Peroxisomal β-oxidation of branched chain fatty acids in human skin fibroblasts

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Abstract Human skin fibroblasts in suspension are able to degrade [1-^{14}C]-labeled α- and γ-methyl branched chain fatty acids such as pristanic and homophytanic acid. Pristanic acid was converted to propionyl-CoA, whereas homophytanic acid was β-oxidized to acetyl-CoA. Incubation of skin fibroblasts with [1-^{14}C]-labeled fatty acids for longer periods produced radiolabeled carbon dioxide, presumably by further degradation of acetyl-CoA or propionyl-CoA generated by β-oxidation. Under the same conditions similar products were produced from very long chain fatty acids, such as lignoceric acid. Inclusion of digitonin (>10 μg/ml) in the incubations strongly inhibited carbon dioxide production but stimulated acetyl-CoA or propionyl-CoA production from fatty acids. ATP, Mg^{2+}, coenzyme A, NAD^+ and L-carnitine stimulated acetyl-CoA or propionyl-CoA production from [1-^{14}C]-labeled fatty acids in skin fibroblast suspensions. Branched chain fatty acid β-oxidation was reduced in peroxisome-deficient cells (Zellweger syndrome and infantile Refsum's disease) but they were β-oxidized normally in cells from patients with X-linked adrenoleukodystrophy (ALD). Under the same conditions, lignoceric acid β-oxidation was impaired in the above three peroxisomal disease states. These results provide evidence that branched chain fatty acid, as well as very long chain fatty acid, β-oxidation occurs only in peroxisomes. As the defect in X-linked ALD is in a peroxisomal fatty acyl-CoA synthetase, which is believed to be specific for very long chain fatty acids, we postulate that different synthetases are involved in the activation of branched chain and very long chain fatty acids in peroxisomes.—**Singh, H., M. Brogan, D. Johnson, and A. Poulos.** Peroxisomal β-oxidation of branched chain fatty acids in human skin fibroblasts. *J. Lipid Res.* 1992. **33:** 1597–1605.

Supplementary key words adrenoleukodystrophy • Zellweger syndrome • rhizomelic chondrodysplasia punctata • mitochondria • peroxisomes

Very long chain fatty acids (VLCFA), i.e., >22 carbons in length, accumulate in several peroxisomal diseases. Recent studies from our laboratory and others strongly suggest that VLCFA are degraded exclusively in peroxisomes (1–8). In some of the peroxisomal diseases, in addition to VLCFA, branched chain fatty acids with α- or β-methyl groups such as pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) also accumulate (9). Due to the β-methyl group, phytanic acid can only be degraded by α-oxidation, i.e., one carbon cleavage. However, the branched chain fatty acids with α- or γ-methyl groups such as pristanic acid and homophytanic acid (4,8,12,16-tetramethylheptadecanoic acid) can theoretically be degraded by β-oxidation. Our in vitro studies with rat liver indicate that mitochondria lack VLCFA-CoA synthetase activity (2, 5). As VLCFA cannot be converted to VLCFA-CoA at the mitochondrial membranes, their oxidation is directed to peroxisomes. In contrast, we find that branched chain fatty acids can be converted to coenzyme A esters at the mitochondrial membranes (H. Singh, K. Beckman, D. Johnson, and A. Poulos, unpublished results), yet these fatty acids accumulate in some peroxisomal diseases (9).

β-Oxidation of VLCFA has been investigated in several laboratories, yet there is limited information on the β-oxidation of branched chain fatty acids. We and others reported (10–12) previously that a VLCFA oxidation defect can be demonstrated in X-linked adrenoleukodystrophy (ALD) using cell suspensions. However, the assay conditions were not investigated and the VLCFA oxidation was measured in the presence (10, 12) or absence (11) of digitonin in the incubations. In order to understand the molecular defect in peroxisomal diseases, we optimized the assay conditions for the measurements of fatty acid oxidation in skin fibroblast suspensions. This is the first report to date providing detailed studies on fatty acid oxidation, including branched chain fatty acids, in cell suspensions. The data clearly indicate that the oxidation of fatty acids in cell suspensions is several-fold greater than that observed in cell culture (6, 13) and the cells in suspension retain the ability to produce carbon dioxide from fatty acids. In the present paper we describe the conditions for separate measurement of water-soluble products

Abbreviations: ALD, adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; LCFA, long chain fatty acids; VLCFA, very long chain fatty acids; TLC, thin-layer chromatography.

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and carbon dioxide from straight chain and branched chain fatty acids. We report that, in addition to VLCFA, branched chain fatty acid β-oxidation is defective in several peroxisomal diseases.

MATERIALS

[1-^{14}C]stearic acid and potassium[^{14}C]cyanide used for radiolabeling of fatty acids were obtained from New England Nuclear, Boston, MA. Reversed-phase KC-18 thin-layer chromatographic (TLC) plates were supplied by Whatman Inc., Clifton, NJ, and TLC silica gel 60 plates by E. Merck, Darmstadt, Germany. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Analytical grade solvents were purchased from May and Baker Australia Pty. Ltd., Melbourne, Australia, or Ajax Chemicals, Sydney, Australia. Basal modified Eagle's medium was purchased from Flow Laboratories, McLean, VA, and fetal calf serum was from GIBCO, New Zealand Ltd., New Zealand. Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and trypsin-versene solutions were obtained from Commonwealth Serum Laboratories, Melbourne, Australia.

Skin fibroblast cultures were established in the Department of Chemical Pathology, The Adelaide Children's Hospital, from skin biopsies of patients with peroxisomal disorders, and from individuals with no prior evidence of a metabolic abnormality. The diagnoses of X-linked ALD, Refsum's disease, infantile Refsum's disease, Zellweger syndrome, and rhizomelic chondrodysplasia punctata (RCDP) were based on case histories and on clinical and biochemical investigations. Bichemical investigations were carried out in our laboratory, which included plasma and skin fibroblast $C_{26:0}/C_{22:0}$ ratios, plasma phytanic acid content, and skin fibroblast phytanic acid oxidation, and dihydroxyacetone 3-phosphate acyl transferase activities.

Radiolabeled lignoceric acid, pristanic acid, and homophytanic acid were synthesized as described in earlier publications (1, 13).

METHODS

Preparation of fibroblasts

Human skin fibroblasts were routinely grown under sterile conditions in tissue culture flasks (75 cm^2) in basal modified Eagle's medium containing 10% (v/v) fetal calf serum. The cells from confluent cultures were harvested with trypsin-versene solution. Briefly, the culture medium was removed from the flasks, the cells were rinsed with Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and incubated at 37°C for 2–5 min with trypsin-versene solution (3 ml/flask). The cells were collected by low speed centrifugation (400 g × 5 min) and the cell pellet was washed three times with 3–4 ml of Dulbecco's phosphate-buffered saline. The washed cell pellet from each flask was dispersed gently in 0.3–0.4 ml of 0.25 M sucrose and used for measurements of fatty acid oxidation. Trypan blue exclusion test on the harvested cells indicated that >95% cells were viable. For measurement of protein content, the fibroblasts were sonicated for 30 sec and protein was estimated as described (14) using human albumin as standard.

Production of radiolabeled water-soluble products from [1-^{14}C]-labeled fatty acids

The [1-^{14}C]-labeled fatty acids were dispersed in α-cyclodextrin or in dipalmitoyl phosphatidylcholine by sonication as described previously (1). The incubation mixture consisted of Tris-HCl buffer, pH 8.0 (50 mM), sucrose (0.25 M), Mg^{2+} (2 mM), dithiothreitol (1 mM), ATP (5 mM), L-carnitine (0.5 mM), NAD^+ (0.4 mM), FAD (60 μM), coenzyme A (120 μM), digitonin (20 μg/ml), [1-^{14}C]-labeled fatty acid (5–10 μM), α-cyclodextrin (2.5 mg/ml) or dipalmitoyl phosphatidyl choline (5 μg/ml) in a total volume of 0.2 ml. The reaction was started by the addition of skin fibroblast suspensions (10–30 μg protein), incubated at 37°C for 60 min and stopped with 4 ml of chloroform–methanol 2:1 (v/v). Twenty μl of 10 M HCl was added, and the two phases were separated by the addition of 0.8 ml water. The lower chloroform phase was discarded and the aqueous phase was washed twice with 2 ml hexane. The washed aqueous phase was made alkaline (pH >10) with 50 μl of 10 M KOH and heated at 60°C for 16 h. The mixture was cooled, acidified (pH 1.0–1.5) with 50 μl of 10 M HCl, and washed twice with 2 ml hexane. The radioactivity retained in the aqueous phase (radiolabeled acetate or propionate) was determined. The recovery of radiolabeled acetate or propionate was >95% under the above procedure.

Radiolabeled CO_2 production from [1-^{14}C]-labeled fatty acids

The assay conditions were the same as described above, except that digitonin was omitted and antibiotics, namely streptomycin and penicillin G (0.75 mg/ml final concentrations), were added to the incubations. The incubations were carried out in 1 × 3.5 cm plastic tubes that were inserted into 1.5 × 5 cm plastic vials containing 400 μl of 1 M KOH. The plastic vials were sealed with parafilm, capped, and incubated at 37°C for 3–4 h. After the incubation, the reaction tubes were removed and the $^{14}CO_2$ trapped in KOH was determined.

RESULTS

Two major radiolabeled water-soluble products were formed upon incubation of [1-^{14}C]-labeled fatty acids with

human skin fibroblast suspensions. One of these compounds was fatty acyl-CoA and the other was propionyl-CoA from pristanic acid, and acetyl-CoA from homophytanic, stearic, and lignoceric acids (for identification of products, see ref. 1). Under these conditions (see Methods) [1-^{14}C]phytanic acid was converted to phytanoyl-CoA and no other water-soluble product was detected. Longer incubation of [1-^{14}C]-labeled fatty acids with human skin fibroblasts, in the absence of digitonin, resulted in the production of radiolabeled CO_2. However, no $^{14}CO_2$ was produced from [1-^{14}C]phytanic acid under these conditions.

Fatty acid β-oxidation to water-soluble products, i.e., acetyl-CoA or propionyl-CoA was linear up to 50 μg protein per incubation and at least up to 60 min incubation period. [1-^{14}C]-labeled fatty acid oxidation to $^{14}CO_2$ was linear at least up to 40 μg protein per incubation period (**Fig. 1**). Similar results were obtained with all the four fatty acids investigated. There was a lag phase in the production of $^{14}CO_2$ from [1-^{14}C]-labeled fatty acids. In the first 30 min of the incubation period little or no $^{14}CO_2$ was produced. The lag phase was longer with lignoceric, pristanic, and homophytanic acids compared to stearic acid (Fig. 1). After 1 h of incubation, the amount of $^{14}CO_2$ production increased with the incubation period up to 4 h (Fig. 1).

Inclusion of digitonin in the assay system stimulated acetate production from [1-^{14}C]-labeled fatty acids (**Fig. 2**). $^{14}CO_2$ production from [1-^{14}C]-labeled fatty acids was strongly inhibited by digitonin. Concentrations of 2 μg/incubation (10 μg/ml, final concentrations) inhibited >90% $^{14}CO_2$ production (Fig. 2). Similar results were obtained using pristanic and homophytanic acids (data not shown). Addition of fatty acid-free bovine serum albumin (0–10 μM), L-malate (0–1 mM), and D-glucose

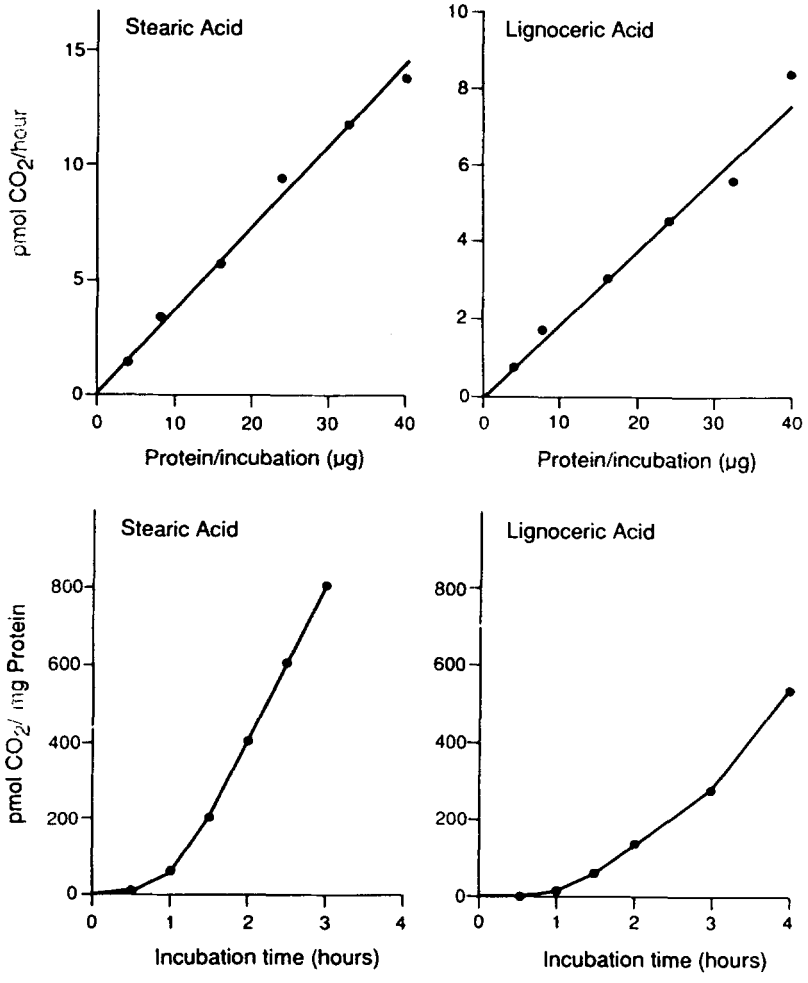

Fig. 1. The effect of increasing concentrations of cellular protein and the incubation period on the production of $^{14}CO_2$ from [1-^{14}C]-labeled fatty acids was determined in skin fibroblasts in suspensions (see Methods). The incubation period was 3 h (for stearic acid) or 4 h (for lignoceric acid) with indicated concentrations of cellular protein. The effect of incubation period on fatty acid oxidation was examined using 20–25 μg of cellular protein per assay. The oxidation assays were performed in duplicate and each point represents the mean of two observations.

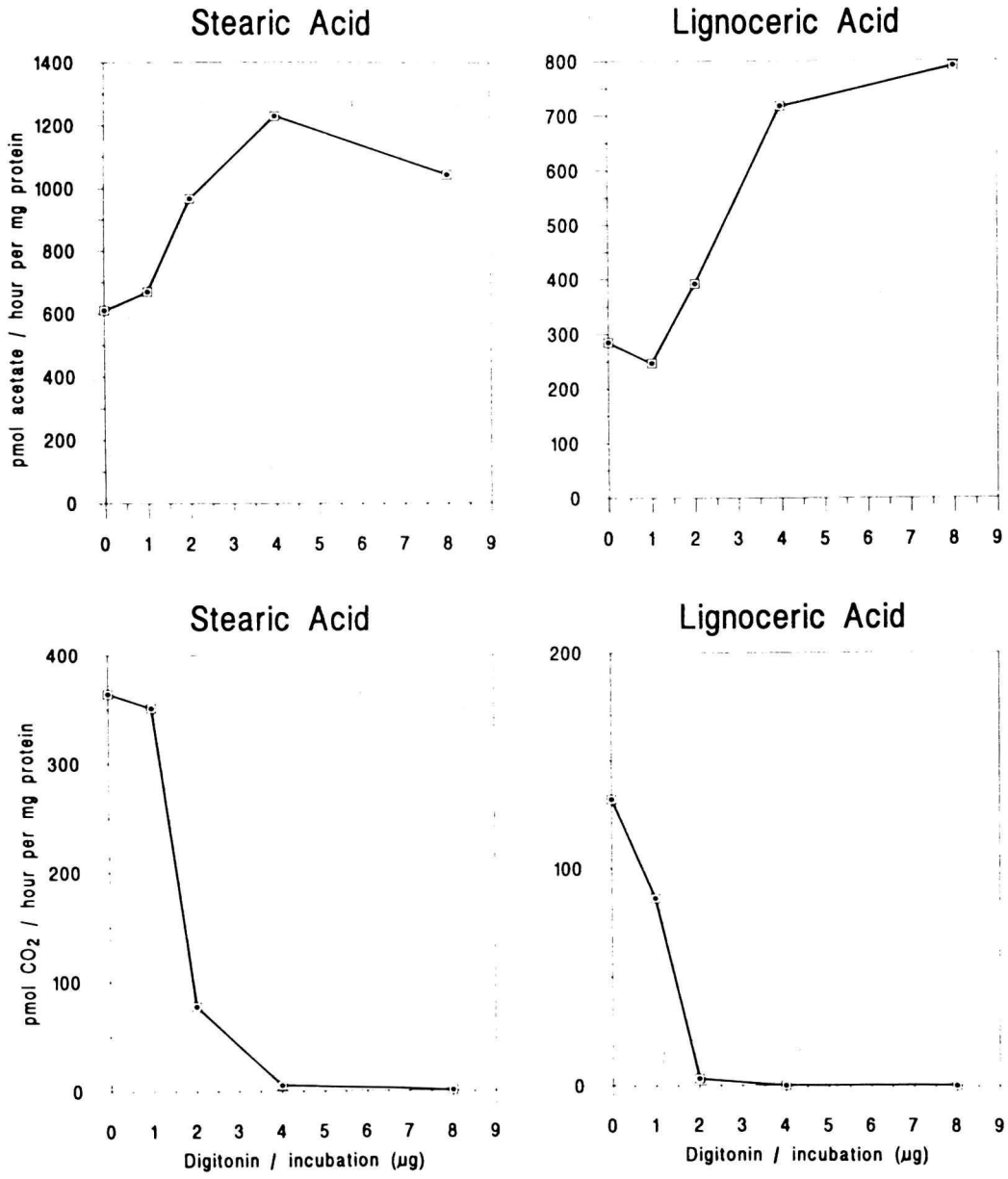

Fig. 2. [1-^{14}C]-labeled fatty acids were incubated with skin fibroblast suspensions (29 μg cellular protein) with indicated amounts of digitonin in the assay medium. For measurements of water-soluble product the incubation period was 60 min at 37°C, whereas for $^{14}CO_2$ measurements the incubations were carried out at 37°C for 3 h (for stearic acid) or 4 h (for lignoceric acid). The assays were performed in duplicate and each point represents the mean of two observations. Three separate experiments were conducted and results were essentially the same.

(0–1 mM) had no effect on radiolabeled acetate or CO_2 production from [1-^{14}C]-labeled fatty acids (data not shown).

We investigated the effect of electron-transport inhibitors on the production of radiolabeled acetate or propionate from [1-^{14}C]-labeled fatty acids (**Table 1**). The assays were performed in the presence of digitonin (see Methods) in the incubations. We found that electron-transport inhibitors, namely, cyanide, antimycin, and rotenone, inhibited fatty acid β-oxidation in control fibroblasts (Table 1). Electron-transport inhibitors markedly inhibited stearic acid β-oxidation compared to pristanic, homophytanic, and lignoceric acid β-oxidation in control fibroblasts (Table 1). Stearic acid β-oxidation to acetate was strongly (>95%) inhibited by electron-transport inhibitors in Zellweger fibroblasts (data not shown). These observations clearly indicate that the electron-transport chain is not affected by low concentrations of digitonin in

TABLE 1. Effect of electron transport inhibitors on fatty acid β-oxidation by skin fibroblasts

Inhibitor	Final Concentration	Stearic Acid	Pristanic Acid	Homophytanic Acid	Lignoceric Acid
		percent inhibition of fatty acid oxidation			
Potassium cyanide	2 mM	54	29	22	36
	5 mM	71	30	27	36
Antimycin	1 μM	78	27	21	22
	10 μM	75	32	28	39
Rotenone	1 μM	67	12	17	35
	10 μM	67	27	28	35

[1-^{14}C]-labeled fatty acids were dispersed in α-cylodextrin and incubated with control fibroblasts (50 μg protein/assay) at 37°C for 60 min. The incubations containing digitonin were performed in duplicate with and without inhibitors and radiolabeled acetate or propionate produced from fatty acids was measured (see Methods). The data are presented as percent inhibition of β-oxidation in the presence of electron-transport inhibitors.

the incubations. In contrast to radiolabeled acetate or propionate production, $^{14}CO_2$ production from [1-^{14}C]-labeled fatty acids was strongly inhibited in the presence of electron-transport inhibitors (**Table 2**) in control fibroblasts, indicating that respiration of cells is completely inhibited under the assay conditions.

The method of presentation of [1-^{14}C]-labeled fatty acid substrate and its oxidation to radiolabeled acetate or propionate, as well as $^{14}CO_2$, was investigated. Stearic, pristanic, and homophytanic acid oxidation to acetate or propionate was essentially the same, whether the fatty acid was dispersed in α-cyclodextrin or dipalmitoyl phosphatidylcholine (**Table 3**). However, lignoceric acid β-oxidation to acetate was at least 10-fold lower in phosphatidylcholine compared to the oxidation measured in α-cyclodextrin (Table 3). A similar pattern was obtained when $^{14}CO_2$ was used as the index for oxidation but the total amount of $^{14}CO_2$ produced was significantly lower than acetate or propionate (data not shown).

Acetate or propionate production from [1-^{14}C]-labeled fatty acid by skin fibroblasts depended on the presence of ATP and Mg^{2+} (**Table 4**). Addition of coenzyme A, L-carnitine, NAD^+, and FAD further stimulated oxidation (Table 4). Radiolabeled CO_2 production from [1-^{14}C]stearic or [1-^{14}C]lignoceric acid was stimulated only by L-carnitine (**Table 5**). Similar results were obtained using [1-^{14}C]pristanic or homophytanic acid (data not shown).

Radiolabeled acetate production from [1-^{14}C]stearic acid was normal or above normal in all the peroxisomal diseases investigated. Propionate production from pristanic acid was <10% of controls in Zellweger syndrome and significant reduction was observed in infantile Refsum's disease compared to control fibroblasts (**Table 6**). However, the oxidation of pristanic acid to propionate was normal in X-linked ALD and Refsum's disease (Table 6). The oxidation of homophytanic acid to acetate was significantly reduced in Zellweger syndrome and infantile Refsum's disease, and the oxidation was normal in the other peroxisomal disorders investigated (Table 6). [1-^{14}C]lignoceric acid oxidation to acetate was deficient in Zellweger syndrome, X-linked ALD, and infantile Refsum's disease, but was nearly normal in Refsum's disease and RCDP (Table 6). The oxidation of [1-^{14}C]lignoceric acid to acetate in infantile Refsum's disease and X-linked

TABLE 2. Effect of electron transport inhibitors on $^{14}CO_2$ production from [1-^{14}C]-labeled fatty acids by skin fibroblasts

Inhibitor	Final Concentration	Stearic Acid	Pristanic Acid	Homophytanic Acid	Lignoceric Acid
		percent inhibition of fatty acid oxidation			
Potassium cyanide	2 mM	71	75	54	67
	5 mM	94	90	86	95
Antimycin	1 μM	99	89	98	98
	10 μM	99	90	98	98
Rotenone	1 μM	99	93	97	99
	10 μM	98	89	98	98

[1-^{14}C]-labeled fatty acids were dispersed in α-cylodextrin and incubated with control fibroblasts (48 μg protein/assay) for 4 h. $^{14}CO_2$ measurements were performed in duplicate in the presence and absence of inhibitors (digitonin was omitted from the incubations, see Methods). The results are expressed as percent inhibition of $^{14}CO_2$ production from [1-^{14}C]-labeled fatty acids by electron-transport inhibitors.

TABLE 3. Method of presentation of substrate and β-oxidation of fatty acids

Treatment	Protein/ Incubation	Radiolabeled Acetate or Propionate Formed from Fatty Acid			
		Stearic Acid	Pristanic Acid	Homophytanic Acid	Lignoceric Acid
	μg	pmol/h			
Substrate dispersed in α-cyclodextrin	14	55.3	17.6	10.2	17.6
	28	84.4	28.1	17.1	30.0
	56	125.8	54.0	28.9	61.9
Substrate dispersed in dipalmitoyl phosphatidylcholine	14	64.9	11.6	5.5	1.9
	28	117.7	18.3	10.3	3.4
	56	156.6	47.4	21.8	3.8

[1-^{14}C]-labeled fatty acid was either dispersed in α-cyclodextrin (20 mg/ml) or dipalmitoyl phosphatidylcholine (40 μg/ml) by sonication, and 25 μl of the substrate suspension was used for β-oxidation assays (see Methods). The incubations (containing digitonin) were performed with indicated levels of cellular proteins per assay. After incubation at 37°C for 60 min the reaction was terminated with chloroform–methanol 2:1 (v/v) and the radiolabeled water-soluble product was determined. The assays were performed in duplicate with skin fibroblasts in suspension and the data are presented as mean values.

ALD was significantly higher than that observed in Zellweger syndrome fibroblasts (Table 6).

Radiolabeled CO_2 production from [1-^{14}C]stearic acid was normal or above normal in all the peroxisomal disease fibroblasts investigated (**Table 7**). The branched chain fatty acids were degraded to CO_2 at a much slower rate compared to stearic acid. CO_2 production from both pristanic and homophytanic acid was reduced in Zellweger syndrome fibroblasts (Table 7). The oxidation of branched chain fatty acids to CO_2 was greater in infantile Refsum's disease compared to Zellweger syndrome (Table 7). $^{14}CO_2$ production from [1-^{14}C]lignoceric acid was greatly reduced in Zellweger syndrome and X-linked ALD fibroblasts (Table 7). The oxidation to CO_2 of both the branched chain fatty acids and lignoceric acid was normal in Refsum's disease and RCDP fibroblasts (Table 7).

DISCUSSION

Fibroblasts in suspension are able to take up and degrade straight chain and branched chain fatty acids by β-oxidation, as is evident from the production of acetyl-CoA or propionyl-CoA. Inclusion of digitonin in the assay system stimulates β-oxidation of fatty acids due to: *a)* solubilization of lipid substrate; *b)* permeabilization of fibroblast plasma membranes and increased uptake of substrate; and *c)* increased uptake of cofactors such as ATP, Mg^{2+}, and coenzyme A which are required at the mitochondrial or peroxisomal membranes. In contrast, $^{14}CO_2$ production from [1-^{14}C]-labeled fatty acids is inhibited by digitonin in human skin fibroblasts. CO_2 is formed from acetyl-CoA in mitochondria via the citric acid cycle. Propionyl-CoA is converted to succinyl-CoA in

TABLE 4. Effect of cofactors on the β-oxidation of fatty acids by skin fibroblasts

Incubation Conditions	β-Oxidation Activity	
	Stearic Acid	Lignoceric Acid
	%	
Complete system	100	100
− Mg^{2+}	14	5
− ATP	6	1
− Coenzyme A	8	38
− L-carnitine	41	69
− NAD^+	44	20
− FAD	74	69

[1-^{14}C]-labeled fatty acid was dispersed in α-cyclodextrin and incubated at 37°C for 60 min with control fibroblasts (28 μg protein/assay). The assays were performed in duplicate with cell suspensions in the presence of digitonin in the incubations and the radiolabeled water-soluble product formed was measured as described under Methods.

TABLE 5. Effect of cofactors on the production of $^{14}CO_2$ from [1-^{14}C]-labeled fatty acids by skin fibroblasts

Incubation Conditions	Oxidation Activity	
	Stearic Acid	Lignoceric Acid
	%	
Complete system	100	100
− Mg^{2+}	99	100
− ATP	90	95
− Coenzyme A	98	106
− L-Carnitine	66	76
− NAD^+	96	106
− FAD	104	100

Radiolabeled stearic acid or lignoceric acid was dispersed in α-cyclodextrin and incubated with control fibroblasts at 37°C for 4 h. The assays were performed in duplicate with cell suspensions (27 μg protein/assay); digitonin was omitted from the incubations and $^{14}CO_2$ produced was determined (see Methods).

TABLE 6. Fatty acid β-oxidation by skin fibroblast suspensions

Clinical Diagnosis	Radiolabeled Acetate or Propionate Produced from [1-^{14}C]-Labeled Fatty Acids			
	Stearic Acid	Pristanic Acid	Homophytanic Acid	Lignoceric Acid
	pmol/h/mg protein			
Zellweger syndrome (n = 5)	2088 ± 466	84 ± 29	97 ± 21	58 ± 13
X-linked ALD (n = 5)	2228 ± 659	1693 ± 244	810 ± 128	244 ± 34
Infantile Refsum's disease (n = 8)	2859 ± 371	223 ± 56	239 ± 47	381 ± 86
Refsum's disease (n = 5)	3535 ± 1154	1243 ± 158	857 ± 117	1087 ± 55
RCDP (n = 4)	1807 ± 366	616 ± 109	417 ± 63	655 ± 78
Control (n = 38)	2081 ± 207	1263 ± 89	724 ± 59	996 ± 62

Radiolabeled fatty acid was dispersed in α-cyclodextrin and added to the fibroblasts. The β-oxidation assays were performed in duplicate in skin fibroblast suspensions in the presence of digitonin in the incubations as described under Methods. The data presented are mean ± SE and (n) is the number of different cell lines tested.

mitochondria by three separate enzyme reactions, namely propionyl-CoA carboxylase, methylmalonyl-CoA racemase, and methylmalonyl-CoA mutase. Succinyl-CoA formed from propionyl-CoA is further converted to CO_2 by citric acid cycle enzymes. As CO_2 formation is mainly mitochondrial, it is reasonable to suggest that digitonin acts on mitochondrial membranes and somehow uncouples mitochondrial β-oxidation from the citric acid cycle. Alternatively, digitonin possibly uncouples the electron-transport chain which in turn affects the citric acid cycle. The data presented in Table 1 clearly indicate that digitonin does not uncouple electron-transport chain and it is further supported by the observations that acetate production from stearic acid is normal in Zellweger syndrome fibroblasts (Table 6). We find that $^{14}CO_2$ production from [2-^{14}C]acetate by skin fibroblasts is completely inhibited by digitonin, indicating that digitonin acts at the level of citric acid cycle.

The results presented in Table 1 clearly indicate that, under the assay conditions described, both mitochondrial and peroxisomal fatty acid β-oxidation are measured. As CO_2 production from fatty acids is completely blocked by digitonin whereas acetate or propionate production is unaffected, we advocate that fatty acid β-oxidation can be reliably measured in the presence of very low concentrations of digitonin. The observations that fatty acid β-oxidation depends on exogenous ATP, Mg^{2+}, and coenzyme A (Table 4) suggest that very low concentrations of digitonin permeabilize plasma membrane of the cells. Low concentrations of digitonin do not uncouple electron-transport chain (Table 1). ATP, Mg^{2+}, and coenzyme A were not required for fatty acid oxidation when digitonin was omitted from the incubations (data not shown, see also Table 5). We speculate that free fatty acids are taken up by the cell and transported to mitochondria or peroxisomes via fatty acid-binding proteins present in the cytosolic compartment of the cell (15).

The results reported in Table 3 are in agreement with our earlier observations using skin fibroblast homogenates where we (1) demonstrated several-fold greater lignoceric acid β-oxidation in the presence of α-cyclodextrin compared to the oxidation measured in the presence of dipalmitoyl phosphatidyl choline. The data presented in Table 3 suggest that either there are separate oxidation

TABLE 7. Oxidation of radiolabeled fatty acids to carbon dioxide by skin fibroblasts

Clinical Diagnosis	Radiolabeled CO_2 Produced from [1-^{14}C]-Labeled Fatty Acids			
	Stearic Acid	Pristanic Acid	Homophytanic Acid	Lignoceric Acid
	pmol/h/mg protein			
Zellweger syndrome (n = 5)	406 ± 113	2 ± 1	13 ± 4	7 ± 1
X-linked ALD (n = 4)	465 ± 149	43 ± 16	57 ± 10	26 ± 6
Infantile Refsum's disease (n = 5)	860 ± 100	10 ± 6	49 ± 9	105 ± 38
Refsum's disease (n = 3)	1063 ± 208	29 ± 6	61 ± 19	228 ± 32
RCDP (n = 5)	714 ± 168	49 ± 6	92 ± 7	166 ± 28
Control (n = 23)	564 ± 36	34 ± 4	64 ± 5	135 ± 11

[1-^{14}C]-labeled fatty acid was dispersed in α-cyclodextrin and added to skin fibroblasts in suspension. The assays were performed in duplicate and digitonin was omitted from the incubations (see Methods); the values represent mean ± SE, and (n) is the number of cell lines examined.

systems for the degradation of LCFA and VLCFA, or at least one of the enzymes involved in the oxidation of these fatty acids is different. Our findings (2) in rat liver peroxisomes and microsomes support the latter possibility.

The observation that stearic acid oxidation is normal whereas pristanic, homophytanic, and lignoceric acid oxidation is defective in Zellweger syndrome fibroblasts in suspension (Tables 6 and 7) suggest that branched chain fatty acid and VLCFA β-oxidation occurs exclusively in peroxisomes. The results on lignoceric acid β-oxidation in Zellweger fibroblasts in suspension are in agreement with the earlier reports using fibroblast homogenates (1, 3, 4, 6, 8). The defective β-oxidation of pristanic and homophytanic acid in Zellweger fibroblasts in suspension also supports our earlier observations with skin fibroblasts in culture (13). It is interesting to note that both CO_2 and acetate production from lignoceric acid are clearly defective in X-linked ALD fibroblasts. These studies differ from our earlier report in cell culture (6) which may relate to slow uptake of lignoceric acid by skin fibroblasts in culture. The defective β-oxidation of lignoceric acid in X-linked ALD is suggested to be due to defective peroxisomal very long chain fatty acyl-CoA synthetase (3, 16). It is thought that in skin fibroblasts, as in rat liver, there are distinct peroxisomal long chain and very long chain fatty acyl-CoA synthetases (2, 17), and the former enzyme is normal in X-linked ALD. As pristanic and homophytanic acid β-oxidation is normal in X-linked ALD it suggests that peroxisomal very long chain fatty acyl-CoA synthetase is different from branched chain fatty acyl-CoA synthetase.

The studies described suggest that branched chain fatty acids and VLCFA are degraded by β-oxidation to acetyl-CoA or propionyl-CoA in peroxisomes. However, the enzymes that degrade acetyl-CoA or propionyl-CoA to CO_2 are present only in mitochondria (see above). Thus, acetyl-CoA or propionyl-CoA formed from fatty acids in peroxisomes must be transported to mitochondria where, presumably after conversion to the free acids by acetyl-CoA hydrolases either in peroxisomes or in the cytosol, the acids can enter the organelle. Alternatively, acetyl-CoA or propionyl-CoA formed by β-oxidation may be converted to carnitine esters by acetyl-CoA carnitine transferases in peroxisomes, and the carnitine esters may be transported from peroxisomes to mitochondria. The longer lag period observed in the production of CO_2 from lignoceric (Fig. 1), pristanic, and homophytanic acids (data not shown) compared to stearic acid (Fig. 1) suggest that acetate or propionate is transported from one organelle to the other in human skin fibroblasts.

Phytanic acid is initially degraded by α-oxidation, and the oxidation is defective in Refsum's disease and RCDP fibroblasts in culture (6, 18–20). The results reported indicate that pristanic acid (formed from phytanic acid by α-oxidation) β-oxidation occurs mainly in peroxisomes in human skin fibroblasts in suspension (Table 6) as well as skin fibroblasts in culture (13). The β-oxidation of pristanic acid is clearly normal in Refsum's disease and RCDP fibroblasts in suspension (Tables 6 and 7). Pristanic acid is one carbon shorter whereas homophytanic acid is one carbon longer than phytanic acid, and yet both the branched chain fatty acids are directed to peroxisomes for degradation in skin fibroblasts (Tables 6 and 7). These observations, together with the reports that Zellweger fibroblasts in culture are unable to degrade [1-^{14}C]phytanic acid (6, 21, 22) suggest strongly that peroxisomes contain α-oxidation enzymes. Thus, it would appear unlikely that in skin fibroblasts α-oxidation of phytanic acid occurs in mitochondria (23) and the product, pristanic acid, is then transported out of mitochondria and further degraded by β-oxidation in peroxisomes. Our in vitro studies with rat liver mitochondria (H. Singh, K. Beckman, D. Johnson, and A. Poulos, unpublished results) indicate that phytanic acid is converted to phytanoyl-CoA at the mitochondrial membranes but cannot be transported inside the mitochondria. Thus, phytanic acid and other branched chain fatty acid oxidation is directed to peroxisomes.

Recent observations suggest that peroxisomal 3-ketoacyl-CoA thiolase is not processed normally in RCDP (12, 24, 25). In RCDP fibroblasts acyl-CoA oxidase and bifunctional protein are transported normally to peroxisomes whereas 3-ketoacyl-CoA thiolase is directed to peroxisomal ghosts' (24, 25). Heikoop et al. (25) claimed that peroxisomal palmitoyl-CoA oxidation is partially defective in RCDP fibroblasts but no quantitative data were presented. Both Balfe et al. (24) and Heikoop et al. (25) were unable to detect immunochemically peroxisomal 3-ketoacyl-CoA thiolase in peroxisomal fractions of RCDP fibroblasts, yet VLCFA, which are known to be degraded only in peroxisomes, do not accumulate in this disease (12, 20). The results reported (Tables 6 and 7) strongly suggest that RCDP skin fibroblast peroxisomes contain sufficient 3-ketoacyl-CoA thiolase activity to degrade not only lignoceric acid but also pristanic and homophytanic acids.

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