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Double Bond Removal from Odd-numbered Carbons during Peroxisomal β-Oxidation of Arachidonic Acid Requires both 2,4-Dienoyl-CoA Reductase and $\Delta^{3,5},\Delta^{2,4}$-Dienoyl-CoA Isomerase*

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The pathway for the peroxisomal β-oxidation of arachidonic acid (5,8,11,14-20:4) was elucidated by comparing its metabolism with 4,7,10-hexadecatrienoic acid (4,7,10-16:3) and 5,8-tetradecadienoic acid (5,8-14:2) which are formed, respectively, after two and three cycles of arachidonic acid degradation. When [1-^{14}C]4,7,10-16:3 was incubated with peroxisomes in the presence of NAD^+ and NADPH, it resulted in a time-dependent increase in the production of acid-soluble radioactivity which was accompanied by the synthesis of 2-*trans*-4,7,10-hexadecatetraenoic acid and two 3,5,7,10-hexadecatetraenoic acid isomers. The formation of conjugated trienoic acids suggests that peroxisomes contain $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase with the ability to convert 2-*trans*-4,7,10-hexadecatetraenoic acid to 3,5,7,10-hexadecatetraenoic acid. When 1-^{14}C-labeled 6,9,12-octadecatrienoic acid or 7,10,13,16-docosatetraenoic acid was incubated without nucleotides, the 3-hydroxy metabolites accumulated, since further degradation requires NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenase. When [1-^{14}C]5,8,11,14-20:4 was incubated under identical conditions, no polar metabolite was detected, but 2-*trans*-4,8,11,14-eicosapentaenoic acid accumulated. When NADPH was added to incubations, 3-hydroxy-8,11,14-eicosatrienoic, 2-*trans*-4,8,11,14-eicosapentaenoic, 2-*trans*-8,11,14-eicosatetraenoic, and 8,11,14-eicosatrienoic acids were produced. Analogous compounds were formed from [1-^{14}C]5,8-14:2. Our results show that the removal of double bonds from odd-numbered carbons in arachidonic acid thus requires both NADPH-dependent 2,4-dienoyl-CoA reductase and $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase. One complete cycle of 5,8-14:2 and 5,8,11,14-20:4 β-oxidation yields, respectively, 6-dodecenoic and 6,9,12-octadecatrienoic acids.

The β-oxidation of unsaturated fatty acids requires both 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase, in addition to the enzymes required for saturated fatty acid degradation (1, 2). Peroxisomes contain a trifunctional enzyme possessing Δ^2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and Δ^3,Δ^2-enoyl-CoA isomerase activities (3). Peroxisomal 2,4-dienoyl-CoA reductase (4) is a different protein from the two mitochondrial isoforms of this enzyme (5). In their comparative studies on peroxisomal β-oxidation of different unsaturated fatty acids, Osmundsen and his colleagues (6, 7) reported that the amount of acid-soluble radioactivity produced from [1-^{14}C]arachidonate was frequently less than from other 1-^{14}C-labeled acids. We also observed that the rate of production of acid-soluble radioactivity from [1-^{14}C]5,8,11,14-20:4 was considerably less than that from [1-^{14}C]9,12-18:2 or [1-^{14}C]7,10,13,16-22:4 (8). When tritium-labeled arachidonic acid was incubated with peroxisomes, Hiltunen *et al.* (6) did not detect any 18-carbon catabolite but a compound did accumulate that they suggested was either 5,8-14:2 or 4,7,10-16:3. We carried out similar studies and were able to characterize both 4,7,10-16:3 and 2-*trans*-4,7,10-16:4 (8). When [U-^{14}C]9,12-18:2 was incubated with peroxisomes, both 7,10-16:2 and 5,8-14:2 accumulated (8).

According to the classical pathways of unsaturated fatty acid degradation, 7,10-16:2 and 3,6,9,12-18:4 would be formed, respectively, after one cycle of 9,12-18:2 and 5,8,11,14-20:4 β-oxidation. However, Tserng and Jin (9) 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 modified to show that it requires NADPH-dependent 2,4-dienoyl-CoA reductase, an enzyme which previously was thought to be required for double bond removal only at even-numbered carbons (1). In addition, a new enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase is required (10, 11). This enzyme has recently been purified from mitochondria by Schulz and co-workers (12), as well as by Tserng and his collaborators (13). In the study reported here, we show that, during the peroxisomal β-oxidation of arachidonate, the removal of odd-numbered double bonds requires both NADPH-dependent 2,4-dienoyl-CoA reductase and $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase.

EXPERIMENTAL PROCEDURES

Materials—ATP, NAD^+, NADPH, CoASH, Hepes, dithiothreitol, TES[1], and essentially fatty acid-free bovine serum albumin were from Sigma. Lactate dehydrogenase and Nycodenz (Accudenz) were obtained, respectively, from Boehringer Mannheim and Accurate Chemicals and Scientific Corp. Arachidonic acid was from Nu-Chek Prep, while the other unlabeled and 1-^{14}C-labeled acids were made by total synthesis (14). The methods described by Stoffel and Pruss (15) were used to synthesize 3-hydroxy-8,11,14-20:3 and 2-*trans*-8,11,14-20:4. Authentic 2-*trans*-4-*cis*-10:2 was isolated from the seed oil of the Chinese Tallow Tree (16).

Isolation of Peroxisomes and Mitochondria from Rat Liver—Male Sprague-Dawley rats were maintained on a chow diet containing 0.5% Clofibrate (prepared by Dyets, Inc., Bethlehem, PA) for 8 days prior to being killed. Peroxisomes were isolated in essence by the method of Das *et al.* (17), as described previously (8). Briefly, liver was homogenized in 0.25 M sucrose, 0.1 mM EDTA, 0.1% ethanol, 10 mM TES, pH 7.5. The

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[1] The abbreviations used are: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; 12-HETE, 12-hydroxy-5,8,10,14-20:4; 15-HETE, 15-hydroxy-5,8,11,13-20:4.

nuclei and mitochondria obtained by centrifugation at 600 × *g* for 10 min and 3,300 × *g* for 10 min were discarded. The light mitochondrial pellet that sedimented at 25,000 × *g* for 15 min was washed with the homogenizing buffer and centrifuged at 25,000 × *g* for 12 min. One more washing was done under the same conditions, except that the centrifugation time was reduced to 10 min. The light mitochondrial fraction was resuspended in the homogenizing medium in a volume corresponding to 1 ml/g liver. Two ml of this suspension was layered on 15 ml of 35% Nycodenz (w/v), containing 10 mM TES, 0.1% ethanol, and 0.1 mM EDTA, pH 7.5, and centrifuged at 56,000 × *g* for 50 min. The peroxisomal pellet was suspended in the incubation medium which contained 130 mM KCl, 20 mM Hepes, pH 7.2, and the protein concentration was adjusted to 3 mg/ml. Protein was assayed with the Coomassie Blue reagent (Pierce), using bovine serum albumin as a standard. Purified mitochondria were isolated according to the procedure of Johnson and Lardy (18), as modified by Broekemeier *et al.* (19). The purity of peroxisomes was determined by marker enzyme analysis as described previously (8). Mitochondrial contamination was less than 1% as determined by measuring succinate cytochrome *c*-reductase in mitochondria and in the Nycodenz-purified peroxisomes (20).

Peroxisomal Metabolism—To measure fatty acid activation, peroxisomes (10 μg of protein) were incubated in a total volume of 0.2 ml, at 37 °C for 2 min in a shaking water bath, in a medium that contained 130 mM KCl, 20 mM Hepes (pH 7.2), 10 mM Mg^{2+}ATP, and 0.2 mM CoASH. Reactions were initiated by addition of the sodium salt of the fatty acid (2 Ci/mol) bound to bovine serum albumin in a 2:1 molar ratio. Substrate concentration varied from 12.5 to 150 μM. Reactions were terminated after 2 min by addition of 1 ml of Dole's reagent (isopropyl alcohol/heptane/0.1 N H_2SO_4, 40:10:1, v/v), followed by 0.35 ml of water and 0.6 ml of heptane (21). The upper layer was discarded, the bottom layer was washed three times with 1-ml aliquots of heptane, by vortexing, and the bottom layer was transferred to scintillation vials and counted in 10 ml of ACS II (Amersham). Maximum rates of activation were calculated from Lineweaver-Burk plots. To measure β-oxidation, peroxisomes (300 μg of protein) were incubated at 37 °C in a shaking water bath in a medium that contained 130 mM KCl, 20 mM Hepes, 0.1 mM EGTA, 0.5 mM NAD^+, 0.1 mM NADPH, 0.1 mM dithiothreitol, 0.4 mM CoASH, 10 mM Mg^{2+}ATP, 20 mM pyruvate, and 2 units of lactate dehydrogenase, pH 7.2 (8, 22). Reactions were initiated by addition of the sodium salt of the fatty acid (2 Ci/mol) that was bound to bovine serum albumin in a 2:1 molar ratio. The final concentration of fatty acid was always 100 μM. Aliquots of 200 μl were removed and added to an equal volume of 5% $HClO_4$. After 30 min at 4 °C, the samples were centrifuged and 200 μl was counted to measure acid-soluble radioactivity.

Metabolite Isolation and Characterization—Large scale incubations were always carried out without NAD^+, but with and without NADPH in order to isolate metabolites for characterization. For each milliliter of incubation medium, 1.7 ml of MeOH and 0.25 ml of 4 N NaOH was added. The contents were stirred overnight, acidified with 0.25 ml of 6 N HCl, and 3.4 ml of $CHCl_3$ was added. The bottom layer was taken to dryness under N_2, and the free fatty acids were esterified by stirring them overnight with 5% anhydrous HCl in MeOH (w/v). The methyl esters were recovered by extraction with hexane. They were separated by reverse phase HPLC by eluting a 0.46 × 25 cm Zorbax ODS column with various concentrations of acetonitrile/water. The effluent (1 ml/min) was mixed with ScintiVerse LC (Fisher) (3 ml/min), and metabolites were detected with a Beckman 171 radioisotope detector. Appropriate fractions were collected from the column effluent, the acetonitrile was removed under N_2, and the metabolites were recovered by extraction with 20% diethyl ether in hexane. Trace amounts of water were removed by passing the combined extract through a Pasteur pipette packed with granular Na_2SO_4. Ultraviolet spectra were measured in MeOH using a Beckman DU-64 spectrophotometer. The methyl esters of 3-hydroxy fatty acids were further derivatized by reacting them with equal volumes of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) and pyridine for 30 min at 60 °C. Mass spectrometry was carried out with a Hewlett-Packard Model 5970 mass selective detector and a 5770 gas chromatograph containing a 30 m × 0.25 mm inside diameter, DB5-ms column (J and W Scientific). Injections were made in isooctane in the splitless mode at 70 °C, and, after 3 min, the oven temperature was programmed to increase, at 20 °C/min to 200 °C.

RESULTS

During the β-oxidation of arachidonate, the synthesis of the acyl-CoA derivatives of 5,8-14:2 and 4,7,10-16:3 occurs by a CoASH-dependent thiolytic cleavage of precursor 3-ketoacyl-CoAs, rather than by ATP-dependent activation. Rates of activation of 5,8-14:2, 4,7,10-16:3, and arachidonate were, respectively, 746, 557, and 283 nmol/min/mg of peroxisomal protein. Since activation rates were high, it was assumed that acyl-CoA formation with all three substrates was not rate-limiting when β-oxidation was assayed.

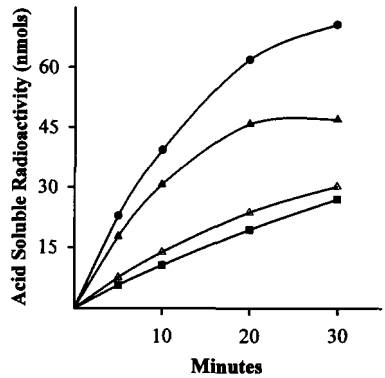

FIG. 1. **The time-dependent β-oxidation [1-^{14}C]5,8-14:2 (●), [1-^{14}C]5,8,11,14-20:4 (■), [1-^{14}C]4,7,10-16:3 without (△) and with (▲) NADPH.** Peroxisomes (300 μg of protein/ml) were incubated with 100 μM levels of fatty acids. At the indicated times, 200 μl was removed and added to 200 μl of cold 5% $HClO_4$. After centrifugation, 200 μl of the supernatant was used to measure acid-soluble radioactivity.

The results in Fig. 1 show that the generation of acid-soluble radioactivity formed during the β-oxidation of [1-^{14}C]5,8-14:2 was greater than for [1-^{14}C]5,8,11,14-20:4. These data suggest that the chain length of the substrate plays a more important role in regulating β-oxidation than does a double bond at position 5. The generation of acid-soluble radioactivity from [1-^{14}C]4,7,10-16:3 requires the participation of NADPH-dependent 2,4-dienoyl-CoA reductase. As shown in Fig. 1, there was a time dependent increase in the rate of β-oxidation when incubations were carried out in the absence of NADPH. When NADPH was added to the incubation, there was an increase in the generation of acid-soluble radioactivity. Similar results were obtained when the rate of β-oxidation of [1-^{14}C]4,7,10,13,16,19-22:6 was compared with and without NADPH (6).

When [1-^{14}C]4,7,10-16:3 was incubated with the NAD^+ generating system and NADPH, the HPLC radiochromatogram in Fig. 2*A* shows that three radioactive metabolites eluted immediately prior to compound 4 which is residual substrate. When [1-^{14}C]4-10:1, a product that would be produced after five cycles of arachidonate β-oxidation, was incubated under identical conditions, it was possible to detect only one metabolite in addition to unreacted substrate (Fig. 3). The metabolite was isolated and its ultraviolet and mass spectra were identical with authentic methyl 2-*trans*-4-*cis*-10:2. When [1-^{14}C]4,7,10-16:3 was incubated without nucleotides, there was an increase in the accumulation of metabolites *versus* when the complete system was used (Fig. 2, *A versus B*). These incubation conditions were then used to accumulate sufficient amounts of metabolites for characterization. The ultraviolet spectrum of the methyl ester of compound 3 (Fig. 4*A*) was identical with that of methyl 2-*trans*-4-*cis*-10:2 (Fig. 4*B*). The mass spectrum of the methyl ester had a base peak of *m/z* 79, a small molecular ion at *m/z* 262 (2%) with other small ions at *m/z* 230 (M − 32; 1%) and *m/z* 231 (M − 31; 1%). Compound 3 is thus methyl 2-*trans*-4,7,10-16:4. The ultraviolet spectrum of compound 2, as shown in Fig. 5, had three absorbance maxima at 257, 267, and 277 nm showing that it contained three conjugated double bonds, none of which were in conjugation with the carbonyl carbon (23). Its mass spectrum differed from compound 3 primarily in that the molecular ion at *m/z* 262 was more intense; *i.e.* 32%, and the base peak was at *m/z* 91. The ultraviolet and the mass

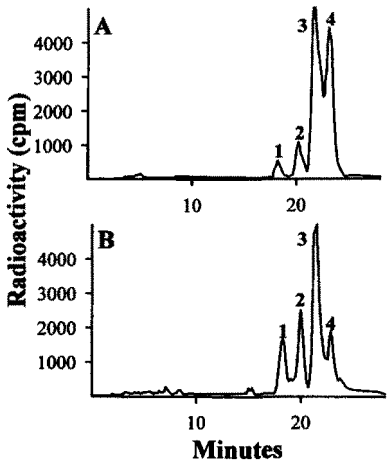

FIG. 2. **Radiochromatograms obtained when 100 μM [1-^{14}C] 4,7,10-16:3 was incubated with 300 μg of peroxisomal protein with (*A*) and without (*B*) NAD$^+$ and NADPH.** After 30 min, the incubations were terminated, and methyl esters were made and separated by HPLC by eluting the column at 1 ml/min with acetonitrile/water (85:15, v/v).

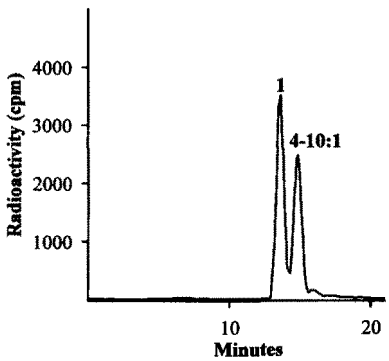

FIG. 3. **A radiochromatogram showing that the metabolism of [1-^{14}C]4-10:1 resulted in the production of a single radioactive catabolite (compound 1).** Peroxisomes were incubated with 100 μM [1-^{14}C]4-10:1 in the presence of NAD$^+$ and NADPH. After 30 min, the reaction was terminated, and methyl esters were made and separated by HPLC by eluting the column at 1 ml/min with acetonitrile/water (70:30, v/v).

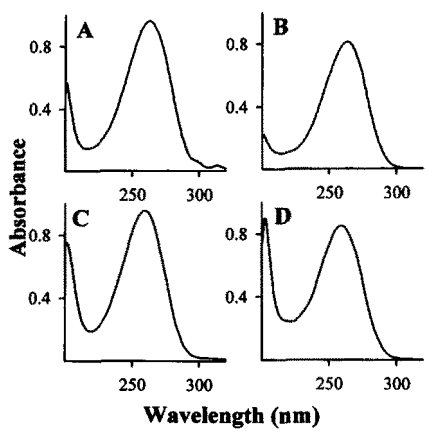

FIG. 4. *A*, the ultraviolet spectra of metabolite 3 from Fig. 2; *B*, authentic methyl 2-*trans*-4-*cis*-10:2; *C*, the major catabolite that was produced when [1-^{14}C]5,8,11,14-20:4 was incubated without NAD$^+$ and NADPH as shown in Fig. 6*A*; and *D*, the major radioactive compounds which co-eluted as shown in Fig. 7*A*.

spectra of compound 1 were similar with compound 2 (data not shown). The initial product formed during the β-oxidation of 4,7,10-16:3 is 2-*trans*-4,7,10-16:4; *i.e.* compound 3. Isomerization of the 2-*trans*-4-*cis*-double bonds, by reversing the reaction catalyzed by $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, would yield 3,5,7,10-16:4. Two isomeric products with this general structure were characterized, but as yet it has not been possible to determine the configuration of the double bonds in compounds 1 and 2 in Figs. 2A and 2B.

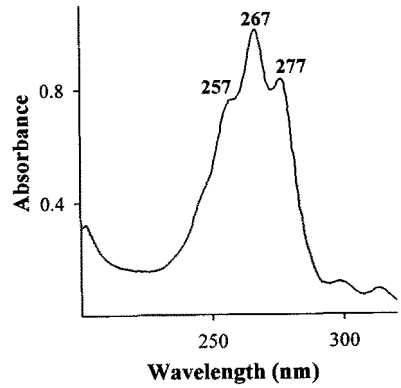

FIG. 5. **The ultraviolet spectrum of compound 2, Fig. 2.**

The above studies suggested that peroxisomes have $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase activity. Experiments were then designed to determine if the double bond at position 5 was removed via the classical pathway of β-oxidation or if $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase was required. Diczfalusy *et al.* (24) reported that 3-hydroxy-9,12-18:2 accumulated when 9,12-18:2 was incubated with peroxisomes in the absence of NAD$^+$. When we incubated [1-^{14}C]6,9,12-18:3 with peroxisomes, without NAD$^+$ and NADPH, a polar metabolite accumulated. The mass spectrum of the methyl ester-trimethylsilyl ether derivative had a weak molecular ion at *m/z* 380 (1%) and a major ion at *m/z* 365 (M − 15; 30%) showing that it is 3-hydroxy-6,9,12-18:3. In a similar way, the mass spectrum of the polar metabolite derived from [1-^{14}C]7,10,13,16-22:4 had a molecular ion at *m/z* 434 (2%) and a major ion at *m/z* 419 (M − 15; 37%). This compound is thus 3-hydroxy-7,10,13,16-22:4.

The above data show that when polyunsaturated fatty acids, with their first double bond at position 6, 7, or 9, are incubated with peroxisomes, in the absence of NAD$^+$, that the expected 3-hydroxy metabolite accumulates. When [1-^{14}C]5,8,11,14-20:4 was incubated under the above conditions, the HPLC radiochromatogram in Fig. 6*A* shows that it was not possible to detect a polar metabolite. Two small radioactive peaks eluted, respectively, at 22.7 and 24 min. The major metabolite, which eluted immediately prior to unmetabolized arachidonate, had an ultraviolet spectrum (Fig. 4*C*) identical with methyl 2-*trans*-4-*cis*-10:2 (Fig. 4*B*). The mass spectrum of the methyl ester had a weak molecular ion at *m/z* 316, which is two mass units less than for methyl 5,8,11,14-20:4. Compound 3 is thus 2-*trans*-4,8,11,14-20:5, which cannot be produced from arachidonate via the classical pathway of β-oxidation since one cycle of β-oxidation would yield 3,6,9,12-18:4, using only the enzymes required for saturated fatty acid degradation. The accumulation of 2-*trans*-4,8,11,14-20:5 further implies that peroxisomes have $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase activity. If the dienoyl-CoA isomerase-NADPH-dependent reductase pathway is required to remove the double bond at position 5 from arachidonate, the synthesis of the 3-hydroxyacyl-CoA would require NADPH. The HPLC radiochromatogram in Fig. 6*B* shows that it was possible to detect seven radioactive metabolites when [1-^{14}C]5,8,11,14-20:4 was incubated with peroxisomes in the presence of NADPH in addition to unmetabolized substrate (compound 5). Under these conditions, it was not possible to detect acid-soluble radioactivity, which would require a complete

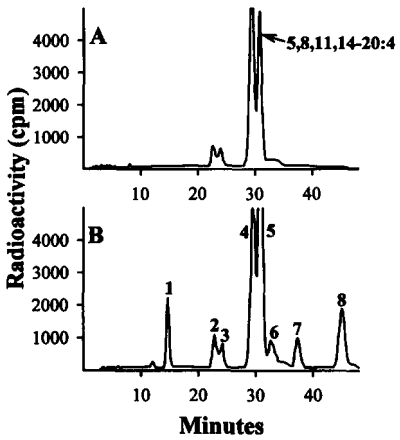

FIG. 6. **HPLC radiochromatograms of the products obtained when 100 μM [1-^{14}C]5,8,11,14-20:4 was incubated without (*A*) and with (*B*) NADPH.** Peroxisomes (300 μg of protein) were incubated for 30 min, the reactions were terminated, and the methyl esters were made and separated by HPLC by eluting the column at 1 ml/min with acetonitrile/water (85:15, v/v).

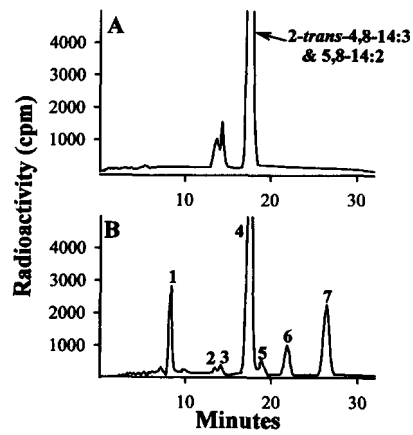

FIG. 7. **HPLC radiochromatograms of the products produced when 100 μM [1-^{14}C]5,8-14:2 was incubated without (*A*) and with (*B*) NADPH.** Peroxisomes (300 μg of protein) were incubated for 30 min, the reactions were terminated, and methyl esters were made and separated by HPLC by eluting the column at 1 ml/min with acetonitrile/water (85:15, v/v).

cycle of β-oxidation. The mass spectrum of the methyl ester-trimethylsilyl ether of compound 1 had a molecular ion at *m/z* 408 (6%) and a major ion at *m/z* 393 (M − 15; 47%). Its spectrum was identical with that of the methyl ester-trimethylsilyl derivative of synthetic 3-hydroxy-8,11,14-20:3. Compound 4 is methyl 2-*trans*-4,8,11,14-20:5. Compounds 7 and 8 were identified by comparing their retention times on HPLC and their mass spectra with authentic standards. They are, respectively, methyl 2-*trans*-8,11,14-20:4 and methyl 8,11,14-20:3.

When [1-^{14}C]5,8-14:2 was incubated without nucleotides, two radioactive metabolites eluted at 13.7 and 14.4 min (Fig. 7*A*). They were collected together and, when analyzed by GC-MS, it was possible to detect two compounds, both of which had molecular ions at *m/z* 236, which is two mass units less than for the molecular ion of methyl 5,8-14:2. The major radioactive compound had a retention time identical with methyl [1-^{14}C]5,8-14:2. When it was isolated and analyzed by GC-MS, two compounds were separated, one of which was unmetabolized substrate while the second compound had a molecular ion at *m/z* 236. The ultraviolet spectrum of this composite sample (Fig. 4*D*) shows that it contains methyl 2-*trans*-4,8-14:3. Since the compounds eluting at 13.7 and 14.4 min also had molecular ions at *m/z* 236, they undoubtedly are 2-*trans*-5,8-14:3 and 3,5,8-14:3. It was not possible to obtain satisfactory mass spectra of the analogous 5,8,11,14-20:4 metabolites, *i.e.* compounds 2 and 3 (Fig. 6*B*); but, most likely, they are methyl 2-*trans*-5,8,11,14-20:5 and 3,5,8,11,14-20:5.

When [1-^{14}C]5,8-14:2 was incubated with peroxisomes in the presence of NADPH, the HPLC radiochromatogram in Fig. 7*B* shows that it was also possible to detect several radioactive metabolites. The mass spectrum of the methyl ester-trimethylsilyl ether of compound 1 had a molecular ion at *m/z* 328 (2%) and an ion at *m/z* 313 (M − 15; 27%) showing that it is 3-hydroxy-8-14:1. Compounds 2 and 3 are 2-*trans*-5,8-14:3 and 3,5,8-14:3. Again, compound 4 was a mixture of unmetabolized methyl 5,8-14:2 and methyl 2-*trans*-4,8-14:3. Compound 5, which corresponds to the uncharacterized arachidonate-derived metabolite (compound 6; Fig. 6*B*) was also not identified. Compounds 6 and 7 had molecular ions respectively at *m/z* 238 and 240. By analogy with the arachidonate-derived metabolites, these undoubtedly are methyl 2-*trans*-8-14:2 and methyl 8-14:1.

DISCUSSION

It is not known *in vivo* how arachidonate distributes itself between mitochondria and peroxisomes for β-oxidation. Several types of evidence suggest that peroxisomes play a major role in this process. Studies using a variety of cells have shown that the lipoxygenase products, 12-hydroxy-5,8,10,14-20:4 (12-HETE) and 15-hydroxy-5,8,11,13-20:4 (15-HETE), are β-oxidized (25–28). With both substrates, the major or the only catabolite that accumulated had its first double bond at position 4, *i.e.* from 12-HETE it was 8-hydroxy-4,6,10-16:3 and from 15-HETE it was 11-hydroxy-4,7,9-16:3. When liver peroxisomes were incubated with 12-HETE, 8-hydroxy-4,6,10-16:3 accumulated as the sole catabolite (29). Neither skin fibroblasts from patients with Zellweger's disease (30) nor Chinese hamster ovary cells with peroxisomal deficiencies β-oxidized 12-HETE (31). When tritium-labeled 5,8,11,14-20:4 was incubated with liver peroxisomes, Hiltunen *et al.* (6) reported that a radioactive catabolite was detected which they suggested was either 5,8-14:2 or 4,7,10-16:3. Using the same incubation conditions, we were able to characterize both 4,7,10-16:3 and 2-*trans*-4,7,10-16:4 (8). When tritium-labeled arachidonic acid was incubated with fibroblasts from control patients, it was possible to detect tritium-labeled 4,7,10-16:3 in the culture medium. It was not possible to detect this catabolite when fibroblasts from a Zellweger's patient were used (32). The above studies document that peroxisomes have the capability of β-oxidizing arachidonate and the lipoxygenase products produced from arachidonate. They also show that a product always accumulates that has its first double bond at position 4.

Studies, particularly by Osmundsen and his colleagues (6, 7), have shown that arachidonate is a relatively poor substrate for peroxisomal β-oxidation when compared with other long chain acids where the first double bond is at different positions. We obtained similar results (8). In the study reported here we show that 5,8-14:2, a catabolite of arachidonate β-oxidation, is more rapidly β-oxidized than is arachidonate. The presence of a double bond at position 5 does not *per se* determine whether an acid will be rapidly β-oxidized by peroxisomes. *In vivo*, it is possible that 5,8-14:2 may never accumulate within peroxisomes if a precursor, such as 4,7,10-16:3, is converted to an acylcarnitine by peroxisomal carnitine acyltransferase (33) and transported out of peroxisomes. The immediate precursor of 5,8-14:2 is 4,7,10-16:3, the β-oxidation of which requires NADPH-dependent 2,4-dienoyl-CoA reductase. When [1-^{14}C]4,7,10-16:3 was incubated with peroxisomes, there was a time-dependent increase in the production of acid-soluble radioactivity that was enhanced by the addition of NADPH. It is not known why peroxisomes generate acid-soluble radioactivity from

[1-^{14}C]4,7,10,13,16,19-22:6 (6) or [1-^{14}C]4,7,10-16:3 when they are incubated in the absence of NADPH. The generation of acid-soluble radioactivity from [1-^{14}C]4,7,10-16:3 or [1-^{14}C]4,7,10,13,16,19-22:6 (6), in the absence of exogenous NADPH, could be explained if peroxisomes contained NADPH, when isolated by Nycodenz centrifugation. This seems unlikely since when [1-^{14}C]5,8,11,14-20:4 was incubated with NAD^+ and either $NADP^+$ (8) or NADPH, the rates of production of acid-soluble radioactivity were similar. The results suggest that when peroxisomes are incubated with NAD^+ and either $NADP^+$ or NADPH, that a transhydrogenase may operate to maintain a level of NADPH sufficient for NADPH-dependent reactions as is required for the first cycle of 4,7,10-16:3 and 5,8,11,14-20:4 β-oxidation.

Even though acids accumulated with their first double bond at position 4, when arachidonate-derived lipoxygenase products or arachidonate were incubated with peroxisomes, our results show that peroxisomes are able to β-oxidize 4,7,10-16:3. It was not possible to assay [1-^{14}C]4-10:1 β-oxidation by measuring acid-soluble radioactivity due to solubility of the substrate in 5% perchloric acid. The characterization of 2-*trans*-4-*cis*-10:2 shows that 4-10:1 is a substrate for fatty acyl-CoA oxidase. Our results show that peroxisomes have the capacity to remove the double bonds at positions 5, 8, and 11 from arachidonate, and, most likely, they are also able to remove the double bond at position 14.

Peroxisomal and mitochondrial pathways of unsaturated fatty acid degradation are identical, but the enzymes are all different. The original observation, that the removal of a double bond at position 5 during mitochondrial fatty acid β-oxidation requires a 5-reductase, has been modified. Both Schulz and his co-workers (10), as well as Tserng and collaborators (11), have shown that NADPH-dependent 2,4-dienoyl-CoA-reductase and a new enzyme, $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase, are required. Both groups of investigators have purified a mitochondrial protein with this enzymatic activity (12, 13).

When we incubated peroxisomes with [1-^{14}C]4,7,10-16:3, it was possible to isolate two 16-carbon acids containing a conjugated triene structure. The implication of this finding is that 2-*trans*-4,7,10-16:4 was isomerized to 3,5,7,10-16:4 via reversal of the $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase-catalyzed reaction. When 5-8:1, a product formed after five cycles of β-oxidation of 9,12,15-18:3, was converted to 3,5-8:2, it was a substrate for mitochondrial dienoyl-CoA isomerase but it was inactive as a substrate with Nycodenz-purified peroxisomes (12). The authors note that the results either indicate that peroxisomes do not have dienoyl-CoA isomerase activity or they do not recognize a short chain substrate. The same investigators reported that only small amounts of 2-*trans*-4-*trans*-8:2 were converted to the 3,5-8:2 isomer by their purified dienoyl-CoA isomerase. It thus appears that the synthesis of the 16:4 isomeric conjugated trienes could not be formed by reversal of the dienoyl-CoA isomerase. There are two important differences in the studies reported by Schulz and colleagues (12) *versus* the studies reported here. The metabolism of 2-*trans*-4,7,10-16:3 to 3,5,7,10-16:4 results in the synthesis of a stable conjugated triene system. When either [1-^{14}C]4,7,10,13,16,19-22:6 or [1-^{14}C]-4,7,10,13,16-22:5 were incubated with peroxisomes, three radioactive metabolites also eluted immediately prior to unmetabolized substrate.[2] When [1-^{14}C]4-10:1, a short chain acid, was incubated with peroxisomes, it was only possible to isolate 2-*trans*-4-*cis*-10:2 suggesting that it was not converted to the 3,5-10:2 isomer. It is not yet known whether the mitochondrial

[2] B. S. Mohammed, D. L. Luthria, and H. Sprecher, unpublished observations.

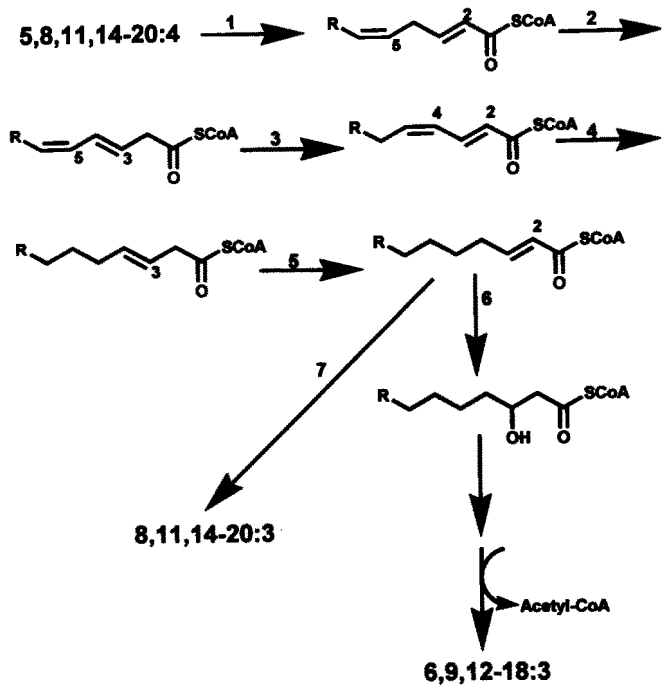

FIG. 8. **The proposed pathway for the peroxisomal β-oxidation of arachidonic acid.** The enzymes in this pathway are fatty acid oxidase (*1*), the trifunctional enzyme with Δ^3,Δ^2-enoyl-CoA isomerase activity (*2*), $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (*3*), NADPH-dependent 2,4-dienoyl-CoA reductase (*4*), the trifunctional enzyme (*5* and *6*), and an NADPH-dependent 2-*trans*-acyl-CoA reductase (*7*).

dienoyl-CoA isomerase has the ability to metabolize appropriate substrates into conjugated trienes.

The metabolic studies with 4,7,10-16:3 provides evidence that peroxisomes have dienoyl-CoA isomerase, and, moreover, the data suggest that the enzyme may be a different protein than is found in mitochondria. More conclusive evidence for a peroxisomal dienoyl-CoA isomerase was obtained by characterizing the products that accumulate when incubations were carried out with and without NADPH. In this regard, both 5,8-14:2 and 5,8,11,14-20:4 are metabolized via identical pathways.

The following observations suggest that arachidonate is degraded as shown in Fig. 8. 1) As expected, when peroxisomes were incubated with 6,9,12-18:3 and 7,10,13,16-22:4, without nucleotides, it was possible to isolate 3-hydroxy-6,9,12-18:3 and 3-hydroxy-7,10,13,16-22:4. 2) When arachidonate was incubated under identical conditions, no polar metabolite was produced, but 2-*trans*-4,8,11,14-20:5 accumulated. This finding implies that 2,4-dienoyl-CoA reductase is required for the first cycle of β-oxidation. 3) When incubations contained NADPH, a polar catabolite accumulated, that was shown to be 3-hydroxy-8,11,14-20:3. 4) When incubations contained NADPH, both 2-*trans*-8,11,14-20:4 and 8,11,14-20:3 were also produced. The 2-*trans*-8,11,14-20:4 serves as a substrate for the trifunctional enzyme with hydratase activity, to yield 3-hydroxy-8,11,14-20:3. In addition, NADPH-dependent reduction will yield 8,11,14-20:3. In 1974, Kunau and Bartnik (34) showed that when mitochondria were incubated with a variety of acids with their first double bond at position 4, that products were produced containing the same number of carbon atoms, but the double bond at position 4 was reduced. They subsequently partially purified a protein with 2-*trans*-reductase activity that was different from 2,4-dienoyl-CoA reductase (35). Our results suggest that peroxisomes have this activity. Horie *et al.* (36) showed that when peroxisomes were incubated with reduced pyridine nucleotides and acetyl-CoA, they had an acetyl-CoA-dependent chain elongation system. The conversion of 2-*trans*-

8,11,14-20:3 to 8,11,14-20:3 is, in essence, the last step in this process.

It should be noted that one complete cycle of arachidonate β-oxidation in peroxisomes yields 6,9,12-18:3. This catabolite, as well as 8,11,14-20:3, produced via NADPH-dependent reduction of 2-*trans*-8,11,14-20:4, are the two immediate precursors for the synthesis of arachidonate in the endoplasmic reticulum. We reported that when 7,10,13,16-22:4 was incubated with microsomes and peroxisomes, it was rapidly β-oxidized to 5,8,11,14-20:4. However, once arachidonate was produced, it was preferentially transported out of peroxisomes and used as a substrate for microsomal acylation into 1-acyl-*sn*-glycero-3-phosphocholine rather than serving as a substrate for continued β-oxidation (8). The possibility thus exists, that when arachidonate is metabolized to 6,9,12-18:3 and 8,11,14-20:3, these acids may move out of peroxisomes for conversion back to arachidonate in the endoplasmic reticulum.

In summary, our results suggest that both odd-numbered double bonds in arachidonate require 2,4-dienoyl-CoA reductase and the dienoyl-CoA isomerase for their removal during peroxisomal β-oxidation. It remains to be determined, *in vivo*, how arachidonate partitions itself between mitochondria and peroxisomes for β-oxidation. Additional mitochondrial studies are also required to determine what fractional amount of arachidonate is degraded via the classical pathway *versus* the newly described pathway that requires the two ancillary enzymes (9–13).

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