Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts

Nanda M. Verhoeven,[1,*] Diane S. Roe,[†] Robert M. Kok,[*] Ronald J. A. Wanders,[§] Cornelis Jakobs,[*] and Charles R. Roe[†]

Department of Clinical Chemistry,* Free University Hospital, 1081 HV Amsterdam, The Netherlands; Institute of Metabolic Disease,[†] Baylor University Medical Center, Dallas, TX; and Departments of Pediatrics and Clinical Chemistry,[§] University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands

Abstract The relationship between peroxisomal and mitochondrial oxidation of the methyl branched fatty acids, phytanic acid and pristanic acid, was studied in normal and mutant human skin fibroblasts with established enzyme deficiencies. Tandem mass spectrometry was used for analysis of the acylcarnitine intermediates. In normal cells, 4,8-dimethylnonanoylcarnitine (C11:0) and 2,6-dimethylheptanoylcarnitine (C9:0) accumulated after incubation with either phytanic acid or pristanic acid. These intermediates were not observed when peroxisome-deficient cells from Zellweger patients were incubated with the same compounds, pointing to the involvement of peroxisomes in the formation of these acylcarnitine intermediates. Similar experiments with fibroblasts deficient in carnitine palmitoyltransferase I, carnitine-acylcarnitine translocase or carnitine palmitoyltransferase II revealed that mitochondrial carnitine palmitoyltransferase I is not required for the oxidation of phytanic acid or pristanic acid, whereas both carnitine-acylcarnitine translocase and carnitine palmitoyltransferase II are necessary. These studies demonstrate that both phytanic acid and pristanic acid are initially oxidized in peroxisomes to 4,8-dimethylnonanoyl-CoA, which is converted to the corresponding acylcarnitine (presumably by peroxisomal carnitine octanoyltransferase), and exported to the mitochondrion. After transport across the mitochondrial membrane and transfer of the acylgroup to coenzyme A, further oxidation to 2,6-dimethylheptanoyl-CoA occurs.**—Verhoeven, N. M., D. S. Roe, R. M. Kok, R. J. A. Wanders, C. Jakobs, and C. R. Roe.** Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J. Lipid Res.* 1998. **39:** 66–74.

Supplementary key words tandem mass spectrometry • peroxisome • mitochondrion • carnitine palmitoyltransferase

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) cannot be degraded by β-oxidation due to its β-methyl group. Instead, a one carbon moiety is split from the molecule by α-oxidation, yielding pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). The site of phytanic acid α-oxidation and the identity of the enzymes involved have been subjects of discussion for a long time. According to some authors, phytanic acid α-oxidation occurs in the endoplasmic reticulum (1) whereas others found evidence for mitochondrial (2) or peroxisomal localization (3, 4). Recently, however, the first step of phytanic acid α-oxidation was elucidated and found to be peroxisomally localized in human liver (5, 6).

Further β-oxidation of pristanic acid has been shown to occur partly in peroxisomes (7, 8). It is not known, however, how many cycles of pristanic acid β-oxidation are peroxisomal. Furthermore, the identity of the product of peroxisomal β-oxidation of pristanic acid, which moves from peroxisomes to mitochondria for further oxidation, has not yet been established.

These questions prompted us to investigate the oxidation of phytanic acid and pristanic acid in intact human fibroblasts using tandem mass spectrometric analysis of acylcarnitine intermediates. In addition to normal fibroblasts, cell lines derived from Zellweger patients which lack peroxisomes were studied to explore the role of peroxisomes. Cell lines obtained from patients with established deficiencies of either CPT I, CPT II, or carnitine-acylcarnitine translocase were studied to explore the involvement of these mitochondrial enzymes in the α-oxidation of phytanic acid and subsequent β-oxidation of pristanic acid.

Abbreviations: CPT I, carnitine palmitoyltransferase I; CPT II, carnitine palmitoyltransferase II; BSA, bovine serum albumin; FCS, fetal calf serum; MEM, minimal essential medium.

[1]To whom correspondence should be addressed.

METHODS

Materials

L-carnitine and defatted bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO); the cell culture media, antibiotics, and fetal calf serum (FCS) were obtained from Gibco BRL (Grand Island, NY). Pristanic acid and phytanic acid were synthesized as described previously (9). [15-methyl-$^2H_3$,16,16,16-$^2H_3$]phytanic acid ([ω-d6]phytanic acid) was synthesized by ARC, Amsterdam, The Netherlands and purified by high performance liquid chromatography. [14-methyl-$^2H_3$,15,15,15-$^2H_3$]pristanic acid ([ω-d6]pristanic acid) was synthesized from [ω-d6]phytanic acid as described for [d3]pristanic acid (9).

[$^2H_3$]octanoylcarnitine and [$^2H_3$]propionylcarnitine were obtained from Cambridge Isotope Laboratories (Cambridge, MA).

Cell culture

Fibroblasts were cultured in Eagle's minimal essential medium containing nonessential amino acids (MEM), 10% FCS, and antibiotics. Studies were conducted using human skin fibroblasts from the 6th to 20th passages. Protein concentrations were determined by the method of Bradford (10) with BSA as standard.

Substrate preparation and fatty acid oxidation assay

Aliquots from stock solutions of phytanic acid and pristanic acid in ethanol were prepared by completely evaporating the ethanol under a stream of dry N_2. The residues were dissolved in a solution of defatted BSA in medium. The molar ratio of fatty acid to BSA was 4:1.

All incubations were performed in triplicate. Skin fibroblasts (0.2–0.6 mg protein) were subcultured in 25-cm^2 flasks and left to grow to confluency, after which the medium was replaced with 3.5 ml of freshly prepared bicarbonate-buffered MEM medium containing 10% FCS, antibiotics, 0.4 mM L-carnitine, and 0.2 mM fatty acid bound to BSA. Cells were incubated for a period of 96 h at 37°C in humidified 5% CO_2/95% air. After the incubation period, the reaction medium was collected. The cells were harvested by trypsinization, washed twice in MEM medium, and resuspended in 300 μl water.

Tandem mass spectrometric analysis of acylcarnitines

To 100-μl aliquots of the reaction medium or the resuspended cells, 20 pmol of [$^2H_3$]propionylcarnitine ($^2H_3$-C3) and 10 pmol [$^2H_3$]octanoylcarnitine ($^2H_3$-C_8) were added as internal standards. The mixture was extracted with 0.8 ml ethanol, centrifuged, and the clear supernatant was dried under nitrogen. The dried aliquots containing acylcarnitines were incubated with 100 μl of 3 M HCl in methanol at 50°C for 15 min in a capped 1-ml glass vial. The esterifying agent was removed by evaporation under nitrogen at 40°C and the methyl esters were dissolved in 50 μl of methanol–glycerol 1:1 (v/v) containing 1% octyl sodium sulfate (matrix).

A QUATTRO tandem quadrupole mass spectrometer (Fisons-VG Instruments, Danvers, MA) equipped with a liquid secondary ionization source and a cesium ion gun was used for the analysis of acylcarnitines. A parent ion scan function of *m/z* 99 enables selective detection of acylcarnitine methyl esters. Approximately 2 μl of sample matrix was analyzed and the data were recorded and processed as previously described (11). The final spectra display the relative intensities of ions corresponding to the molecular weights of the individual acylcarnitine methyl esters. Concentrations of C9:0 and C11:0 were measured relative to the concentration of the internal standard [$^2H_3$]octanoylcarnitine.

Gas chromatography–mass spectrometric analysis of fatty acids

To verify the structures of the acylmoieties of the acylcarnitines C11 and C9, we subjected the media from [ω-d6]pristanic acid incubations to hydrolysis. Hereafter, the pentafluorobenzyl esters of the free fatty acids were formed by addition of 100 μl 7% pentafluorobenzylbromide (PFB-Br) in acetonitrile and 10 μl triethylamine. After extraction to hexane, the extract was analyzed by gas chromatography negative chemical ionization mass spectrometry, using two different mass spectrometric techniques. First, selected ion monitoring was performed, monitoring the carboxylate anions of d6 labeled C11 (*m/z* −191) and d6 labeled C9 (*m/z* −163) (Hewlett-Packard Engine 5989B). Second, high energy collision and constant B/E ratio linked scanning, producing the daughter spectrum of the carboxylate anion of the fatty acid, was performed on a two sector Kratos Concept 1H mass spectrometer. The daughter spectrum of the charge remote fragmentation type was recorded in one single scan (1 sec/decade) over the eluting gas chromatographic peak.

Source of cells

Information on the origins as well as enzyme activities of the different cell lines used can be found in **Table 1**.

RESULTS

Oxidation of branched chain fatty acids in normal fibroblasts

Figure 1 depicts the acylcarnitine profile in medium from normal human fibroblasts incubated with pris-

TABLE 1. Production of 2,6-dimethylheptanoylcarnitine (C9:0) and 4,8-dimethylnonanoylcarnitine (C11:0) in human fibroblasts after incubation with 0.2 mm pristanic acid or 0.2 mm phytanic acid and 0.4 mm l-carnitine

		Pristanic Acid		Phytanic Acid	
Type	Ref	C9	C11	C9	C11
CPT I deficiency					
Patient 1	13, 14	2.0 ± 0.5	1.1 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
Patient 2	15	3.8 ± 0.3	1.6 ± 0.2		
CPT II deficiency					
Patient 1[a]		nd	5.6 ± 0.1	nd	1.2 ± 0.5
Patient 2	16	nd	13.5 ± 2.0	nd	1.6 ± 0.5
Patient 3[b]		nd	4.8 ± 0.8	nd	1.3 ± 0.2
Patient 4	17	nd	9.3 ± 2.9	nd	1.5 ± 0.4
Translocase def.					
Patient 1	18	0.5 ± 0.1	6.2 ± 0.9	nd	0.6 ± 0.2
Patient 2	19	0.8 ± 0.1	7.2 ± 0.5	0.2 ± 0.0	2.1 ± 0.1
Patient 3	13	0.5 ± 0.2	5.1 ± 0.9	0.1 ± 0.1	1.6 ± 0.2
Zellweger syndrome					
Patient 1[c]		nd	nd	nd	nd
Patient 2[d]		nd	nd	nd	nd
Patient 3[d]		nd	nd	nd	nd
Controls					
Control 1[e]		1.6 ± 0.1	3.6 ± 0.2	0.2 ± 0.0	0.2 ± 0.0
Control 2[e]		2.9 ± 0.2	1.6 ± 0.3	0.9 ± 0.1	0.3 ± 0.1
Control 3[f]		3.6 ± 1.1	0.9 ± 0.3	0.4 ± 0.1	0.4 ± 0.1

Results are expressed as nmol/96 h per mg protein ± SD; nd, not detectable.
[a]CPT II activity fibroblasts: 1.7 nmol/min · mg protein (controls 15.4 ± 3.3).
[b]CPT II activity fibroblasts: 0.08 nmol/min · mg protein (controls 0.85–2.31).
[c]Obtained from the American Cell Culture Repository (GM 00228A).
[d]Patients were not described before but showed all biochemical abnormalities found in Zellweger syndrome.
[e]Obtained from patients not affected with a disorder affecting peroxisomal or mitochondrial oxidation.
[f]Obtained from the American Cell Culture Repository (GM 0833A).

tanic acid. Unlike in the medium of cells incubated with unbranched fatty acids such as palmitate or linoleate, acylcarnitines with an odd number of carbons (C9:0 and C11:0) were observed.

The acylcarnitines corresponding to C3 and C5 (propionylcarnitine, isovalerylcarnitine, and 2-methylbutyrylcarnitine) are derived from amino acid degradation. The acylcarnitines corresponding to C4, butyryl- and isobutyrylcarnitine, are derived from fat and valine degradation, respectively. Signals corresponding to butyryl- and hexanoyl-, palmitoyl-, and linoleylcarnitine are also observed in the absence of added pristanic acid, suggesting that these originate from the fatty acids in the medium containing fetal calf serum (12).

The same profile with C9:0 and C11:0 was observed in medium from control cells incubated with phytanic acid, although, due to the low α-oxidation activity as compared to the β-oxidation activity, their concentrations were lower than those observed with pristanic acid (Table 1).

In addition to medium, acylcarnitines in the cells were analyzed. The acylcarnitine profile only showed the presence of C2, C3, C4, and C5 acylcarnitines, not originating from phytanic acid or pristanic acid.

Oxidation of branched chain fatty acids in peroxisome-deficient cells

Figure 1 also shows the results of incubating Zellweger cells, which lack peroxisomes, with either phytanic acid or pristanic acid. The profile only revealed acylcarnitines originating from degradation of amino acids. There was no evidence for the odd numbered carbon species, C9:0 and C11:0, observed in control cells.

Oxidation of branched chain fatty acids in mutant fibroblasts with deficiencies at the level of CPT I, carnitine-acylcarnitine translocase or CPT II

CPT I-deficient fibroblasts are not able to generate acylcarnitines from long chain fatty acids. **Figure 2** illustrates the absence of signals corresponding to these fatty acids and intermediates expected to be derived from them. However, when incubated with pristanic acid, C9:0 and C11:0 acylcarnitines were observed in concentrations comparable to those of control cells. The same profile was observed when CPT I-deficient cells were incubated with phytanic acid, but the levels of C9:0 and C11:0 were lower (Table 1).

After incubation with pristanic acid, the carnitine-

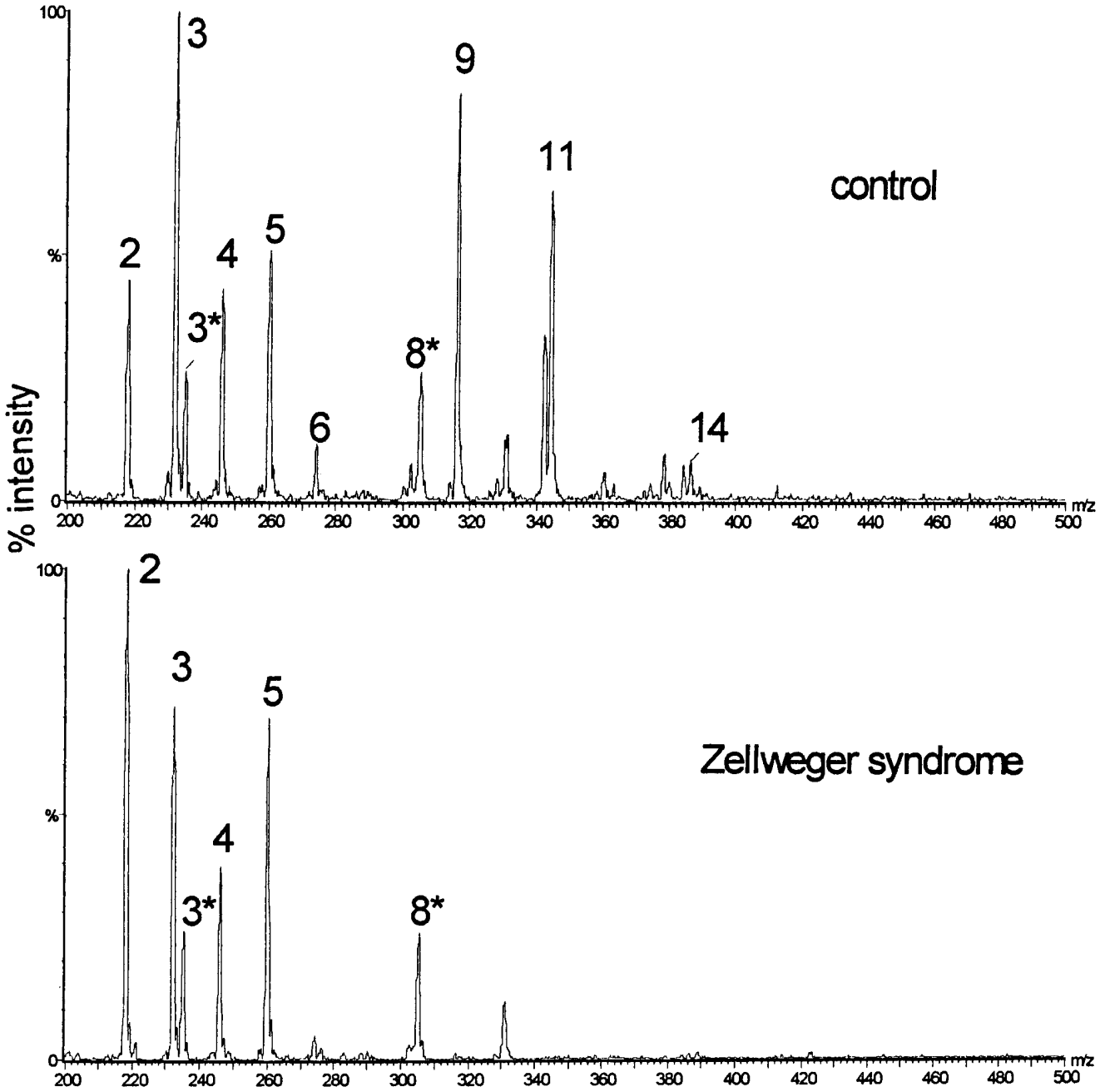

Fig. 1. Acylcarnitine profile produced upon incubation of normal and Zellweger syndrome fibroblasts with pristanic acid. The signals corresponding to the acylcarnitine methyl esters are numbered according to the carbons in the acyl moiety; *indicates deuterated internal standards for C3 and C8.

acylcarnitine translocase-deficient cell lines revealed increased signals corresponding to palmitoylcarnitine and oleoylcarnitine, derived from the long chain fatty acids in the FCS. The concentration of C11:0 was increased, while the level of C9:0 was decreased relative to normal cells. This phenomenon was also observed with phytanic acid; the concentration of C11:0 was lower than that seen with pristanic acid.

Cells deficient in CPT II also showed low amounts of long chain acylcarnitines (C16:0 and C18:1) expected from degradation of fatty acids from the medium. As with translocase-deficient cells, C11:0 was greatly increased while C9:0 was virtually absent. With phytanic acid and pristanic acid similar profiles were obtained, although C11:0 was increased more in the pristanic acid incubation.

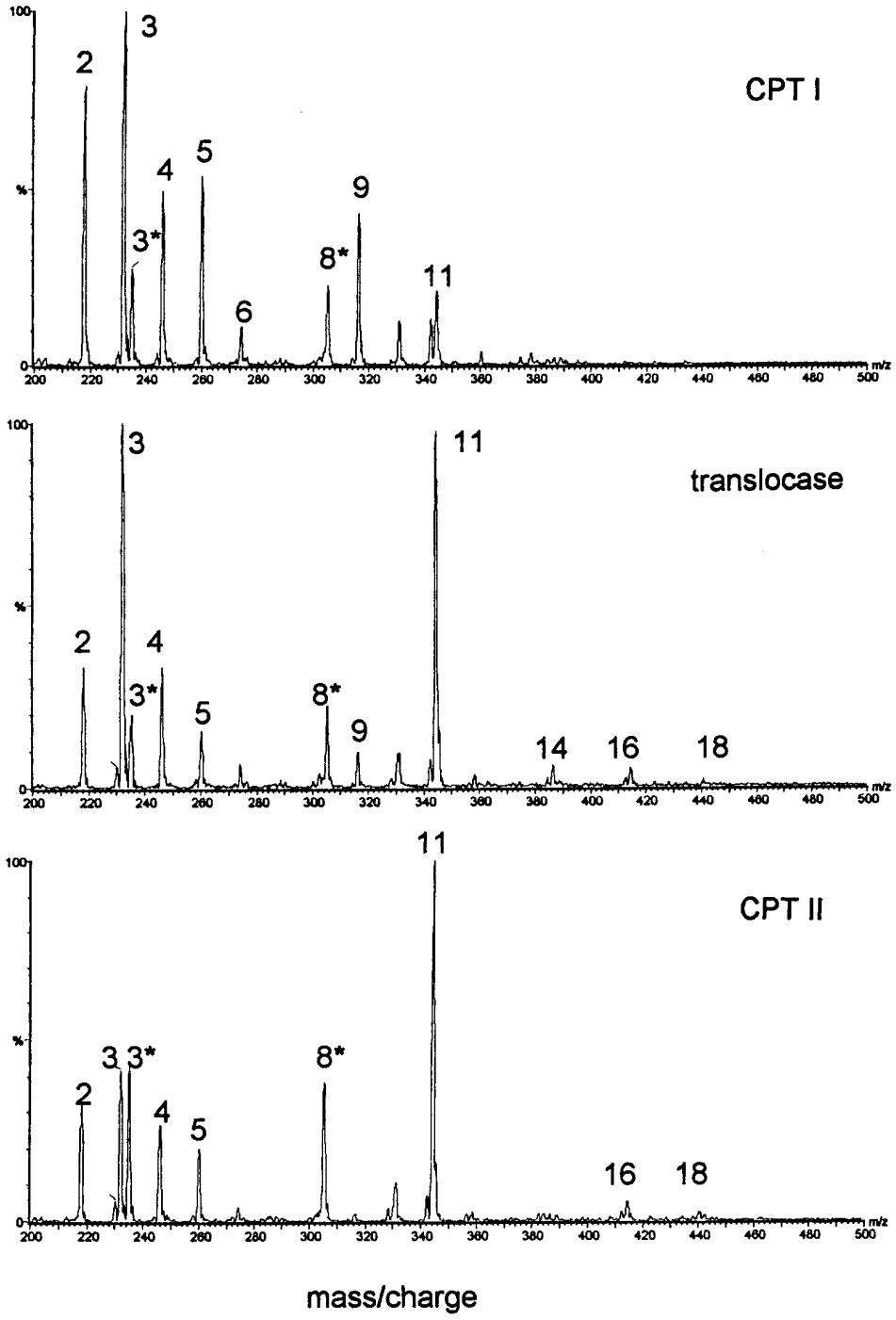

Fig. 2. Acylcarnitine profile produced upon incubation of fibroblasts deficient in CPT I, carnitine-acylcarnitine translocase, or CPT II with pristanic acid. For nomenclature see Fig. 1.

Incubations with [ω-d6]phytanic acid and [ω-d6]pristanic acid

To verify that the C11:0 and C9:0 observed in the acylcarnitine profiles originate from the substrates phytanic acid and pristanic acid, ω-labeled substrates were used. The C11:0 and C9:0 observed after incubation with these substrates were found to be labeled, as their masses were 6 units higher than when unlabeled substrates were used. No labeled acylcarnitines with shorter chain length than C9 were observed.

Gas chromatography selected ion monitoring mass spectrometry analysis of the fatty acids present in the medium after hydrolysis showed the presence of d6-labeled C11 and d6-labeled C9 fatty acids in the controls. These fatty acids were present in very low concentrations in medium of the Zellweger cell lines. High concentrations of C11 were found in the media from CPT II- and carnitine-acylcarnitine translocase-deficient cells, whereas the C9 concentrations were very low. The retention time of the C11 in the medium was the same as the retention time of an authentic standard of 4,8-dimethylnonanoic acid, but different from the retention time of straight chain undecanoic acid.

Using high energy collision and constant B/E ratio linked scanning, daughter spectra of the charge remote fragmentation type are obtained. In this way, the spectrum of a straight chain fatty acids shows, next to the carboxylate anion, a smooth pattern of all fragments formed after $C_nH_{2n}+H_2$ losses. The daughter spectrum of $C_nH_{2n}+H_2$ losses from the parent anion of d6-labeled C11 in the medium (m/z −191) showed absence of anions resulting from the loss of ethane (resulting in m/z −158) and heptane (resulting in m/z −85) (**Fig. 3C**). This points to the presence of methyl substitution on the 4 and 8 carbon of a C9 aliphatic acid. Furthermore, it was concluded from the loss of CHD_3 and $C_3H_2D_6$ from the parent anion that deuterium labeling was on the 8′ and 9 carbon atom of the C11 in the medium. The spectrum was compared with spectra of unlabeled synthetic undecanoic acid (Fig. 3A) and 4,8-dimethylnonanoic acid (Fig. 3B). The daughter spectrum of undecanoic acid differed from the spectrum of [ω-d6]C11 in the medium (Fig. 3A). In contrast, 4,8-dimethylnonanoic acid showed, apart from the deuterium labeling, the same daughter spectrum from its carboxylate anion m/z −185 as [ω-d6]C11 in the medium (Fig. 3B).

In addition, the daughter spectrum of the d6-labeled C9 in the medium (m/z −163) was taken. The spectrum showed absence of anions resulting from losses of ethane and heptane, proving that this compound also has a branched chain structure (2,6-dimethylheptanoic acid)(results not shown).

DISCUSSION

Fatty acid degradation is known to occur in both mitochondria and peroxisomes of human cells. Short, medium, and long chain fatty acids are mainly degraded in mitochondria while oxidation of fatty acids of greater chain length (>20 carbons) or methyl branched fatty acids, such as phytanic acid and pris-

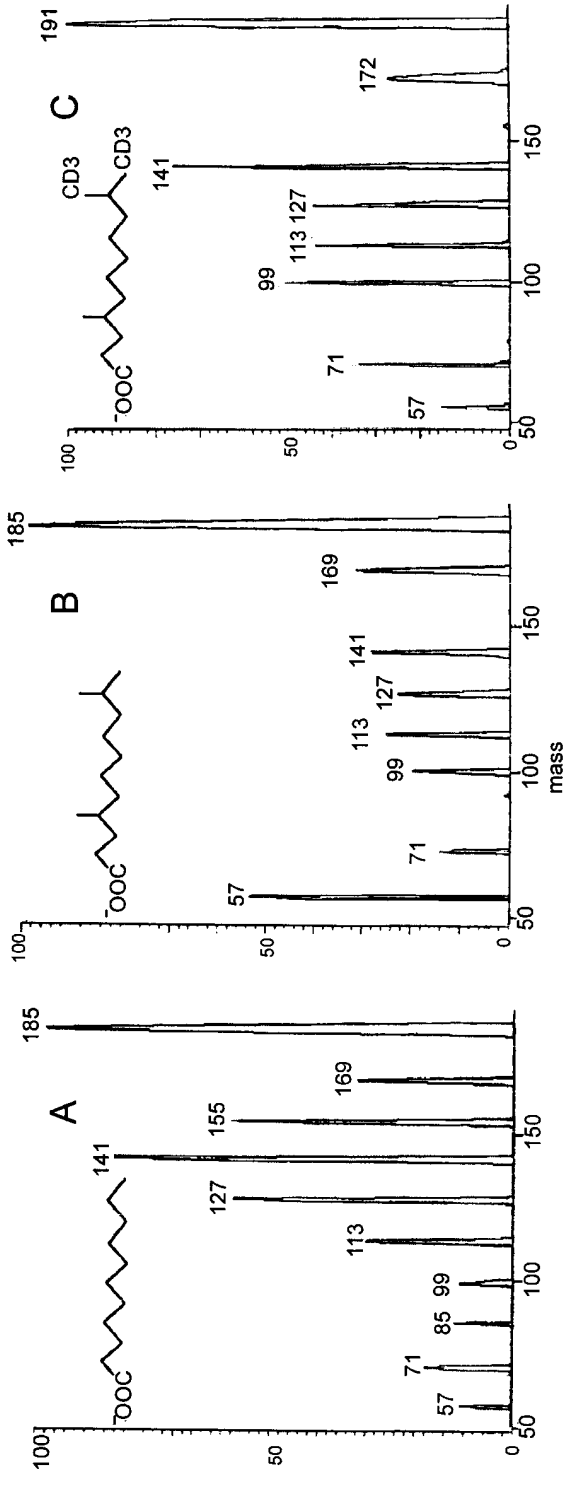

Fig. 3. The daughter spectra showing $C_nH_{2n}+H_2$ losses from the parent anions of A: undecanoic acid, B: 4,8-dimethylnonanoic acid, and C: d6-labeled C11 in the medium of cells incubated with [ω-d6]pristanic acid.

tanic acid, occurs in peroxisomes (5, 7, 8). The mechanism and intracellular localization of the complete degradation of phytanic acid have long remained unclear, but have been partly elucidated recently. Before oxidation, phytanic acid is activated to phytanoyl-CoA by phytanoyl-CoA synthetase in the peroxisomal outer membrane (20, 21). Subsequently, phytanoyl-CoA is converted to 2-hydroxyphytanoyl-CoA by phytanoyl-CoA hydroxylase, a dioxygenase type of enzyme. This conversion is localized in peroxisomes and deficient in Refsum disease, rhizomelic chondrodysplasia punctata, and Zellweger syndrome (5, 6, 22). The remainder of the pathway to pristanic acid is known to include a decarboxylation step in which formyl-CoA is released (23) and pristanal is formed (24). However, the enzymes involved are still to be resolved.

The subsequent oxidation of pristanic acid is known to be peroxisomal. This was concluded from the fact that oxidation of [1-^{14}C]pristanic acid is deficient in peroxisome-deficient cells obtained from patients affected with the Zellweger syndrome (7), suggesting that at least the first cycle of pristanic acid β-oxidation, yielding a 16 carbon branched chain fatty acid, occurs in peroxisomes. It is generally assumed that peroxisomal β-oxidation of fatty acids is incomplete because peroxisomal acyl-CoA oxidase does not accept shorter chain acyl-CoAs as substrates (25–27).

In this study, we investigated the oxidation of phytanic acid and pristanic acid in cultured human fibroblasts. It was shown that control human fibroblasts are able to degrade both phytanic acid and pristanic acid to 4,8-dimethylnonanoyl-CoA followed by one step of β-oxidation yielding 2,6-dimethylheptanoyl-CoA. Both these intermediates are converted to their corresponding carnitine esters by the cells. The observation that C9:0 and C11:0 were not detectable in the cells suggests rapid excretion and/or further metabolism of these intermediates.

Confirmation that the C9:0 and C11:0 intermediates originate from the substrates phytanic acid and pristanic acid was obtained by using highly purified ω-d6-labeled substrates and gas chromatography mass spectrometry analysis of free fatty acids in the medium.

Further information about the mechanism and subcellular localization of the oxidation of phytanic acid and pristanic acid was obtained by using cell lines with specific enzyme defects. Incubations of cell lines with a deficiency of CPT I showed that CPT I is not required for degradation of phytanic acid and pristanic acid, as the amounts of C11:0 and C9:0 in the media were equivalent to the controls.

Peroxisome-deficient cells, obtained from patients affected with a generalized peroxisomal disorder, showed the absence of C11:0 and C9:0 after incubation with phytanic acid or pristanic acid. This confirms the involvement of peroxisomes in the oxidation of these branched chain fatty acids.

The observation that in the medium of cell lines with a deficiency of carnitine-acylcarnitine translocase an increased amount of C11:0 and a decreased amount of C9:0 were found shows that this enzyme is not involved in formation of C11:0, but is indispensable for further oxidation of C11:0 to C9:0.

Combining the results obtained with the CPT I-deficient cell lines and the carnitine–acylcarnitine

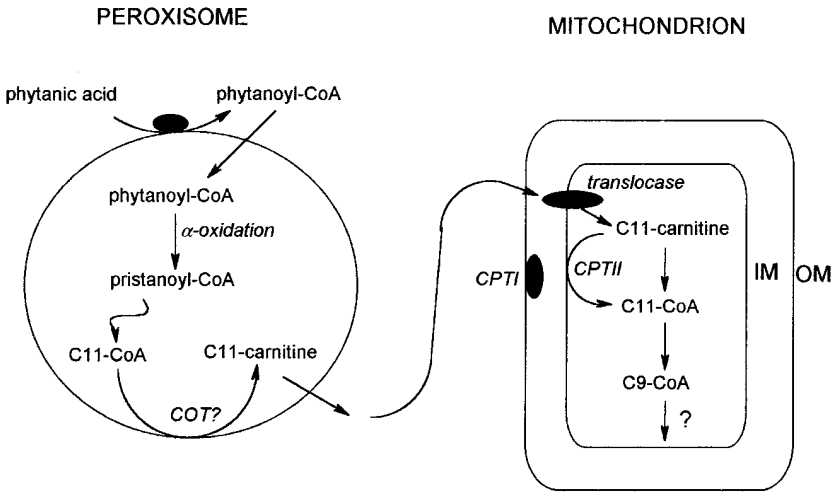

Fig. 4. Model of the functional and physical organization of oxidation of phytanic acid and pristanic acid in human fibroblasts. COT: carnitine octanoyltransferase, translocase: carnitine-acylcarnitine translocase, CPT I: carnitine palmitoyltransferase I, CPT II: carnitine palmitoyltransferase II, IM: mitochondrial inner membrane, OM: mitochondrial outer membrane.

translocase-deficient cell lines, we conclude that 4,8-dimethylnonanoyl-CoA is formed in peroxisomes, after which it is converted to its carnitine ester. This acylcarnitine formation is possibly catalyzed by the peroxisomal carnitine octanoyltransferase which preferably accepts acyl-CoAs of 8–10 carbon atoms (28). The C11:0 is exported to the mitochondrion, where it is degraded to 2,6-dimethylheptanoyl-CoA by one cycle of mitochondrial β-oxidation.

For this to be true, CPT II would also be required for the activation and thus further oxidation of C11:0 and the production of the C9:0 intermediate. The observation that only the C11:0 intermediate was detected after incubation of CPT II-deficient cells with both phytanic acid and pristanic acid supports this conclusion.

The proposed sequence of oxidation of phytanic acid involving initial peroxisomal α-oxidation to pristanic acid and subsequent peroxisomal and mitochondrial β-oxidation of pristanic acid is depicted in **Fig. 4.** Peroxisomal oxidation of pristanic acid proceeds efficiently for 3 cycles, yielding the 11 carbon intermediate 4,8-dimethylnonanoyl-CoA which is apparently converted to the carnitine ester by peroxisomal carnitine octanoyltransferase (not CPT I) and exported from the peroxisome. Further oxidation first requires the carnitine-acylcarnitine translocase in the mitochondrial inner membrane for mitochondrial import and CPT II for activation of C11:0 to C11:0-CoA. Hereafter, at least one cycle of mitochondrial β-oxidation occurs, resulting in formation of C9:0-CoA. The rapid equilibration between CoA esters and their corresponding acylcarnitines within a cell can explain the formation of C9:0 acylcarnitine from C9:0-CoA. Further mitochondrial metabolism of C9:0-CoA would yield C6:0 and C4:0 compounds. In the acylcarnitine profile, however, there was no indication for d6 labeled C4:0 or C6:0 acylcarnitines derived from [ω-d6]pristanic acid. This suggests absence of further metabolism of C9:0-CoA. Future experiments, focussing on the intra-mitochondrial fate of pristanic acid-derived C9:0-CoA may substantiate this observation.

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