Metabolism of saturated and polyunsaturated very-long-chain fatty acids in fibroblasts from patients with defects in peroxisomal β -oxidation

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The metabolism of [$1-^{14}\text{C}$]lignoceric acid ($\text{C}_{24:0}$) and [$1-^{14}\text{C}$]tetracosatetraenoic acid ($\text{C}_{24:4,n-6}$) was studied in normal skin fibroblast cultures and in cultures from patients with defects in peroxisomal β -oxidation (but normal peroxisomal numbers). Cells from X-linked adrenoleukodystrophy (ALD) patients with a presumed defect in a peroxisomal acyl-CoA synthetase, specific for fatty acids of carbon chain lengths greater than 22 (very-long-chain fatty acids; VLCFA), showed a relatively normal production of radiolabelled CO_2 and water-soluble metabolites from [$1-^{14}\text{C}$] $\text{C}_{24:0}$. However, the products of synthesis from acetate *de novo* (released by β -oxidation), i.e. C_{16} and C_{18} fatty acids, were decreased, and carbon chain elongation of the fatty acid was increased. In contrast, cell lines from two patients with an unidentified lesion in peroxisomal β -oxidation (peroxisomal disease, PD) showed a marked deficiency in CO_2 and water-soluble metabolite production, a decreased synthesis of C_{16} and C_{18} fatty acids and an increase in carbon chain elongation. The relatively normal β -oxidation activity of ALD cells appears to be related to low uptake of substrate, as a defect in β -oxidation is apparent when measurements are performed on cell suspensions under high uptake conditions. Oxidation of [$1-^{14}\text{C}$] $\text{C}_{24:4}$ was relatively normal in ALD cells and in the cells from one PD patient but abnormal in those from the other. Our data suggest that, despite the deficiency in VLCFA CoA synthetase, ALD cells retain a near normal ability to oxidize both saturated and polyunsaturated VLCFA under some culture conditions. However, acetate released by β -oxidation of the saturated VLCFA and, to a much lesser degree, the polyunsaturated VLCFA, appears to be used preferentially for the production of CO_2 and water-soluble products, and acetate availability for fatty acid synthesis in other subcellular compartments is markedly decreased. It is likely that the increased carbon chain elongation of the saturated VLCFA which is also observed reflects the increased availability of substrate ($\text{C}_{24:0}$) and/or an increase in microsomal elongation activity in ALD cells.

INTRODUCTION

Peroxisomes are subcellular organelles found in all eukaryotic species and, with few exceptions, in all cells. Numerous studies in recent years have shown that peroxisomal activity is essential in many catabolic pathways, including the β -oxidation of very-long-chain saturated and unsaturated fatty acids containing more than 22 carbon atoms, the degradation of prostaglandins, dicarboxylic acids and xenobiotics, and also the conversion of cholestanoic acid to bile acids (Wanders *et al.*, 1988a; Poulos, 1989). Peroxisomes contain the full complement of enzymes necessary for β -oxidation: acyl-CoA synthetase, acyl-CoA oxidase, the bifunctional protein with both enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities, and 3-oxo-acyl CoA thiolase (Hashimoto, 1987) as well as additional enzymes required for the degradation of unsaturated fatty acids (Schulz & Kunau, 1987). A number of disorders of peroxisomal function have been recognized. These may be characterized by a generalized loss of peroxisomal function as in Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum's disease, or by loss of a single peroxisomal function such as in hyperoxaluria Type I, X-linked adrenoleukodystrophy (ALD), pseudo-Zellweger syndrome and Refsum's disease.

As yet, the metabolic basis for the existence of two separate β -oxidation pathways remains unknown, although the oxidation of some fatty acids, in particular those with carbon chain lengths greater than 22 (VLCFA) appears to be confined to peroxisomes. Based on our recent studies with cells from patients with Zellweger's syndrome, a rare inherited neurodegenerative dis-

order characterized by a near total deficiency of peroxisomes in all tissues thus far examined, we proposed that a major function of the peroxisomal pathway is the generation of acetyl-CoA which can be used for a variety of synthetic processes in the cell. However, although Zellweger syndrome cells have been shown to have a deficiency in the entire peroxisomal β -oxidation pathway, they are also deficient in a number of other metabolic pathways (Wanders *et al.*, 1988a). To explore more fully the biochemical role of peroxisomal β -oxidation in fatty acid metabolism, we have therefore employed cells from patients with specific defects in peroxisomal β -oxidation but who show no evidence of an abnormality in peroxisomal biogenesis. These include patients with ALD and others who have normal peroxisomes but with biochemical evidence for disturbances in peroxisomal β -oxidation. Recent studies suggest that the defect in ALD is in a peroxisomal lignoceroyl-CoA ligase or synthetase (Wanders *et al.*, 1988b; Lazo *et al.*, 1988), whereas in other disease states the defects are in specific proteins along the β -oxidation pathway, e.g. thiolase and acyl-CoA oxidase (Schram *et al.*, 1987; Poll-The *et al.*, 1988). The disturbance in peroxisomal β -oxidation in these various patients is expressed biochemically by the accumulation of VLCFA in plasma and fibroblasts and forms the basis for the diagnosis of these disorders.

MATERIALS AND METHODS

All solvents used were from May and Baker Australia Pty. Ltd., Victoria, or from Ajax Chemicals, Sydney, NSW, Australia. Reversed-phase KC-18 t.l.c. plates were purchased from What-

Abbreviations used: VLCFA, very-long-chain fatty acids; BMEM, basal modified Eagle's medium; DCF, dichlorofluorescein; ALD, X-linked adrenoleukodystrophy; $\text{C}_{24:4}$, tetracosatetraenoic acid; $\text{C}_{24:0}$, lignoceric acid; PD, peroxisomal disease.

man Inc., Clifton, NJ, U.S.A., and thin-layer silica gel 60 plates were from E. Merck, Darmstadt, Germany. Basal modified Eagle's medium (BMEM) and fetal calf serum were purchased from Flow Laboratories, North Ryde, NSW, Australia. Dulbecco's phosphate-buffered saline (Ca^{2+} - and Mg^{2+} -free) was from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia, and BSA (fatty-acid free) and Dowex-50W strongly acid cation-exchange resin (12% cross-linked; dry mesh 200–400) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Bio-Rad protein assay dye reagent concentrate and anion-exchange resin AG2 $\times$ 8 (200–400 mesh), chloride form, were from Bio-Rad Laboratories, Richmond, CA, U.S.A. LKB Ultrafilm (^3H -labelled) was from LKB-Produkter AB, Bromma, Sweden, and 10% palladium on carbon catalyst was from Riedel-de-Haen AG, Hanover, Germany. NCS solubilizer for liquid scintillation counting was purchased from Amersham Australia Pty. Ltd., North Ryde, NSW, Australia.

[1- ^{14}C]Lignoceric ($\text{C}_{24:0}$) and [1- ^{14}C]tetracosatetraenoic acid ($\text{C}_{24:4, n-6}$) were prepared as described previously (Singh & Poulos, 1986; Street *et al.*, 1989). Purity was monitored using gas chromatography, h.p.l.c. and reversed-phase t.l.c.

Patients

The diagnosis was based on clinical history, examination and results of laboratory investigation (Poulos *et al.*, 1985, 1987) as described below.

ALD. Diagnosis of ALD was confirmed by demonstration of increased plasma and fibroblast $\text{C}_{26:0}/\text{C}_{22:0}$ fatty acid ratios both in the patients and in the patients' mothers (Moser *et al.*, 1980, 1981). DB and RM exhibited the typical pattern of mental and motor deterioration coupled with adrenal insufficiency. DC and JR were diagnosed because of affected siblings, and JR succumbed to acute adrenal insufficiency. Post-mortem findings on RM and JR confirmed diagnosis.

Other patients. The diagnosis of peroxisomal disease (PD) in two other patients (JG and AE) was suggested by the demonstration of elevated plasma and fibroblast $\text{C}_{26:0}/\text{C}_{22:0}$ fatty acid ratios, normal plasma phytanic acid levels and normal activities of dihydroxyacetone phosphate acyltransferase. Particle-bound catalase was normal in the fibroblasts of both patients, suggesting that peroxisomal biogenesis was not affected (Goldfischer *et al.*, 1986). Immunocytochemical analysis of a liver biopsy from one of the patients (AE), using antibodies to catalase and the peroxisomal bifunctional protein as probes, confirmed the presence of peroxisomes. The clinical, ultrastructural and biochemical findings were consistent with the diagnosis of pseudo-Zellweger syndrome (Goldfischer *et al.*, 1986). However, due to the unavailability of peroxisomal thiolase antibody, we were unable to confirm this diagnosis (Schram *et al.*, 1987).

Fibroblasts

Cultured skin fibroblasts from normal subjects and from patients with X-linked ALD or PD were grown in tissue culture flasks (25 cm^2) in BMEM containing 10% (v/v) fetal calf serum for 7–18 days until confluent. The culture medium was removed and the cells were incubated in BMEM without fetal calf serum for 16 h before the addition of substrate. Normal ($n = 8$) and X-linked ALD ($n = 4$) cell lines were used with each substrate. Two different PD cell lines were used and the time course carried out on two separate occasions.

Preparation of substrates

[1- ^{14}C] $\text{C}_{24:4}$ (55 mCi/mmol) was heated with 5 μl of 0.6 M-NaOH and then incubated at 37 °C for 30–60 min with fetal calf

serum to bind fatty acids, and diluted with BMEM to give a fetal calf serum concentration of 1–2% (v/v). The substrate solution was sterilized by passage through a Millex-GS filter (0.22 μm pore size) and gassed with CO_2 , and 3 ml of the filtrate [containing 4.5–5.5 nmol of fatty acid; $(0.55\text{--}0.65) \times 10^6$ d.p.m.] was added to each fibroblast culture. Under these conditions, 70% of the substrate bound to fetal calf serum and passed through the filter.

[1- ^{14}C] $\text{C}_{24:0}$ (58 mCi/mmol), which does not bind efficiently to fetal calf serum, was bound to BSA (fatty-acid free) as follows. The substrate was incubated in 0.6 M-NaOH (5 μl) and ethanol (300 μl) at 75 °C for 15 min. After cooling to room temperature, 1 ml of fatty-acid free BSA (50 mg/ml) was added slowly and the substrate was incubated at 37 °C for 60 min. Fetal calf serum and BMEM were added, and the substrate was sterilized and further prepared as described above. A 3 ml portion of the filtrate (containing 4.5–5.5 nmol of labelled fatty acid, 1–2% fetal calf serum, 20–40 μl of ethanol and 1–2 mg of albumin/ml) was added to each culture flask. Under these conditions, 50% of the substrate bound to protein and passed through the filter.

Incubation of 1- ^{14}C -labelled fatty acids with fibroblasts in culture

Radiolabelled fatty acids were added to confluent cultures of normal and mutant cells, and the flasks were sealed with sterilized rubber stoppers. Corresponding control incubations were done in culture flasks without cells. After incubation at 37 °C for periods between 1 and 3 days, the released $^{14}\text{CO}_2$ was quantified as described previously (Poulos, 1981). The medium was removed and the cells were washed with Dulbecco's phosphate-buffered saline (Ca^{2+} - and Mg^{2+} -free) before harvesting in 3 ml of 0.1% (v/v) Triton X-100. The harvested cells were disrupted by ultrasonication and a sample (100 μl) was assayed for protein as described by Bradford (1976). All substrates and cell lines were incubated under similar conditions with similar amounts of added radioactivity.

Incubation of radiolabelled fatty acids with cell suspensions

Fibroblasts were harvested from tissue culture flasks with trypsin as described earlier (Singh & Poulos, 1986). [1- ^{14}C]Stearic acid or [1- ^{14}C] $\text{C}_{24:0}$ were dispersed in α -cyclodextrin by sonication (Singh & Poulos, 1986). Cell suspensions (20–60 μg of protein) were incubated at 37 °C for 60 min in 0.2 ml of the reaction mixture containing sucrose (0.2 M), Tris/HCl buffer, pH 8.0 (50 mM), ATP (5 mM), MgCl_2 (2 mM), dithiothreitol (1 mM), L-carnitine (0.5 mM), NAD^+ (0.2 mM), coenzyme A (160 μM), FAD (60 μM), digitonin (40 $\mu\text{g}/\text{ml}$) and radiolabelled fatty acid (5–10 μM) dispersed in α -cyclodextrin. The reaction was stopped with 4 ml of chloroform/methanol (2:1, v/v), and radiolabelled water-soluble metabolites were determined as described by Singh & Poulos (1986).

Lipid extraction and analysis

Lipids were extracted from the media and cell suspensions by the Bligh & Dyer (1959) method. After partitioning of media and cell extracts, radiolabelled product in the upper layer (water-soluble product) was measured. Lipid hydrolysis and methylation of the liberated radiolabelled fatty acids in the lower phase (> 95% of total radioactivity) were carried out in methanol/18 M- H_2SO_4 (200:3, v/v) at 75 °C for 3 h. The methyl esters were extracted with 3 ml of hexane and purified by t.l.c. on a silica gel 60 plate in dichloromethane. The zone corresponding to the fatty acid methyl esters was revealed under u.v. light after spraying with dichlorofluorescein (DCF) (0.1%, w/v) in methanol/water (19:1, v/v). The scraped regions of gel were extracted with 3 ml of chloroform/methanol (2:1, v/v). The DCF was removed by partitioning with 1 drop of 2 M- NH_4OH

and 600 μ l of 0.1 M-KCl. The bottom layer was washed with 500 μ l of synthetic upper phase (Folch *et al.*, 1957). Extracted fatty acid methyl esters were applied to reversed-phase KC-18 plates and developed twice in the same direction in acetonitrile/tetrahydrofuran (9:1, v/v). Appropriate standards were co-chromatographed to assist identification of the products. Autoradiographs were prepared by exposing 3 H-labelled Ultrafilm to the plates for 5–10 days. Radioactive zones were scraped from the plate and the radioactivity was determined by counting in 10 ml of Beckman Ready Value scintillation fluid.

Argentation t.l.c.

Argentation t.l.c. was used to separate fatty acid groups on the basis of the degree of unsaturation. Silica-gel 60 plates (20 cm $\times$ 20 cm, 0.25 mm) were sprayed with AgNO₃ as described previously (Inomata *et al.*, 1982) and fatty acid methyl esters were chromatographed in hexane/ether (19:1, v/v). Mixtures of C₁₆–C₂₄ saturated, monounsaturated and polyunsaturated fatty acids were co-chromatographed to assist identification of the products. After spraying the plate with DCF, bands were revealed under u.v. light and extracted as described above. The saturated, monounsaturated and polyunsaturated fatty acid groups were further separated by reversed-phase t.l.c. as described above.

Hydrogenation

Fatty acids were hydrogenated in order to gain information about fatty acid chain length. The extracts were diluted to 5 ml with hexane, and palladium catalyst (0.1 g) was added. The solution was stirred vigorously under hydrogen for 7 h at room temperature, filtered and the product was chromatographed by reversed-phase t.l.c. as described above.

H.p.l.c.

Fatty acid methyl esters were separated by h.p.l.c. in order to facilitate identification of elongation and desaturation products as follows. PD fibroblasts were incubated with C_{24:4}, harvested, extracted, hydrolysed and methylated as described. The fatty acid extract was dissolved in 80 μ l of methanol and chromatographed at a flow rate of 1 ml/min in a gradient of acetonitrile/water (9:1, v/v) to acetonitrile over 50 min on a 250 mm $\times$ 4 mm ODS (5 μ m) column (SGE, Ringwood, Victoria, Australia) using an LKB 2150 h.p.l.c. pump with 11300 Ultrograd mixer and 2152 system controller (LKB, Sydney, NSW, Australia). Fractions (1 ml) were collected using an LKB 2212 Helirac fraction collector and pooled if necessary in order to isolate substrate and metabolites. Pooled fractions were hydrogenated and chromatographed on reverse-phase t.l.c. plates as described above.

Saturated, monounsaturated and polyenoic fatty acids in plasma

Plasma saturated (C_{24:0}, C_{26:0}), monounsaturated (C_{26:1}) and polyenoic (C_{24:5}, C_{26:5}) VLCFAs were measured in the two PD patients (AE, JG) and the ratios of these fatty acids to docosanoic acid (C_{22:0}) were determined as described previously (Poulos *et al.*, 1989) and compared with previously published data for normal children and ALD patients.

Characterization of water-soluble products

Initial experiments to characterize the water-soluble products have been described elsewhere (Street *et al.*, 1989). However, the high concentrations of amino acids and succinic acid in the BMEM interfered with identification of the product by t.l.c. and ion-exchange chromatography. Therefore normal fibroblast cultures were grown and starved as described above. The cells were then washed with Hanks buffer and grown in the presence of radiolabelled substrate (C_{24:4}) in Hanks buffer with 1% fetal

calf serum for 6 h. Cells and media were harvested and extracted as described above. The upper aqueous phase extract resulting from the extraction of conditioned medium was dried under N₂. The radiolabelled product (approx. 100 000 c.p.m.) was dissolved in 2 $\times$ 10 ml of distilled water adjusted to pH 2 with 0.1 M-HCl and applied to a Dowex 50 column (7 cm $\times$ 1 cm) prepared in water. The column was washed with 40 ml of water, and basic compounds, including amino acids, were eluted with 20 ml of 2 M-ammonia. The non-retained fraction was adjusted to pH 7 with 1 M-KOH and applied to an AG2 $\times$ 8 column (7 cm $\times$ 1 cm) prepared in water. The column was washed with 5 ml of water to remove natural compounds such as sugars, and the acidic compounds including organic acids were eluted with 15 ml of 40% formic acid. No neutral compounds were recovered by this procedure from two different normal cell lines. The acidic fractions were analysed by anion-exchange h.p.l.c. (Bio-Rad HPX 87 in 6 mM-H₂SO₄ at a flow rate of 0.5 ml/min) with fractions collected for liquid scintillation counting and t.l.c. as described by Kawamura & Kishimoto (1981). The basic fraction (amino acids) was separated on a cation exchange column using lithium citrate buffers and a Dionex amino acid analyser. Detection of endogenous amino acids was carried out by post-column derivatization using o-phthalaldehyde in borate buffer, pH 10.4, and fractions were collected for liquid scintillation counting. Retention times of individual amino acids were determined using Pierce amino acid standards mixtures. Amino acids were also analysed by t.l.c. on silica gel 60 plates (Kawamura & Kishimoto, 1981).

RESULTS

Uptake and incorporation into lipids

The degree of uptake and incorporation of [1-¹⁴C]C_{24:4} and [1-¹⁴C]C_{24:0} added to normal cell cultures has been reported earlier (Street *et al.*, 1989). In a 3 day period, 39% and 75% of the label from C_{24:4} was recovered in the lipid fraction isolated from the two PD lines compared with up to 30% recovery for the control

Table 1. Distribution of label from [1-¹⁴C]C_{24:0} and [1-¹⁴C]C_{24:4} in skin fibroblasts in culture

	Radioactivity recovered (nmol of label/mg of protein)			
	PD		X-linked ALD	Control
	AE	JG		
C _{24:0} :				
Cell lipid	8.96	2.87	2.03	2.05
radioactivity			(1.24–3.02)	(1.02–4.21)
Total substrate	6.37	2.39	1.69	1.67
remaining			(1.12–2.43)	(0.93–3.19)
Total fatty acid	2.61	0.49	0.37	0.38
metabolites			(0.12–0.58)	(0.09–1.02)
C _{24:4} :				
Cell lipid	20.70	30.40	4.45	7.00
radioactivity			(1.49–7.03)	(1.38–15.58)
Total substrate	13.46	22.88	1.87	1.63
remaining			(0.49–3.38)	(0.17–3.23)
Total fatty acid	7.08	7.51	2.60	5.37
metabolites			(1.05–3.68)	(1.21–12.36)

Fig. 1. Time course for synthesis of $^{14}\text{CO}_2$ and ^{14}C -labelled water-soluble products from $[1\text{-}^{14}\text{C}]\text{C}_{24:0}$

Flasks of confluent cells from four X-linked ALD, two PD and seven normal cell lines were incubated with labelled substrate as described in the Materials and methods section. One flask of cells was used to measure $^{14}\text{CO}_2$ and ^{14}C -water-soluble product at each time point. The same cell line was used for each time course shown. The range of normal values is shown as a shaded area. (a) X-linked ALD; (b) PD.

and ALD lines. Label from $\text{C}_{24:0}$ was recovered from cell lipids in smaller amounts than that from $\text{C}_{24:4}$ (up to 20% of the label), but the amounts recovered from normal cell and mutant cell lipids were similar (Table 1). It should be emphasized, however, that the actual uptake of label into the cells is greater than that suggested by Table 1, because substantial amounts of label were converted into CO_2 and water-soluble products. The latter were subsequently secreted into the culture medium. Most of the cellular lipid radioactivity remained as fatty acid esterified to lipids, with only a small amount (< 5%) converted to other metabolites such as cholesterol.

CO_2 and water-soluble product

Previous studies in this laboratory (Street *et al.*, 1989) and others (Kondrup & Lazarow, 1985; Christensen *et al.*, 1986) have shown that analysis of $^{14}\text{CO}_2$ alone may not be an adequate index of β -oxidation of fatty acids, since up to 30% of added $[^{14}\text{C}]\text{VLCFA}$ may be transformed into radiolabelled water-soluble product. Therefore values for combined $^{14}\text{CO}_2$ and ^{14}C -water-soluble product formation should give a more accurate index of β -oxidation. Using this index, we found that β -oxidation of lignoceric acid was normal in three out of four ALD fibroblast cultures (Fig. 1a). These results reflect a normal conversion to water-soluble product with moderately decreased formation of $^{14}\text{CO}_2$ in two of the ALD patients (Table 2). In contrast the two patients with PD had a greatly decreased activity for β -oxidation

Table 2. Oxidation of $[1\text{-}^{14}\text{C}]\text{C}_{24:0}$ and $[1\text{-}^{14}\text{C}]\text{C}_{24:4}$ into CO_2 by skin fibroblasts in culture

Skin fibroblasts were incubated with $1\text{-}^{14}\text{C}$ -labelled fatty acid for 24 or 72 h and CO_2 was collected as described in the text.

Clinical diagnosis	Patient	$^{14}\text{CO}_2$ produced (pmol/h per mg of protein)			
		$\text{C}_{24:0}$		$\text{C}_{24:4}$	
		24 h	72 h	24 h	72 h
PD	AE	0.9	1.3	26.8	27.0
	JG	0.5	0.5	4.3	13.5
ALD	RM	3.2	5.6	31.5	27.3
	DC	1.7	4.5	72.2	46.2
	DB	3.1	4.3	47.2	42.2
	JR	2.2	3.4	118.4	80.0
Controls ($n = 6$)		3.1–5.3	5.2–10.8	21.3–88.2	15.6–45.5

Fig. 2. Time course for synthesis of $^{14}\text{CO}_2$ and ^{14}C -labelled water-soluble products from $[1\text{-}^{14}\text{C}]\text{C}_{24:4}$ by skin fibroblast cultures from X-linked ALD and PD patients

Conditions were similar to those described in Fig. 1. (a) X-linked ALD; (b) PD.

of $C_{24:0}$, and the production of both $^{14}CO_2$ and ^{14}C -water-soluble product (Table 2 and Fig. 1b) fell within the range previously observed for patients with Zellweger syndrome (Street *et al.*, 1989).

With $[1-^{14}C]C_{24:4}$ as substrate, total β -oxidation activity expressed as the sum of CO_2 and water-soluble product was normal or elevated in all X-linked ALD patients (Fig. 2a). β -Oxidation activity towards $C_{24:4}$ was also normal in one of the patients with PD, but was decreased in the other (Fig. 2b).

The data suggest that with $[^{14}C]C_{24:4}$ as substrate, ^{14}C -labelled water-soluble product is synthesized with much greater rapidity than is $^{14}CO_2$. With controls and X-linked ALD fibroblast cultures, most of the ^{14}C -water-soluble product from both substrates was found in the medium (71–96% of the total water-soluble product formed) and the proportion of the total radioactivity in the water-soluble product found in the medium consistently increased with incubation time (results not shown). Labelled substrates added to control incubations without cells were recovered unchanged and no significant $^{14}CO_2$ or ^{14}C -labelled water-soluble product formation was observed.

Elongation and desaturation

Label from $C_{24:4}$ was detected in $C_{26:4}$ in all control cell cultures. The proportion of this elongation product was increased in fibroblasts from PD patients. Trace amounts of an even longer elongation product, $C_{28:4}$, was also detected in PD cell lines. A significant proportion of the label in the metabolites formed (up to 43%) was present in PD lines in a fatty acid we have tentatively identified as $C_{24:5}$, a product of Δ -6 desaturation. This product was either not seen in ALD or control cells, or was present only in trace amounts. Substantial amounts of the labelled substrate remained (mostly in an esterified form), particularly in PD lines, even after a 3 day incubation period (Table 1). The percentage contribution of each fatty acid metabolite to the total fatty acid profile after 3 days of incubation is shown in Table 3.

$C_{24:0}$ was elongated to $C_{26:0}$ by all control cell lines. Increased elongation was observed in fibroblasts from all X-linked ALD and PD patients with an increased proportion of $C_{26:0}$, and, in many cases, small amounts of $C_{28:0}$. The percentage contribution of each fatty acid metabolite to the total fatty acid profile after 3 days of incubation is shown in Table 4.

Saturated and monounsaturated fatty acid synthesis

Significant amounts of labelled C_{14} – C_{24} saturated fatty acids and small amounts of monounsaturated fatty acids were observed with normal fibroblast cell lines incubated with $C_{24:4}$ (Table 3). However, a marked decrease in the proportion of C_{14} , C_{16} and C_{18} fatty acids was observed in cells from PD but not X-linked ALD patients.

The incorporation of label from $C_{24:0}$ into saturated C_{16} and C_{18} fatty acids was undetectable in PD and a pattern of labelled saturated fatty acids similar to that seen in Zellweger fibroblast cultures was observed (Table 4) (see Table 1 from Street *et al.*, 1989). The proportion of labelled $C_{16:0}$ and $C_{18:0}$ fatty acids in fibroblast cultures from ALD patients was decreased (Table 4).

Plasma VLCFA ratios

Plasma fatty acid ratios for the PD patients are shown in Table 5 and are compared with previously published values for normal children and ALD patients. It is of interest that AE, whose skin fibroblasts exhibited normal β -oxidation activity towards $C_{24:4}$ (Fig. 2b), did not accumulate abnormal amounts of polyenoic VLCFA in plasma as seen in Zellweger syndrome (Poulos *et al.*, 1989). In contrast, fibroblast cultures from JG were unable to β -oxidize $C_{24:4}$ normally and there was an increase in the levels of

Table 3. Individual fatty acid metabolites from $[1-^{14}C]C_{24:4}$: distribution in cells from normal, X-linked ALD and PD skin fibroblast cultures

Fibroblast cell cultures were incubated with $[1-^{14}C]C_{24:4}$ for 72 h and products were identified as described in the text. Values are a range from four normal and four ALD cell lines and are expressed as a percentage of total recovered radioactivity. Values are shown for individual PD cell lines.

Fatty acids	Distribution (% of total)			
	Normal	X-linked ALD	PD	
			AE	JG
$C_{14:0}$	2.8–10.0	2.4–4.0	0.9	1.4
$C_{16:0}$	32.2–49.5	29.5–43.2	9.8	3.2
$C_{18:0}$	19.9–34.1	10.4–16.5	2.8	0.8
$C_{20:0}$	0–1.5	0–0.9	0	0
$C_{22:0}$	0–2.5	0–1.9	0	0
$C_{24:0}$	1.9–4.3	2.1–5.9	2.0	0
$C_{26:0}$	0–0.3	0–3.1	0	0
$C_{28:0}$	0	0	0	0
$C_{14:1}$	0–0.4	0	0	0
$C_{16:1}$	0–1.4	0–0.7	0	0
$C_{18:1}$	1.9–5.8	0–3.9	0.9	0
$C_{20:1}$	0–0.7	1.0–1.6	0	0
$C_{22:1}$	0	0–3.6	0	0
$C_{24:1}$	0–1.3	1.9–2.1	0	0
$C_{26:4}$	3.4–8.2	10.1–11.5	63.4	47.0
$C_{28:4}$	0	0.7–1.1	4.9	2.4
$C_{24:5}$	0–1.5	0–2.9	16.1	43.7
Unknown polyunsaturates	2.5–14.8	5.2–22.6	0	2.8

Table 4. Individual fatty acid metabolites from $[1-^{14}C]C_{24:0}$: Distribution in cells from normal, X-linked ALD and PD skin fibroblast cultures

Fibroblast cell cultures were incubated with $[1-^{14}C]C_{24:0}$. Conditions were similar to those described for Table 3.

Fatty acids	Distribution (% of total)			
	Normal	X-linked ALD	PD	
			AE	JG
$C_{16:0}$	42.2–54.8	8.3–23.5	0	0
$C_{18:0}$	22.9–33.3	4.7–6.9	0	0
$C_{26:0}$	18.4–34.9	69.2–72.0	91.1	94.8
$C_{28:0}$	0	0–7.3	8.9	5.2

polyenoic VLCFA in plasma. However, polyenoic fatty acids with carbon chains longer than C_{26} were not detected in plasma, whereas C_{28} and C_{30} polyenoics are detectable in Zellweger syndrome (Poulos *et al.*, 1989).

Characterization of water-soluble product

The identification of glutamate as the principal water-soluble metabolite produced from the β -oxidation of VLCFA proposed in our earlier studies (Street *et al.*, 1989) was confirmed in extracts of cells and media. The principal water-soluble product from $C_{24:4}$ co-chromatographed with the same retention time as unlabelled glutamate on an ion-exchange amino acid column. A substantial fraction of the total labelled product (15–50%) was acidic and was bound to an anion-exchange column. Preliminary h.p.l.c. analysis indicated that the acidic compounds were citric acid cycle intermediates, mainly citric and succinic acids. Less than 2% of the water-soluble product were neutral compounds.

Table 5. VLCFA in plasma of ALD and PD patients

Fatty acid ratios were determined in plasma from normal, PD and ALD subjects as described in the text.

Disease state	Fatty acid ratios				
	$C_{24:0}/C_{22:0}$	$C_{26:0}/C_{22:0}$	$C_{26:1}/C_{22:0}$	$C_{24:5}/C_{22:0}$	$C_{26:5}/C_{22:0}$
PD: JG	2.39	0.70	0.53	0.21	0.083
AE	1.63	0.31	0.33	< 0.02	< 0.02
X-linked ALD ($n = 36$)	1.46–2.07	0.07–0.12	< 0.02	< 0.02	< 0.02
Controls ($n > 50$)	< 1.15	< 0.03	< 0.01	< 0.02	< 0.02

Table 6. Oxidation of $[1-^{14}C]C_{24:0}$ and $[1-^{14}C]C_{18:0}$ by fortified cell suspensions to water-soluble products

Fibroblast suspensions were incubated with $1-^{14}C$ -labelled fatty acid and the amount of water-soluble product formed was determined as described in the text.

	Water-soluble product (pmol/h per mg of protein)	
	$C_{24:0}$	$C_{18:0}$
Normal cells ($n = 6$)	803 ± 88 (642–891)	1566 ± 326 (1145–1992)
ALD cells ($n = 7$)	131 ± 38 (98–221)	1278 ± 497 (680–1913)

Incubation of cell suspensions with $[1-^{14}C]C_{24:0}$

Suspensions of normal fibroblasts converted radiolabelled $C_{24:0}$ into water-soluble products at a much faster rate than did the corresponding cells grown under culture conditions. In 1 h, up to 4.5% of the fatty acid was converted into water-soluble product, most of which appeared to be acetyl-CoA, by normal cells. Little labelled CO_2 was produced under these conditions. ALD cells incubated under the same conditions showed a marked decrease in the acetyl-CoA produced (approx. 0.5% conversion of substrate), despite the fact that oxidation of shorter-chain fatty acids (e.g. stearic acid) was near normal (Table 6).

DISCUSSION

Accumulation of VLCFA in association with inherited disease was first described by Igarashi *et al.* (1976) when elevated levels of saturated VLCFA were found in brain and adrenals of patients with X-linked ALD. Later studies also showed that accumulation occurred in cultured skin fibroblasts (Moser *et al.*, 1981) and plasma (Moser *et al.*, 1980). Saturated VLCFA are also known to accumulate in plasma from patients with pseudo-Zellweger syndrome (Goldfischer *et al.*, 1986) as well as other peroxisomal disorders including Zellweger syndrome (Poulos *et al.*, 1989). Oxidation of the saturated VLCFA $C_{24:0}$ in Zellweger syndrome has been shown to be strongly deficient (Poulos *et al.*, 1986; Singh *et al.*, 1987; Street *et al.*, 1989). The absence of peroxisomes in Zellweger syndrome patients is associated with a deficiency in the three peroxisomal β -oxidation enzyme proteins acyl-CoA oxidase, bifunctional protein and thiolase (Tager *et al.*, 1985; Suzuki *et al.*, 1986). These findings provide an explanation for the accumulation of VLCFAs in Zellweger syndrome. In ALD and at least two other clinical phenotypes, however, recent studies have suggested that a single enzyme defect in the β -oxidation pathway may cause accumulation of VLCFA (Schram *et al.*, 1987; Wanders *et al.*, 1988b; Lazo *et al.*, 1988; Poll-The *et al.*, 1988).

We find normal or near normal oxidation of $C_{24:0}$ in ALD

when measurements are performed under normal culture conditions, indicating that these cells possess sufficient oxidation activity to cope with the amount of exogenous fatty acid. Our data may therefore be consistent with the known biochemical and clinical expression of the disease. Despite a very low residual activity of lignoceryl-CoA synthetase towards $C_{24:0}$ and/or $C_{26:0}$ reported to occur in fibroblast peroxisomes of X-linked ALD patients (Lazo *et al.*, 1988; Wanders *et al.*, 1988b), accumulation of VLCFA, particularly $C_{26:0}$, in plasma and cultured skin fibroblasts is less apparent in X-linked ALD than in Zellweger syndrome and neonatal ALD (Moser *et al.*, 1984; Poulos *et al.*, 1985, 1986). Onset of the disease occurs later in life and the course is milder. Moser *et al.* (1984) have also reported the existence of persons with the biochemical changes observed in X-linked ALD but who are asymptomatic or mildly symptomatic. Two cases with a fluctuating course have also been described (Walsh, 1980).

The data we have presented suggest that, despite a defect in peroxisomal VLCFA CoA synthetase, the residual enzyme activity in the ALD cell may be able to accommodate the exogenous fatty acid levels. The abnormality becomes more apparent when measurements are carried out using fortified cell suspensions. Uptake of the fatty acid is increased greatly under these conditions, and this is almost certainly the basis for the marked increase in oxidation activity in normal as well as in ALD cells. It would seem likely, therefore, that the much lower residual activity of ALD cells measured under these conditions reflects their inability to cope with this increased uptake.

It is worth noting that, despite the apparently normal β -oxidation of saturated VLCFA, ALD cells show an increase in carbon chain elongation as well as a decreased incorporation of label from $[1-^{14}C]C_{24:0}$ into shorter-chain saturated fatty acids. Our data support the earlier work of Tsuji *et al.* (1984), who reported that microsomal fatty acid elongation activity was increased significantly in ALD fibroblasts. Whereas the increase in carbon chain elongation products in ALD cells incubated with $C_{24:0}$ is most likely due to either an increase in microsomal elongation activity or an increase in substrate (i.e. $C_{24:0}$) availability, it is difficult to explain why synthesis of C_{16} and C_{18} fatty acids is affected even though β -oxidation activity is relatively normal (Fig. 1a). One possible explanation is that the acetate formed in peroxisomes from the oxidation of substrate is more easily accessible to mitochondria where, presumably, its conversion to water-soluble products takes place. It is possible, therefore, that the decreased labelling of C_{16} and C_{18} fatty acids reflects the diminished availability of acetate for cytoplasmic fatty acid synthesis.

Our data are consistent with those of Christensen *et al.* (1989), who reported that CO_2 and water-soluble products are formed in relatively normal amounts from $[2-^{14}C]$ adrenic acid ($C_{22:4,n-6}$) by ALD cells. It is of interest, however, that we observed a slight increase in elongation of $C_{24:4}$ in X-linked ALD (Table 3).

With respect to the two PD patients, heterogeneity is apparent. The cells from one patient (AE) were able to produce relatively normal amounts of CO₂ and water-soluble products from C_{24:4} whereas cells from the other (JG) had much lower activity, a finding reflected in the plasma VLCFA profiles (Table 5). However, despite the apparently normal oxidation activity in AE, these cells show a marked increase in elongation products and a decreased production of labelled C₁₆ and C₁₈ fatty acids (Tables 3 and 4). These data suggest that even in some PD lines there may be sufficient residual peroxisomal β -oxidation activity to produce near-normal amounts of CO₂ and water-soluble products even though the labelling of the presumed products of synthesis *de novo* and chain elongation is clearly grossly abnormal. It is of interest, however, that the production of water-soluble products from C_{24:0} was markedly decreased in the two PD patients, but was normal in X-linked ALD.

Our studies have confirmed that one of the metabolites in normal cells is the amino acid glutamate. The identification of some of the other metabolites is more tentative but our data suggest that other amino acids (including glycine and aspartic acid) and citric acid cycle intermediates are also produced and secreted into the culture medium. The secretion of the labelled amino acids occurs even in medium containing very low concentrations of these compounds, e.g. Hanks medium, suggesting that their transport out of the cell is not related to the presence of high extracellular levels of unlabelled amino acids. At present it is not known which subcellular compartments are involved in the process. Presumably the acetate produced from C_{24:4} arises only from the peroxisomes, but whether some of the various metabolites are formed in this organelle is not clear. Plant glyoxysomes, organelles which appear to be related to peroxisomes, possess the necessary synthetic machinery to convert acetyl-CoA to some of the products we have observed. However, although these pathways have not been described in animal cells, human liver does contain a peroxisomal glyoxylate:alanine aminotransferase, suggesting that glyoxylate can be metabolized in human peroxisomes (Danpure & Jennings, 1986). It is tempting to speculate, therefore, that the synthesis of some of the non-lipid metabolites, in particular the amino acids, may take place in peroxisomes.

Although the data obtained with ALD cells suggest that the peroxisomal pathway for the β -oxidation of C_{24:0} is functioning in culture, there is clear evidence that, under the same conditions, this particular pathway is defective in the two PD cell lines. The metabolism of C_{24:0} in the latter cells is similar to that found in Zellweger cells, indicating that the increased carbon chain elongation, decreased incorporation of label into shorter-chain fatty acids and amino acids, and decreased synthesis of fatty acids *de novo*, are a direct consequence of the deficiency in peroxisomal β -oxidation rather than the defect in peroxisomal biogenesis. Our data suggest that acetate generated from peroxisomal β -oxidation may be available for cytoplasmic and possibly microsomal biosynthesis of fatty acids, and may also enter the mitochondrial tricarboxylic acid cycle, but the mechanism of this transfer of acetate remains unknown. It is likely that further studies with cell lines with specific defects at different points along the β -oxidation pathway will contribute significantly to our understanding of the peroxisomal role in fatty acid metabolism and its relationship to pathways present in other subcellular compartments.

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