Docosahexaenoic acid synthesis in human skin fibroblasts involves peroxisomal retroconversion of tetracosahexaenoic acid[1]

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Abstract The purpose of this study was to determine whether the formation of docosahexaenoic acid in human cells occurs through a pathway that involves 24-carbon n-3 fatty acid intermediates and retroconversion. Normal human skin fibroblasts synthesized radiolabeled docosahexaenoic acid from [1-^{14}C]18:3n-3, [3-^{14}C]22:5n-3, [3-^{14}C]24:5n-3, and [3-^{14}C]24:6n-3. The amount of docosahexaenoate formed was reduced in fibroblasts defective in peroxisomal biogenesis, by 90–100% in Zellweger's syndrome and by 50–75% in infantile Refsum's disease. Fatty acid elongation and desaturation were intact in these mutant cells. No decrease in radiolabeled docosahexaenoic acid production occurred in mutant fibroblasts defective in peroxisomal α-oxidation or mitochondrial β-oxidation, or in normal fibroblasts treated with methyl palmoxirate to inhibit mitochondrial β-oxidation. Therefore, the retroconversion step in docosahexaenoic acid formation occurs through peroxisomal β-oxidation in normal human cells. These results demonstrate that the pathway for docosahexaenoic acid synthesis in human cells involves 24-carbon intermediates. The limited ability to synthesize docosahexaenoic acid may underlie some of the pathology that occurs in genetic diseases involving peroxisomal β-oxidation.—**Moore, S. A., E. Hurt, E. Yoder, H. Sprecher,** and A. A. Spector. Docosahexaenoic acid synthesis in human skin fibroblasts involves peroxisomal retroconversion of tetracosahexaenoic acid. *J. Lipid Res.* 1995. **36:** 2433–2443.

Supplementary key words omega-3 fatty acid • essential fatty acid • polyunsaturated fatty acid • fatty acid β-oxidation • Zellweger's syndrome • infantile Refsum's disease

Docosahexaenoic acid (DHA or 22:6), the most abundant n-3 fatty acid normally present in human and animal tissues, has important effects on membrane structure and function (1, 2). Phospholipids that contain DHA form structural domains in the lipid bilayer that are optimal for the function of certain integral proteins involved in signal transduction or membrane transport (3–5). Therefore, it is essential to understand the factors that regulate the production and availability of DHA for tissue lipid synthesis.

Like other essential polyunsaturated fatty acids, DHA cannot be synthesized de novo by human or animal tissues. It is either obtained preformed in the diet or synthesized from n-3 fatty acid precursors such as linolenic acid (18:3) and eicosapentaenoic acid (20:5) (6–8). Synthesis from these precursors involves a series of chain elongation and desaturation reactions (9). The final reaction in this pathway has long been thought to be the conversion of docosapentaenoic acid (22:5n-3) to DHA mediated by an acyl-CoA 4-desaturase. However, recent studies with rat liver preparations suggest that this conversion may instead occur through a more circuitous route in which tetracosapentaenoic acid (24:5n-3) and tetracosahexaenoic acid (24:6n-3) are formed, followed by retroconversion of the 24:6 to 22:6 (10, 11).

As the direct studies of this new pathway have been restricted to rat liver and indirect supportive evidence limited to studies in the cat (12), the present study was designed to determine whether 24:5 and 24:6 also are intermediates in DHA synthesis in intact human cells and, if so, to further determine how 24:6 is converted to DHA. Human skin fibroblasts were chosen for this work because mutant cell lines deficient in peroxisomal and

Abbreviations: DHA, docosahexaenoic acid; FBS, fetal bovine serum; HPLC, high performance liquid chromatography. Fatty acids are abbreviated as number of carbon atoms:number of double bonds. To avoid confusion in the case of certain unsaturated fatty acids, the suffix n-3 or n-6 is added to indicate the positions of the double bonds.

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mitochondrial fatty acid oxidation are available for comparative studies. Patients with genetic peroxisomal biogenesis disorders (13), especially Zellweger's syndrome (14), have very low levels of DHA in the plasma and tissues (15–17). The present results demonstrate that DHA is synthesized from n–3 fatty acid precursors in a normal human cell, that 24:5 and 24:6 are intermediates in the pathway, and that the retroconversion of 24:6 to DHA occurs through peroxisomal β-oxidation. Furthermore, they show that DHA synthesis is severely limited in Zellweger's syndrome fibroblasts because the retroconversion reaction is defective in these peroxisomal deficient mutants.

METHODS

Materials

Fatty acids and fatty acid methyl esters were purchased from NuChek Prep (Elysian, MN) or synthesized as previously described (10). Radiolabeled [1-^{14}C]18:3n–3 and [1-^{14}C]20:5n–3 were purchased from New England Nuclear (DuPont, Boston, MA), while [3-^{14}C]22:5n–3, [3-^{14}C]24:5n–3, and [3-^{14}C]24:6n–3 were synthesized as previously described (10). Each radiolabeled fatty acid had a specific activity of approximately 55 mCi/mmol. Methyl palmoxirate (2-tetradecyloxirane carboxylate) was a generous gift from the R. W. Johnson Pharmaceutical Research Institute.

Cell culture

Normal human skin fibroblasts (GM08333) and human fibroblasts deficient in mitochondrial very long-chain acyl-CoA dehydrogenase (GM06127), which cannot carry out mitochondrial long-chain fatty acid β-oxidation, were purchased from the American Type Culture Collection (Rockville, MD). Additional normal, Zellweger's syndrome, which are defective in peroxisomal biogenesis, and adult Refsum's disease fibroblasts, which are defective in peroxisomal α-oxidation, were obtained from the University of Iowa Pediatrics Cytogenetics Laboratory. Infantile Refsum's disease fibroblasts, a less severe form of peroxisomal biogenesis defect, and additional Zellweger's syndrome fibroblasts (complementation group I) were provided by the Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts from two normal and two Zellweger's syndrome patients were tested; other mutant fibroblast types were from single patients. Each fibroblast type was grown in Eagle's medium supplemented with 10% FBS, glutamine, nonessential amino acids, vitamins, and penicillin/streptomycin. Cells were subcultured with trypsin and used between passages 7 and 22.

Experimental protocol

The uptake, elongation, and desaturation of essential fatty acids was studied in confluent cultures of fibroblasts grown in tissue culture flasks. Incubations were carried out in Eagle's culture medium containing 10% FBS, 2 μM ^{14}C-labeled fatty acid, and 0–40 μM unlabeled fatty acid. Cells were maintained at 37°C in a 5% CO_2 incubator during all of the incubations.

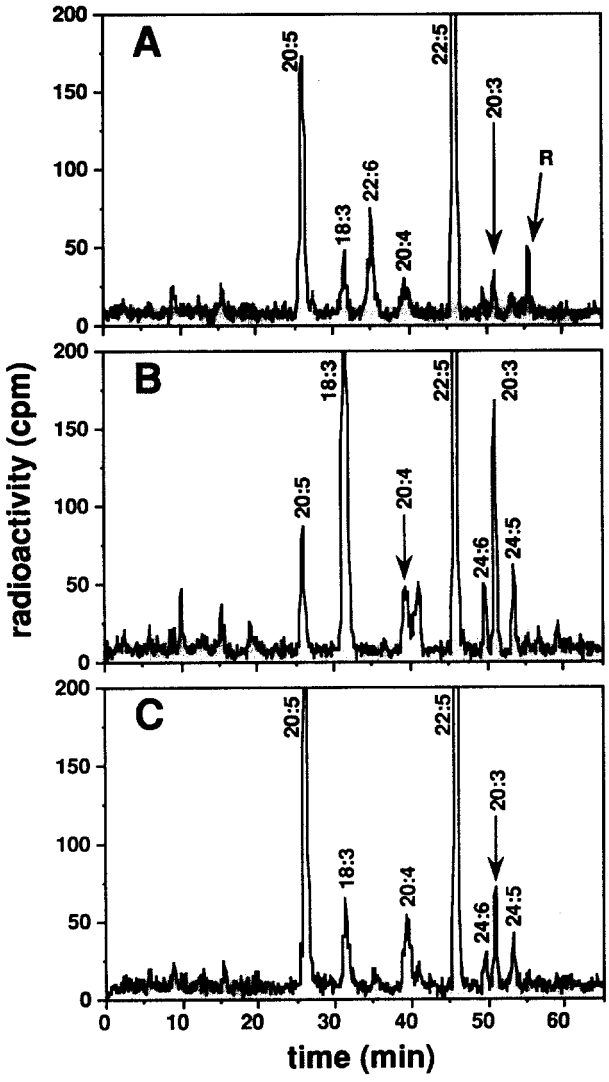

Fig. 1. Linolenic acid utilization. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after a 65-h incubation of human skin fibroblasts with [1-^{14}C]18:3n–3; (A) normal; (B) Zellweger's syndrome; and (C) infantile Refsum's disease. Chromatograms representative of at least three separate cultures of each fibroblast type are shown here and in all other HPLC figures. The acetonitrile was 76% for the first 45 min, then increased to 100% over 1 min, and held there until 60

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TABLE 1. n-3 Fatty acids produced by human fibroblast cultures from radiolabeled precursors

Fatty Acid Substrate	Fibroblast Type	Amount of Radiolabeled Fatty Acid Detected						
		18:3	20:3	20:5	22:5	24:5	24:6	22:6
[1-^{14}C]18:3n-3	Normal	188 ± 22	97 ± 9	720 ± 8	1704 ± 4	65 ± 10	89 ± 14	349 ± 55
	Zellweger's	965 ±150	462 ± 43	371 ± 81	1094 ± 104	130 ± 6	179 ± 21	ND
	Infantile Refsum's	296 ± 36	188 ± 7	984 ± 22	1338 ± 36	105 ± 9	167 ± 38	110 ± 11
[3-^{14}C]22:5n-3	Normal			552 ± 35	2163 ± 33	49 ± 22	67 ± 7	533 ± 40
	Zellweger's			41 ± 7	2521 ± 19	223 ± 2	456 ± 11	ND
	Infantile Refsum's			229 ± 1	2728 ± 10	61 ± 1	228 ± 17	251 ± 3
[3-^{14}C]24:5n-3	Normal				423 ± 5	228 ± 10	150 ± 15	1984 ± 75
	Zellweger's				83 ± 21	583 ± 12	1815 ± 3	140 ± 8
	Infantile Refsum's				161 ± 26	366 ± 12	1272 ± 12	561 ± 6
[3-^{14}C]24:6n-3	Normal				98 ± 20		201 ± 12	2910 ± 91
	Zellweger's				ND		2359 ± 49	182 ± 26
	Infantile Refsum's				ND		1571 ± 25	740 ± 16

Radiolabeled fatty acid substrates (2 μM) were incubated 65 h with confluent cultures of human skin fibroblasts derived from normal subjects or from patients with infantile Refsum's disease or Zellweger's syndrome. The methyl esters of total radiolabeled fatty acids (combined medium and cell lipids) were separated by reverse phase HPLC. Cultures were studied in triplicate and the mean ± SEM radiolabeled n-3 fatty acid values are shown; ND, not detected.

Lipid analyses

Lipids were extracted from the incubation medium by a modification of the procedure of Folch, Lees, and Sloane Stanley (18). The medium was centrifuged to remove cellular debris, then extracted with a 2:1 (v/v) mixture of chloroform–methanol containing 1% acetic acid. The chloroform phase was dried under N_2 and the residue was resuspended in acetonitrile.

To isolate cell lipids, the incubation medium was removed and the cells were washed with serum-free medium containing 10 μM fatty acid-free bovine serum albumin, scraped into methanol, and transferred to a siliconized screw-top glass test tube (19). One volume of chloroform was added, and after vortexing and adding one volume of 0.88% KCl, each sample was centrifuged at 1300 *g* for 10 min to separate the phases. After washing the aqueous phase with one volume of chloroform, the two chloroform layers were combined, dried under N_2, and resuspended in chloroform–methanol 2:1 (v/v).

Aliquots of the total cell lipid, total medium lipid, or pooled medium and cell lipid extracts were combined with an excess of 14% BF_3 in methanol and heated to 100°C for 15 min to produce fatty acid methyl esters (20). After the addition of water, the methyl esters were extracted in heptane, dried under N_2, and resuspended in acetonitrile.

High performance liquid chromatography (HPLC)

Radioactive methyl esters prepared from the cell lipids or incubation medium were separated by reverse phase HPLC by a modification of the method of Aveldaño, VanRollins, and Horrocks (21). Briefly, a 4.6 × 150 mm C18 reverse phase Beckman HPLC column with 5 μm spherical packing was used with a mobile phase of water and acetonitrile in a two-step isocratic elution. The more polar fatty acid methyl esters were eluted with either 76% or 86% acetonitrile, while the less polar esters were eluted with 100% acetonitrile. The 76% acetonitrile protocol was used for those samples containing both radiolabeled 18:3n-3 and 22:6n-3, as these two fatty acids co-elute at 86% acetonitrile. The 86% acetonitrile protocol was necessary to reliably separate the 24-carbon n-3 fatty acids from 20:3n-3 and from 16- to 20-carbon saturated and monounsaturated fatty acids. Radioactivity was monitored by mixing the column effluent with scintillator solution at a 1:3 ratio and passing the mixture through an on-line Radiomatic Instruments Flo One-β radioactivity detector (Radioanalytic, Tampa, FL). The systems were standardized with methyl esters of the following radiolabeled fatty acids: 16:0, 16:1, 18:1, 18:3n-3, 20:4n-6, 20:5n-3, 22:5n-3, 24:5n-3, 24:6n-3, and 22:6n-3.

RESULTS

Linolenic acid utilization

The radiolabeled fatty acids contained in cell cultures after incubation of normal and peroxisomal-deficient human skin fibroblasts with [1-^{14}C]18:3n-3 are shown in the HPLC tracings illustrated in **Fig. 1.** After a 65-h

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incubation with normal fibroblasts, 10% of the radioactivity was found in 22:6. Radioactivity was present in other n–3 fatty acids, including 20:3, 20:5, 22:5, 24:5 and 24:6 (Fig. 1A and **Table 1**). An additional radiolabeled component, R, contains a mixture of 16- and 18-carbon saturated and monounsaturated fatty acids (8).

As opposed to the normal cells, the peroxisomal-deficient Zellweger's syndrome fibroblasts formed no radiolabeled 22:6 even though the [1-^{14}C]18:3 was taken up and converted to other n–3 fatty acid intermediates in the biosynthetic pathway, including 4% conversion to 24:5 and 5% to 24:6 (Fig. 1B and Table 1). Several other differences were apparent in the Zellweger cell cultures. They contained relatively large amounts of unmodified 18:3 and 20:3, an elongation product that is not an intermediate in the 22:6 biosynthetic pathway (10), and they retained a relatively small amount of radiolabeled 20:5 even though a sizable quantity of radiolabeled 22:5 was formed.

An intermediate result was obtained with infantile Refsum's disease fibroblasts, which have a less severe peroxisomal deficiency than the Zellweger cells (22). The infantile Refsum cells converted 70% less 18:3 to 22:6 than normal fibroblasts, but they also retained less unmodified 18:3 and formed less 20:3, 24:5 and 24:6 than the Zellweger cells (Fig. 1C and Table 1).

Several small unknown peaks were also present in these chromatograms and in the chromatograms of later figures. In aggregate, they usually represented less than 5%, and never exceeded 10%, of total radioactivity in a given sample. No attempts were made at identifying these minor metabolites.

Docosapentaenoic acid utilization

A similar difference between normal and Zellweger fibroblasts was observed in studies with [3-^{14}C]22:5n–3. After incubation for 65 h, the normal fibroblasts produced large quantities of radiolabeled 22:6 and 20:5, as well as a small amount of 24:5 and 24:6 (**Fig. 2A** and Table 1). The radiolabeled component R containing a mixture of 16- and 18-carbon saturated and monounsaturated fatty acids was also detected. A different pattern was observed after incubation of the Zellweger fibroblasts with [3-^{14}C]22:5. These mutants accumulated large amounts of 24:5 and 24:6 (5- to 8-times more than observed with the normal fibroblasts), but they produced no radiolabeled 22:6 and almost no radiolabeled 20:5 from the [3-^{14}C]22:5 (Fig. 2B and Table 1). They also produced three radiolabeled unknown compounds, designated X, Y, and Z, with longer retention times than the reutilization peak R. An intermediate result was observed with the infantile Refsum cells. They converted small amounts of the [3-^{14}C]22:5 to 22:6 and 20:5, less than half of that observed with the normal

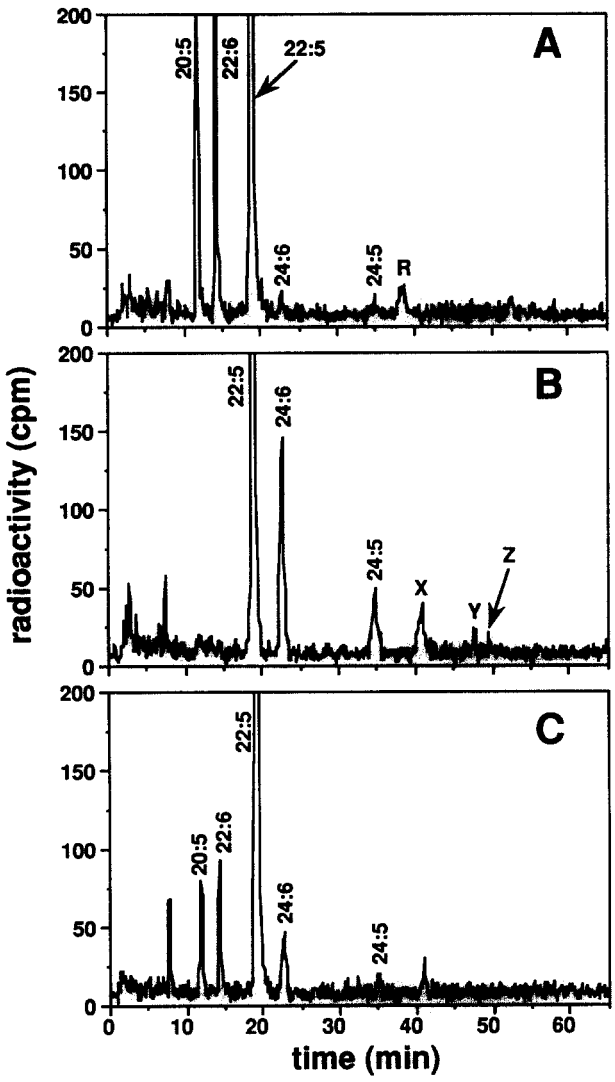

Fig. 2. Docosapentaenoic acid utilization. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after a 65-h incubation of human skin fibroblasts with [3-^{14}C]22:5n–3; (A) normal; (B) Zellweger's syndrome; and (C) infantile Refsum's disease. The acetonitrile was 86% for the first 35 min, then increased to 100% over 2 min, and held there until 50 min when the system was returned to 86%.

fibroblasts, and they accumulated 30–50% less radiolabeled 24:5 and 24:6 than the Zellweger cells (Fig. 2C and Table 1).

Tetracosapentaenoic acid utilization

The results with [1-^{14}C]18:3 and [3-^{14}C]22:5 suggested that, similar to rat liver (10), the pathway for DHA synthesis from n–3 fatty acid precursors in human fibroblasts involves the formation of 24-carbon polyunsaturated intermediates, followed by retroconversion of 24:6 to 22:6. To more directly evaluate the putative retroconversion reaction, cells were incubated with [3-

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^{14}C]24:5n-3. The normal fibroblasts converted more than half of the radiolabeled 24:5 to 22:6, and they produced a substantial amount of 22:5 and some 24:6 (**Fig. 3A** and Table 1). Radioactivity was also recovered in peak R, indicating reutilization of some of the labeled acetyl-CoA generated from the 24:5 substrate for fatty acid synthesis.

As opposed to these findings, the Zellweger fibroblasts converted only very small amounts of [3-^{14}C]24:5 to 22:6 or 22:5, 7% and 20% of the values obtained with the normal fibroblasts, respectively (Fig. 3B and Table 1). Instead, they formed large amounts of 24:6, which comprised half of the fatty acid radioactivity, retained more unmodified 24:5, and produced three radiolabeled unknown compounds, designated X, Y, and Z, which have longer retention times than the reutilization component, peak R. While the infantile Refsum cells also accumulated 24:6 and unknown peaks X, Y, and Z, they still were able to produce a moderate amount of radiolabeled 22:6 from the [3-^{14}C]24:5, 28% of the value obtained with the normal fibroblasts (Fig. 3C and Table 1).

Tetracosahexaenoic acid utilization

Figure 4 shows that similar results were obtained with [3-^{14}C]24:6n-3, the direct precursor of 22:6. Normal fibroblasts converted 24:6 primarily to 22:6 (Fig. 4A and Table 1), whereas the Zellweger fibroblasts produced very little radiolabeled 22:6, only 6% as much as the normal fibroblasts. Much of the [3-^{14}C]24:6n-3 in the Zellweger cell cultures remained as unmodified 24:6, but a considerable amount was converted to unknown peaks X and Y, 25% and 4% of total radioactivity, respectively (Fig. 4B and Table 1). While the infantile Refsum fibroblast cultures also retained unmodified 24:6 and formed the unknown peaks X and Y, they were able to produce a substantial amount of 22:6 from [3-^{14}C]24:6, 25% of the value obtained with the normal fibroblasts (Fig. 4C and Table 1).

Competition experiment to test for acyl-CoA 4-desaturase activity

To determine whether the more direct acyl-CoA 4-desaturase pathway might be present in addition to the retroconversion pathway, normal human fibroblasts were incubated with 2 μM [3-^{14}C]22:5n-3 in the presence of 0–40 μM unlabeled 24:5n-3, 24:6n-3, or 18:1. **Figure 5** (top panel) demonstrates that the conversion of radiolabeled 22:5 to 22:6 was reduced when either unlabeled 24:5n-3 or 24:6n-3 was added to the medium. This was not due to inhibition of retroconversion because labeled 20:5 production from [3-^{14}C]22:5 was not reduced; it was increased when unlabeled 24:5 or 24:6 were present (Fig. 5, bottom panel). Addition of unlabeled 18:1 had

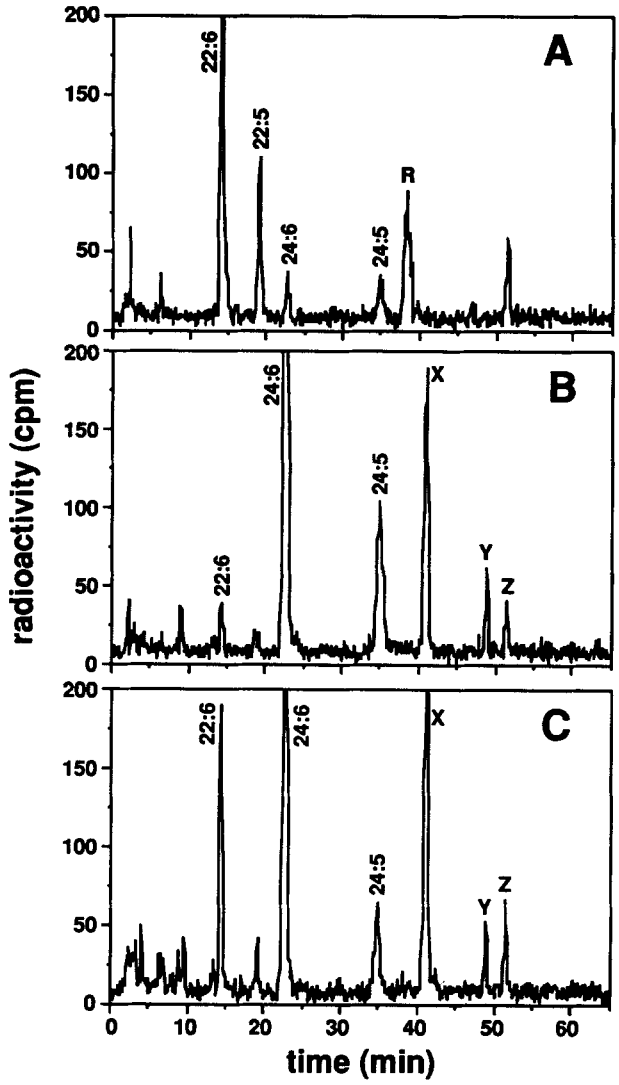

Fig. 3. Tetracosapentaenoic acid utilization. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after a 65-h incubation of human skin fibroblasts with [3-^{14}C]24:5n-3; (A) normal; (B) Zellweger's syndrome; and (C) infantile Refsum's disease. The separation gradient is the same as Fig. 2.

minimal effect on either process, indicating specificity for the 24-carbon intermediates (10). A likely explanation for the decrease in radioactive 22:6 formation is that the specific radioactivity of the 24-carbon intermediates formed from [3-^{14}C]22:5 is lower because of mixing with the added unlabeled 24:5 or 24:6. This is consistent with a mechanism in which 24:5 and 24:6 are intermediates in the conversion of 22:5 to 22:6. It also seems likely that 22:5 conversion to the 24-carbon intermediates is reduced by the presence of preformed 24:5 or 24:6, for this would explain the increased availability

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of [3-^{14}C]22:5 for retroconversion to 20:5 when unlabeled 24:5 or 24:6 are added.

Mechanism of retroconversion

Taken together, the data obtained with [3-^{14}C]22:5, [3-^{14}C]24:5, and [3-^{14}C]24:6 indicate that in human skin fibroblasts, 24-carbon n–3 fatty acids are intermediates in 22:6 biosynthesis. The inability of the Zellweger fibroblasts to produce DHA from these radiolabeled precursors indicates that the retroconversion of 24:6 to 22:6 either occurs in the peroxisomes or is somehow dependent on normal peroxisomal function.

To obtain more information regarding the mechanism of retroconversion, [3-^{14}C]24:6n–3 was incubated with normal human skin fibroblasts in the presence of methyl palmoxirate, a carnitine palmitoyltransferase-I inhibitor (23). The incubation was shortened to 18 h to prevent cytotoxicity that might result from prolonged exposure to a metabolic inhibitor. Even at this early time, a substantial amount of 24:6 was converted to 22:6 when the inhibitor was not present (**Fig. 6A**). The addition of methyl palmoxirate did not appreciably reduce the conversion of 24:6 to 22:6, 44 ± 2% in the controls as compared with 43 ± 3% when methyl palmoxerate was added (Fig. 6B), even though the inhibitor blocks mitochondrial β-oxidation under these conditions (24). Therefore, mitochondria appear to be excluded as the site of retroconversion. The methyl palmoxerate data and this conclusion are corroborated by studies with mutant human fibroblasts that cannot carry out mitochondrial long-chain fatty acid oxidation. **Figure 7A** illustrates that cells deficient in the mitochondrial very long-chain acyl-CoA dehydrogenase convert large amounts of [3-^{14}C]24:6n–3 to 22:6 during a 65-h incubation. The amount converted to 22:6, 76%, is similar to the 81% conversion to 22:6 obtained with normal fibroblasts incubated for the same length of time (Fig. 4A and Table 1). Taken together, these data indicate that the retroconversion occurs through peroxisomal oxidation.

As the Zellweger fibroblasts are deficient in peroxisomal α- and β-oxidation, another peroxisomal mutant was used to distinguish between these possibilities. The conversion of [3-^{14}C]24:6n–3 to 22:6 was tested during 65-h incubations with adult Refsum's disease fibroblasts which are only deficient in fatty acid α-oxidation. The adult Refsum cells converted 24:6 to 22:6 in amounts similar to normal fibroblasts (Fig. 7B), indicating that the retroconversion of 24:6 to 22:6 does not occur through fatty acid α-oxidation.

Radioactivity from [3-^{14}C]24:6 was incorporated into the acetyl-CoA reutilization component, peak R, in the normal human skin fibroblasts (Fig. 4) as well as in those deficient in mitochondrial long-chain fatty acid β-oxidation and fatty acid α-oxidation (Fig. 7), but not in the

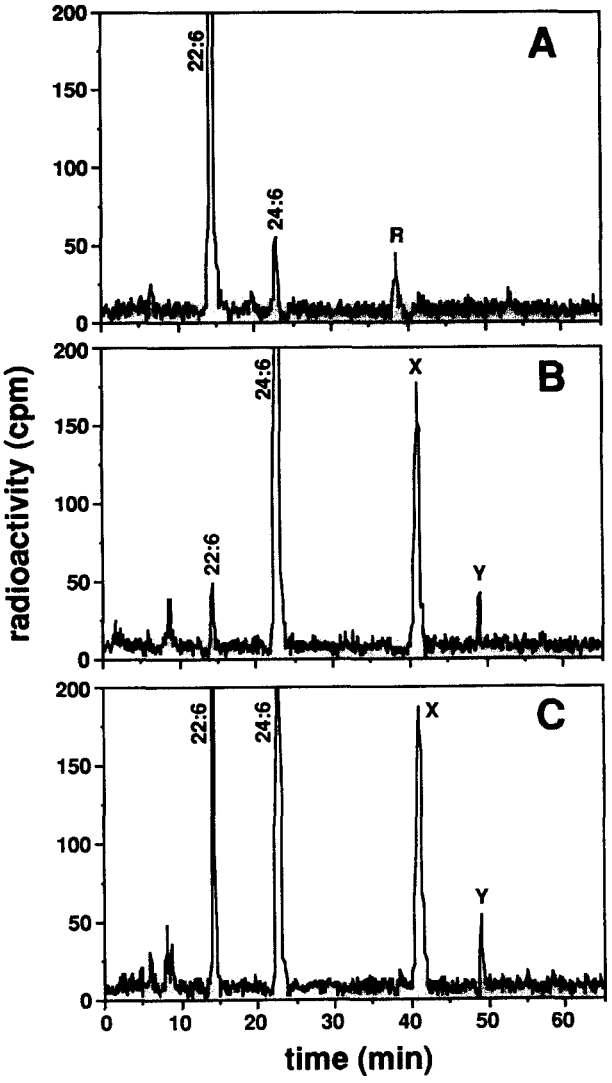

Fig. 4. Tetracosahexaenoic acid utilization. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after a 65-h incubation of human skin fibroblasts with [3-^{14}C]24:6n–3; (A) normal; (B) Zellweger's syndrome; and (C) infantile Refsum's disease. The separation gradient is the same as Fig. 2.

Zellweger and infantile Refsum cells (Fig. 4). This finding is in agreement with previous results indicating that acetyl-CoA generated by peroxisomal β-oxidation can be reutilized for fatty acid synthesis (25, 26). Additional studies indicate that human fibroblasts are also capable of retroconverting 22:6n–3 by peroxisomal β-oxidation. In separate incubations of normal fibroblasts with [4,5-^{3}H]22:6n–3, substantial amounts of radiolabeled 20:5 and 22:5 were detected by HPLC (data not shown).

Uptake of n–3 fatty acids

Because some of the observed differences in n–3 fatty acid metabolism might be explained by differences in

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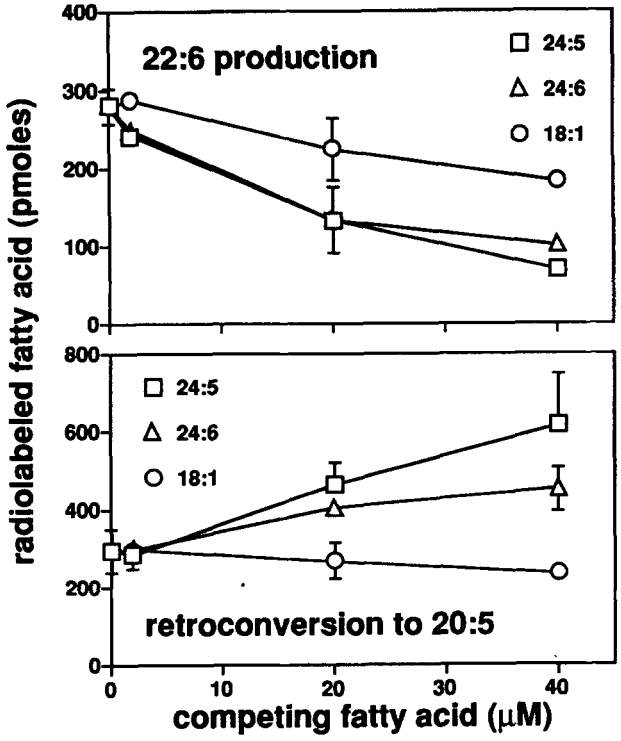

Fig. 5. Synthesis of radiolabeled DHA from [3-^{14}C]22:5n-3: competition by other fatty acids. Normal human skin fibroblasts were incubated 48 h in 2 μM [3-^{14}C]22:5n-3 with or without increasing concentrations of unlabeled 24:5, 24:6, or 18:1. Pooled cell and medium fatty acid methyl esters were separated and assayed by HPLC. The upper panel shows radiolabeled 22:6 production and the lower panel the radiolabeled 20:5 production. Data points are the mean ± SEM from triplicate cultures. Where error bars are omitted, they are too small to be visible.

the uptake of fatty acids between the normal human fibroblasts and the genetic mutants, this parameter was closely monitored in all experiments. In all cases, uptake varied by less than 10% within a given experiment among the normal, peroxisomal-deficient, α-oxidation-deficient, and mitochondrial β-oxidation-deficient cells. In the experiment presented in Table 1, for example, the uptake of 18:3 by normal, infantile Refsum's, and Zellweger's fibroblasts was 75%, 81%, and 73%, respectively, and the uptake of 22:5 was 72%, 76%, and 69%, respectively.

Comparison of fibroblasts from different subjects

To determine whether individual variation among subjects from which the fibroblasts were cultured might explain differences observed in n-3 fatty acid metabolism, some incubations were performed in fibroblasts from two normal subjects and from two patients with Zellweger's syndrome. The amount of radiolabeled 22:6 produced from [1-^{14}C]18:3n-3 by the normal human fibroblasts was 308 ± 48 and 349 ± 55 pmol during 65-h incubations, and the variation in the amount of the other radiolabeled n-3 fatty acid products formed was also small. Comparably close agreement was obtained with [3-^{14}C]24:5n-3 as the substrate; 1463 ± 128 and 1984 ± 75 pmol 22:6 was produced during 65-h incubations with the two normal fibroblast cultures. A comparison of Zellweger's syndrome fibroblasts from two patients yielded 22:6 values of 131 ± 47 and 140 ± 8 pmol after 65-h incubations with [3-^{14}C]24:6n-3. The fibroblast cultures from neither of the Zellweger's patients produced a detectable amount of radiolabeled 22:6 from [1-^{14}C]18:3n-3, but they produced very similar amounts of other n-3 fatty acids; for example, 1094 ± 104 and 1222 ± 244 pmol of 22:5.

Distribution of radiolabeled n-3 fatty acid products between cell lipids and culture medium

After incubation with radiolabeled substrates, the medium and cell lipids were extracted and analyzed separately in some experiments to determine whether some n-3 fatty acid products were selectively retained or released by the cells. The data indicate that some n-3 fatty acids were retained in the cell lipids, while others

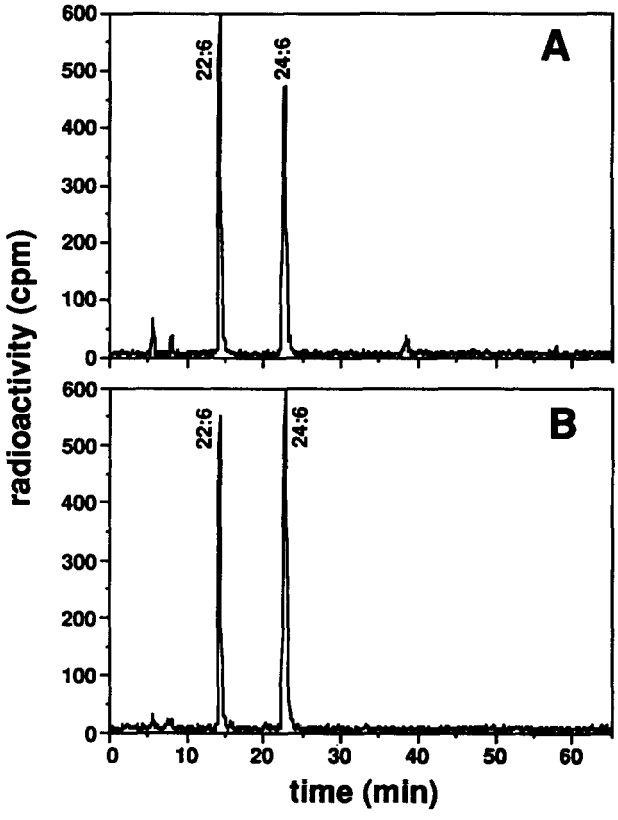

Fig. 6. Effect of methyl palmoxirate on docosahexaenoic acid synthesis from tetradocohexaenoic acid. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after an 18-h incubation of normal human skin fibroblasts with [3-^{14}C]24:6n-3; (A) control; (B) 200 μM methyl palmoxirate added to the incubation medium. The separation gradient is the same as Fig. 2.

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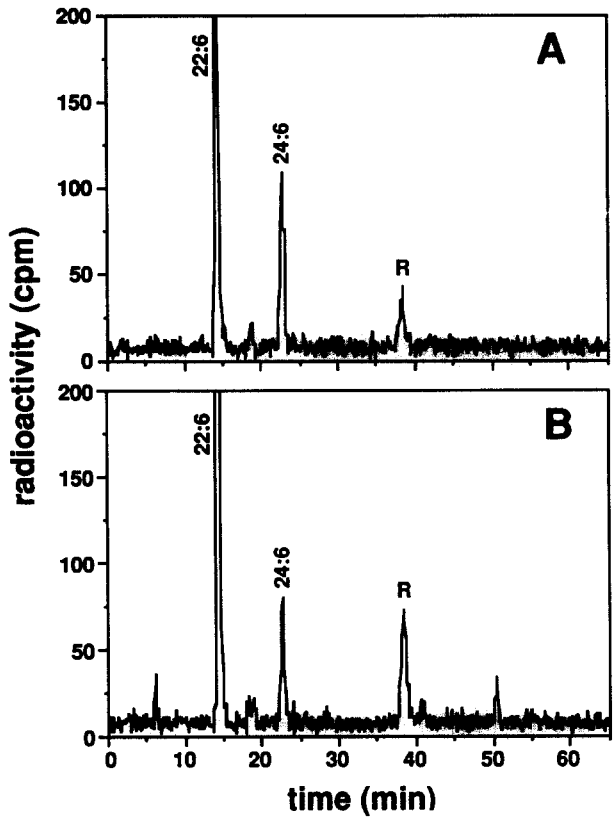

Fig. 7. Docosahexaenoic acid synthesis by fatty acid α-oxidation and mitochondrial β-oxidation mutants. These radiochromatograms were obtained by reverse phase HPLC analysis of pooled medium and cell lipid fatty acid methyl esters after a 65-h incubation of human skin fibroblasts with [3-^{14}C]24:6n-3; (A) mitochondrial very long-chain acyl-CoA dehydrogenase deficiency; (B) adult Refsum's disease. Chromatograms are representative of at least three separate cultures of each fibroblast type. The separation gradient is the same as Fig. 2.

were selectively released into the medium. The most striking examples are illustrated in **Fig. 8.** The top two radiochromatograms compare the cell lipid (Fig. 8A) and culture medium (Fig. 8B) fatty acid profiles of adult Refsum's disease fibroblasts after 65 h of incubation with [1-^{14}C]18:3n-3. There was an 8-fold greater amount of radiolabeled 22:5 in cell lipids than in the culture medium. Furthermore, in seven separate experiments with different fibroblast cultures and either [1-^{14}C]18:3n-3 or [1-^{14}C]20:5n-3 as substrate, more of the labeled 22:5 formed was retained in the cell lipids; ratios of labeled 22:5 in cell lipids:medium ranged from 2:1 to 8:1 (average ratio 4.7:1). In contrast to the preferential retention of 22:5 in cell lipids, labeled 18:4 was detected only in the medium (Fig. 8B). The only other labeled n-3 fatty acid to distribute preferentially to the medium was 24:6 (data not shown). In twelve separate experiments, there was an average 1.7-fold greater amount of 24:6 in the medium.

The radiochromatograms in Fig. 8C and D were obtained after 8-h incubations of Zellweger fibroblasts with [3-^{14}C]24:6n-3. Unknown radiolabeled metabolites X and Y distributed preferentially in the cell lipids (Fig. 8C) and were undetectable in the culture medium (Fig. 8D). Only after longer incubations (more than 16 h) were these labeled products released to the medium in detectable amounts (data not shown), but even then, their ratios in the cell lipids:medium was greater than 2:1.

Additional studies indicated that the distribution of labeled n-3 fatty acid products between the cells and medium was similar in the normal fibroblasts and three mutants, Zellweger's syndrome, adult Refsum's disease, and very long-chain acyl-CoA dehydrogenase deficiency fibroblasts. Therefore, the selective retention or release of the products was not affected appreciably by these peroxisomal or mitochondrial mutations.

DISCUSSION

DHA, the most abundant n-3 fatty acid present in human and animal tissues, has been thought to be formed directly from its precursor, 22:5n-3, by the action of a microsomal acyl-CoA 4-desaturase (9). However, recent studies with rat hepatocytes suggest that the mechanism of this conversion may be more complicated (10). According to these findings, 22:5 is elongated to 24:5, this undergoes 6-desaturation to form 24:6, and the 24:6 is then retroconverted to 22:6. Based on our studies with normal and Zellweger fibroblasts, this newly proposed mechanism for DHA synthesis is operative in human cells. When the Zellweger fibroblasts were exposed to [3-^{14}C]22:5n-3, substantial amounts of radioactivity were recovered in 24:6 even though 22:6 was not radiolabeled (Fig. 2B). Therefore, 22:6 is not an intermediate in the conversion of 22:5 to 24:6. As 24:5 was also radiolabeled, the sequence that occurs in the normal human fibroblast appears to be: elongation of 22:5 to 24:5, followed by desaturation of 24:5 to 24:6, and retroconversion of 24:6 to 22:6 (Fig. 4A). By contrast, the Zellweger fibroblasts are defective in this retroconversion reaction (Fig. 4B). This is the first demonstration that DHA formation is a peroxisomal-dependent process. Consistent with this observation, the retroconversion of 22-carbon unsaturated fatty acids to their 20-carbon products also is peroxisome-dependent (27).

A substantial decrease in DHA content occurs in the plasma and tissues of patients with Zellweger's syndrome (15–17). This has been attributed to a deficiency of the acyl-CoA 4-desaturase thought to mediate the direct conversion of 22:5n-3 to 22:6 (28). However, acyl-CoA 4-desaturase activity has never been demon-

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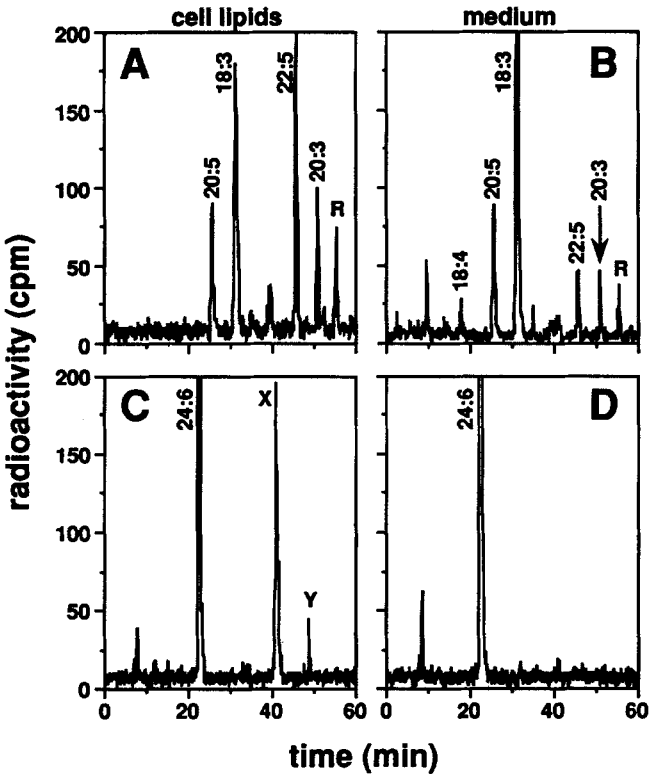

Fig. 8. Distribution of radiolabeled n–3 fatty acids between cell lipids and culture medium. These radiochromatograms of the fatty acid methyl esters were obtained by reverse phase HPLC analysis after separation of the cells from the culture medium: (A, B) after a 65-h incubation of adult Refsum's disease fibroblasts with [1-^{14}C]18:3n–3, and (C, D) after an 8-h incubation of Zellweger's syndrome fibroblasts with [3-^{14}C]24:6n–3. Chromatograms A and C show the distribution of the labeled products in the cell lipids, while B and D show the distribution in the culture medium. These distributions are representative of those observed in at least three separate cultures of each fibroblast type. The methyl esters were separated using 76% acetonitrile in A and B, 86% acetonitrile in C and D as described in Figs. 1 and 2, respectively.

strated experimentally in mammalian tissues (10). Furthermore, acyl-CoA desaturation occurs in the microsomes (10), whereas all of the known defects in Zellweger's syndrome involve peroxisomal functions (13). These inconsistencies are resolved by the present findings. As the conversion of 24:5 to 24:6 takes place in the Zellweger fibroblasts (Fig. 3B), there is no defect in the insertion of the sixth double bond in an n–3 fatty acid in this disease. What is defective is the retroconversion of 24:6 to 22:6, a peroxisomal-dependent process that results in a shift of this double bond from the Δ6- to the Δ4-position in the fatty acyl chain. Therefore, the DHA decrease in Zellweger's syndrome can be explained without having to invoke any additional defect besides the absence of peroxisomal function.

A possible explanation for the small amount of radiolabeled DHA formed by the Zellweger fibroblasts from [3-^{14}C]24:5n–3 (Fig. 3B) and [3-^{14}C]24:6n–3 (Fig. 4B) is that some retroconversion may occur through mitochondrial β-oxidation. However, a major quantitative role for mitochondrial β-oxidation is unlikely because fibroblasts deficient in very long-chain acyl-CoA dehydrogenase, as well as normal fibroblasts exposed to methyl palmoxirate, convert large amounts of 24:6 to 22:6 (Figs. 7A and 6B, respectively). These results indicate that retroconversion must be a peroxisomal process, but they do not distinguish between α- and β-oxidation because both of these processes are deficient in the Zellweger fibroblasts (13). Recent evidence suggests that α-oxidation probably plays a role in the retroconversion of polyunsaturated fatty acids in rat seminiferous tubules (29). However, α-oxidation as a major quantitative pathway for the retroconversion of 24:6 to 22:6 in human cells is excluded by the findings with the adult Refsum fibroblasts. These human cells, which are deficient in α-oxidation but have intact peroxisomal β-oxidation (30), converted substantial amounts of 24:6 to 22:6 (Fig. 7B).

The finding that infantile Refsum's disease fibroblasts, which like Zellweger fibroblasts are deficient in peroxisomal biogenesis (31), produce a moderate amount of 22:6 from 24:6 does not negate the conclusion that retroconversion occurs primarily through peroxisomal β-oxidation. Infantile Refsum's disease is a milder form of generalized peroxisomal dysfunction than Zellweger's syndrome (13), peroxisomal functions are not as severely depressed, and the children who inherit this variant of the disease have a longer lifespan (22). Therefore, it is not surprising that the infantile Refsum cells retain a greater capacity to retroconvert 24:6 than the Zellweger cells.

In agreement with the findings of Christensen et al. (27), we observed that [3-^{14}C]22:5n–3 was retroconverted to 20:5 in normal fibroblasts (Fig. 2A). This suggests that two processes contribute to endogenous 20:5 formation in human cells, synthesis from 18:3 and retroconversion from 22:5. Studies with n–6 fatty acids in rat seminiferous tubules also indicate that elongation and retroconversion both contribute to formation of corresponding fatty acids, including 20:4n–6 (29). As compared with normal fibroblasts where synthesis and retroconversion are intact (Fig. 1A), relatively little radiolabeled 20:5 accumulated when the Zellweger cells were incubated with [1-^{14}C]18:3n–3 even though large amounts of labeled 22:5 were formed (Fig. 1B). This must be due to the lack of input from retroconversion, and it suggests that retroconversion from 22:5 makes an important contribution to the 20:5 content in human tissue.

Unknowns X, Y, and Z were detected when the Zellweger and infantile Refsum fibroblasts were incubated

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with [3-^{14}C]22:5n-3, [3-^{14}C]24:5n-3, or [3-^{14}C]24:6n-3 (Figs. 2, 3, and 4, respectively). These radiolabeled compounds were not formed by the normal fibroblasts. Based on their HPLC retention times, it is likely that these compounds are elongation products of 24:5 or 24:6. This is consistent with the finding that very long-chain, highly unsaturated n-6 fatty acids, including 26:5, 28:5, 30:5, and 30:6, are present in the plasma of Zellweger's syndrome patients (32). Corresponding n-3 fatty acids have not been reported in Zellweger's syndrome, but this may be due to the fact that more n-6 than n-3 fatty acid precursors are contained in the plasma and tissues of these patients (17). Alternatively, in the peroxisomal-deficient state, there may be a shift to significant mitochondrial β-oxidation of these very long-chain n-3 fatty acids that keeps them from accumulating in the tissues of Zellweger's patients.

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