, and 5,11,14,17-20:4, respectively. At that time, we concluded that rat liver microsomes did not contain an 8-desaturase, and that 6,9,12-18:3 was an obligatory intermediate in the biosynthesis of arachidonate (2). Our results were corroborated by Dhopeshwarkar and Subramanian (3,4) who showed that brain also lacked an 8-desaturase. At about the same time, Albert et al. (5) incubated [1-^{14}C]11,14-20:2 with testes microsomes and isolated the radioactive trienoic acids. When they degraded them, they found radioactive 5- and 8-carbon aldehydo methyl esters, suggesting that rat testes contained an 8-desaturase. More recently, Cook et al. (6) reported that cultured C6 glioma cells metabolized deuterium-labeled fatty acids via a number of pathways, one of which could proceed via desaturation at position 8.

Acyl-CoA-dependent desaturase reactions are routinely assayed by incubating a radioactive fatty acid with microsomes in the presence of NADH, Mg^{2+}ATP, CoASH, and ATP. The introduction of a double bond, at a given position, is frequently assumed to indicate that microsomes contain a position-specific desaturase. However, relatively little is known about the proteins that catalyze position-specific desaturation reactions. The 9-desaturase that catalyzes the conversion of stearoyl-CoA to oleoyl-CoA has been purified from rat liver. It is a single polypeptide of 53,000 Da. The introduction of a double bond at position 9 is an aerobic process and

it requires cytochrome b_5, cytochrome b_5 reductase and the desaturase (7). It is now known that two different 9-desaturases are expressed in tissue-specific ways in mice (8,9). A partially purified 6-desaturase from rat liver also requires cytochrome b_5 and cytochrome b_5 reductase for activity (10). There are no reported attempts to purify a hepatic acyl-CoA-dependent 5-desaturase. It is not known how many desaturases are expressed in mammalian tissue, or if any given protein can accept multiple substrates, or perhaps even introduce double bonds at different positions. For example, both 8,11,14-20:3 and 11,14-20:2 are desaturated at position 5. Does a single enzyme desaturate both substrates? In this regard, it is interesting to note that Gurr et al. (11) reported that *cis*-12-18:1 was desaturated at position 9, to yield linoleate, in a number of animal and plant species. It seems very unlikely that the synthesis of arachidonate, via 11,14-20:2, is a major pathway. If 11,14-20:2 is desaturated at position 8, it remains to be determined if liver expresses an 8-desaturase, or whether another protein is simply not absolutely position specific.

When rats are fed a chow diet, their membrane lipids generally contain large amounts of both linoleate and arachidonate but only low levels of 18:3(n-6) and 20:3(n-6). Microsomal reaction rates have frequently been used as a predictor of what types of fatty acids are made for subsequent esterification into membrane lipids. Desaturation of linoleate to 6,9,12-18:3 by a 6-desaturase is generally recognized as the rate-limiting step in the synthesis of arachidonate (12). The biosynthesis of unsaturated fatty acids and their subsequent incorporation into membrane lipids are processes that are both localized primarily in the endoplasmic reticulum. The rate of chain-elongation of 18:3(n-6) to 20:3(n-6) was 2.5 nmol/min/mg of microsomal protein (12), whereas the rate of acylation of 6,9,12-18:3 into 1-acyl-*sn*-glycero-3-phosphocholine was 28 nmol/min/mg of microsomal protein (13). If reaction rates *per se* are predictors of membrane fatty acid composition, it would be predicted that 6,9,12-18:3 should be preferentially esterified rather than being chain-elongated to 8,11,14-20:3. *In vivo*, it is possible that linoleate may be channeled to arachidonate, so that 6,9,12-18:3 and 8,11,14-20:3 are not readily made available for acylation reactions. Alternatively, a more complex type of fatty acid metabolism may take place. When we incubated [1-^{14}C]6,9,12-18;3 or [1-^{14}C]6,9,12,15-18:4 with hepatocytes, both substrates were initially incorporated into phospholipids. After about 60 min, all of the exogenous substrate had been metabolized. However, from 60 to 120 min, there was a continued increase in the amount of esterified arachidonate and 20:5(n-3). The continued synthesis of these acids was accompanied by a decrease in the amount of esterified 18:3(n-6) and 18:4(n-3). We suggested that the 18-carbon acids may have entered a very labile phospholipid pool and that they were rapidly hydrolyzed by a phospholipase A_2 for further metabolism to arachidonate (14). Many tissues, including liver (15), contain a CoASH-dependent, ATP-independent pathway for remodeling the fatty acid composition of membrane lipids. It is thus possible that when 6,9,12-18:3 is produced *in vivo*, it is initially incorporated into membrane lipids. If the CoASH-dependent pathway was highly fatty acid-specific, the esterified 6,9,12-18:3 might simply be transferred to CoASH for subsequent metabolism to 8,11,14-20:3 and then to arachidonate. If this pathway is operative, it implies that when 6,9,12-18:3 is produced, it is initially esterified, but sufficient amounts do not

accumulate to detect when the fatty acid composition of membrane lipids is quantified.

It is well established that many physiological processes are modified by the types of unsaturated fatty acids found in membrane lipids. Three basic different nutritional models have been used to determine how changes in membrane fatty acid composition alter function. When animals are fed no fat or a diet devoid of essential fatty acids, their membrane lipids accumulate 5,8,11-20:3. The essential fatty acid-deficient model has been used extensively to study the role of essential fatty acids as they relate to a variety of parameters. The second dietary model may be called a fatty acid replacement strategy. In this dietary model, a fat is added to a basal diet that already contains an adequate level of essential fatty acids. The addition of fish oils to the diet is an example of this type of dietary intervention. When a typical Western diet is consumed, the membrane lipids contain only trace amounts of 20:5(n-3). When fish oil is added to the diet, some of the esterified arachidonate is replaced by 20:5(n-3). This type of dietary intervention has been most frequently used to determine how eicosanoid-mediated processes, such as platelet aggregation, and inflammation, are altered. The third dietary model has as its basic premise the addition to the diet of an oil containing a fatty acid that is beyond the rate-limiting step in a biosynthetic sequence. The addition of oils containing 6,9,12-18:3 to the diet is an example of this type of strategy, because the rate-limiting 6-desaturase step is circumvented. However, it has never been determined to what extent the addition of 6,9,12-18:3 to the diet affects the metabolism of linoleate to arachidonate. We carried out a number of feeding studies, using deuterium-labeled fatty acids, to quantify how they modified both the mass amounts of esterified arachidonate and its isotopic composition. In these studies, male weanling rats were fed a modified AIN-76 diet for 4 wk in which the fat content was 3.3% by weight, consisting of 2.1% ethyl oleate, 1% ethyl linoleate and 0.2% ethyl linolenate. After 4 wk on this diet, the rats were changed to one in which all of the linoleate was replaced by an equal amount of 17,17,18,18-d_4-18:2(n-6). The rats were killed 4 d later, and the molar fraction of esterified deuterium-labeled 20:4(n-6) in liver phospholipids, as determined by mass spectrometry, was found to be 33.9%. The second group of rats was preconditioned by feeding them an identical diet, except for a reduction of oleate content to 1.9%, with 0.2% ethyl 6,9,12-18:3 included in the diet. After 4 wk, all of the linoleate was again replaced by the deuterium-labeled analog. Now the molar fraction of deuterium-labeled arachidonate was reduced to 27.1%. The third group of animals was fed the same diet as group 2, but now the unlabeled 18:3(n-6) was replaced by an equal amount of 17,17,18,18-d_4-18:3(n-6). The molar fraction of labeled 20:4(n-6) in phospholipids was 24.6%. In these studies, the amount of 18:3(n-6) that was fed was 20% of the level of linoleate. However, the molar fraction of esterified labeled 20:4(n-6) derived from 18:3(n-6) was 70% of that produced from 18:2(n-6), i.e., 27.1/33.9 = 70%. When 18:3(n-6) was added to the diet, it depressed the amount of d_4-18:2(n-6) metabolized to esterified d_4-20:4(n-6). However, it can be calculated that the amount of esterified labeled 20:4(n-6) that was produced from the combined metabolism of 18:2(n-6) plus 18:3(n-6) was greater than what was produced from linoleate alone. When 18:2(n-6) was the only dietary (n-6) acid, 33.9% of the arachidonate was labeled. When unlabeled 18:3(n-6) was added to the diet, this value was reduced to 27.1%. However, it can be calculated that when 0.2% 18:3(n-6) was added to a diet containing 1% 18:2(n-6), the molar fraction of labeled 20:4(n-6) in liver phospholipid was 51.7%, i.e., 27.1% plus 24.6%. None of these dietary manipulations altered the actual amount of arachidonate esterified in phospholipids. It can thus be concluded that dietary supplements of 18:3(n-6) increase the production of 20:4(n-6), but they do not alter the amount of this acid esterified in phospholipids. Obviously, additional studies are required to determine if this type of phenomenon is observed when other levels of linoleate and 18:3(n-6) are fed.

Intracellular Movement in Fatty Acid Biosynthesis

It has generally been accepted that 7,10,13,16-22:4 and 7,10,13,16,19-22:5 are desaturated at position 4 in the endoplasmic reticulum to yield 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6, respectively. When we incubated [1-^{14}C]7,10,13,16,19-22:5 with rat liver microsomes and appropriate cofactors, the substrate was not desaturated. However, when NADPH and malonyl-CoA were added to the incubation, the substrate was initially chain-elongated to yield 9,12,15,18,21-24:5, which was subsequently desaturated at position 6 to yield 6,9,12,15,18,21-24:6. When we incubated [1-^{14}C]7,10,13,16,19-22:5 and [3-^{14}C]labeled 9,12,15,18,21-24:5 or 6,9,12,15,18,21-24:6 with hepatocytes, all three substrates were metabolized to yield esterified 4,7,10,13,16,19-22:6 (16). Comparable studies have shown that 7,10,13,16-22:4 is metabolized to 4,7,10,13,16-22:5 via an analogous pathway (17).

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 is independent of an acyl-CoA-dependent 4-desaturase. Indeed, the synthesis of these two acids implies that there is intracellular communication between the endoplasmic reticulum and a site for partial β-oxidation. Both 6,9,12,15,18,21-24:6 and 6,9,12,15,18-24:5 are made in the endoplasmic reticulum. These acids must then move to a site for β-oxidation where they are chain-shortened, to 4,7,10,13,16,19-22:6 and 4,7,10,13,16-22:5, respectively, followed by their esterification into membrane lipids. It is well recognized that peroxisomes chain-shorten fatty acids, and these catabolites may then move to mitochondria where the process is completed (18). It is also possible that peroxisomes partially β-oxidize long-chain acids, and that the chain-shortened products move to the endoplasmic reticulum where they are esterified. We used 7,10,13,16-22:4 as a model substrate to study this partial β-oxidation esterification process. The rationale for using this acid as a model substrate is based on three types of studies. When 7,10,13,16-22:4 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 injected into the tail vein of rats fed chow, 96% of the esterified radioactivity in liver phospholipids was arachidonate with the substrate accounting for only 4% (20). When [3-^{14}C]7,10,13,16-22:4 was incubated with fibroblasts, it was metabolized to yield esterified arachidonate. However, when it was incubated with fibroblasts from patients with Zellweger's disease, who lack peroxisomes, it was not possible to detect any esterified arachidonate (21).

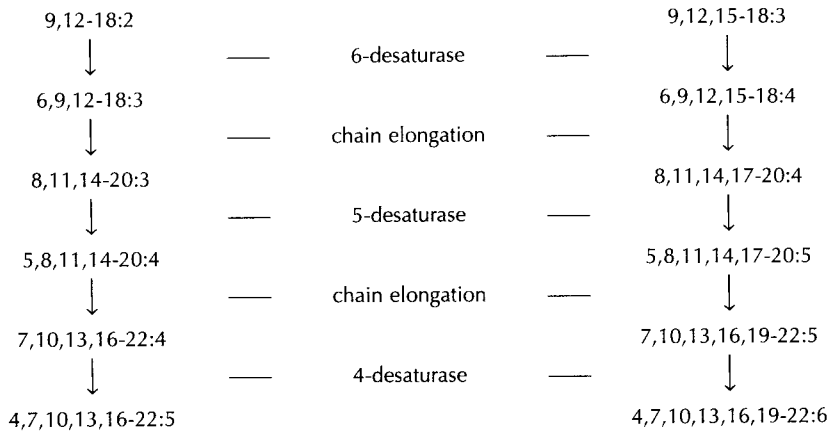

Fig. 2.1. The pathways, as commonly depicted, for showing how dietary linoleate and linolenate are metabolized to 4,7,10,13,16-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid, respectively, in the endoplasmic reticulum.

When we incubated [1-^{14}C]7,10,13,16-22:4 with rat liver peroxisomes, its rate of β-oxidation, as measured by the generation of acid-soluble radioactivity, was not depressed when microsomes and 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) were included in the incubation. Conversely, when [3-^{14}C]7,10,13,16-22:4 was incubated with peroxisomes, its rate of β-oxidation, as measured by the generation of acid-soluble radioactivity, was depressed when 1-acyl-GPC and microsomes were included in the incubation. The data show that the preferred metabolic rate of [1-^{14}C]5,8,11,14-20:4, when it was produced via β-oxidation, was to move out of peroxisomes to the endoplasmic reticulum where it was esterified into the acceptor, rather than serving as a continued substrate for β-oxidation. When [1-^{14}C]7,10,13,16-22:4 was incubated alone with peroxisomes, its rate of β-oxidation was about fourfold greater compared with [1-^{14}C]7,10,13,16-22:4. When the acyl-CoAs of 5,8,11,14-20:4 and 7,10,13,16-22:4 were incubated with 1-acyl-GPC and rat liver microsomes, their rates of acylation were 159 and 18 nmol/min/mg of microsomal protein, respectively. These studies show that there is an inverse relationship between rates of β-oxidation and acylation. Indeed, the studies suggest that competition between peroxisomal β-oxidation and microsomal acylation reactions may be an important *in vivo* control for determining what types of polyunsaturated fatty acids are incorporated into membrane lipids (22).

Figure 2.1 shows the classical pathway whereby dietary linoleate and linolenate are metabolized to 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6, respectively. Figure 2.2 shows a revised pathway for the biosynthesis of those two acids. There are three major differences between the two reaction sequences. According to the revised pathway, both 6,9,12,15,18-24:5 and 6,9,12,15,18,21-24:6 are made in the endoplasmic reticulum. These acids must then move to peroxisomes where they are chain-shortened by two carbon atoms. The resulting products presumably must then move back to the endoplasmic reticulum where they are esterified into acceptors. It

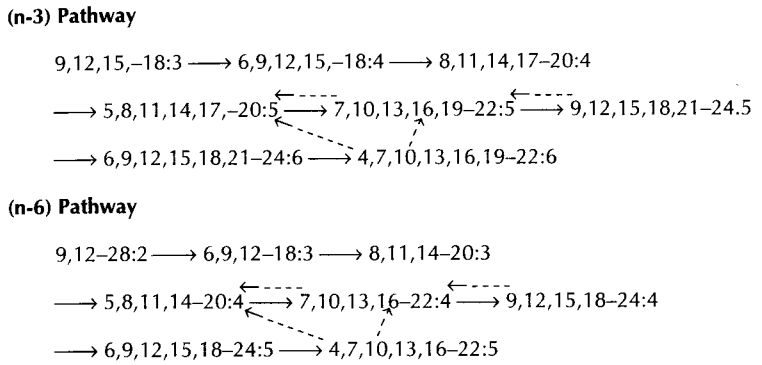

Fig. 2.2. Revised pathways for the biosynthesis of 4,7,10,13,16-docosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid. The solid arrows denote reaction taking place in the endoplasmic reticulum. The dashed arrows show acids that are substrates for partial β-oxidation followed by esterification of chain-shortened products into membrane lipids.

remains to be determined what regulates this intracellular movement. According to Figure 2.1, two acids in each sequence are chain-elongated. According to the revised pathway, three acids in each pathway are chain-elongated and two of these reactions take place in sequence. In a recent review Cinti and his colleagues (23) summarize the evidence suggesting that separate malonyl-CoA-dependent condensing enzymes are used for saturated vs. unsaturated primers. The resulting β-ketoacyl-CoA derivatives are then channeled into a common set of enzymes to complete the chain-elongation process. It would seem unlikely that a single condensing enzyme could accept six different primers, as would be required by the revised pathway. Clearly, additional studies are required to determine what regulates microsomal fatty acid chain-elongation and how many enzymes are present. Desaturation at position 6 has generally been recognized as the rate-limiting step in the biosynthesis of arachidonate. According to the revised pathway, four different fatty acids must be desaturated at position 6. In a series of competitive substrate studies, we were unable to obtain any conclusive evidence that microsomes contain chain length-specific 6-desaturases (24). Further studies are also clearly required to determine what regulates desaturation at position 6 as it now relates to the biosynthesis of both 4,7,10,13,16-22:5 and 4,7,10,13,16,19-22:6.

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