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Pseudo Infantile Refsum's Disease: Catalase-Deficient Peroxisomal Particles with Partial Deficiency of Plasmalogen Synthesis and Oxidation of Fatty Acids

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ABSTRACT. Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease are genetic disorders characterized by the virtual absence of catalase-positive peroxisomes and a general impairment of peroxisomal functions. Recent studies in these three disorders have provided morphologic evidence of peroxisomal "ghosts" of density 1.10 g/cm^3 that contain membrane proteins but lack a majority of the matrix enzyme activities. We report here the biochemical studies in a female infant with clinical features of infantile Refsum's disease whose liver and fibroblasts contained cytosolic catalase but no catalase-positive peroxisomes. Oxidation of phytanic and pipecolic acids was severely impaired, whereas oxidation of very-long-chain fatty acids and dihydroxyacetone phosphate acyltransferase activity were only partially decreased. Immunoblot analysis showed that the three peroxisomal β-oxidation enzymes (acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase) were detectable in liver tissues. The 3-ketoacyl-CoA thiolase was of the mature form (41 kD), in contrast with other peroxisomal disorders with multiple enzyme deficiencies. The majority of these peroxisomal enzyme activities were associated with two subcellular membrane vesicle fractions lacking catalase: one had the density of normal peroxisomes (1.17 g/cm^3), the other, yet undescribed, a lower density (1.137 g/cm^3). This suggests that peroxisomes (density = 1.17 g/cm^3) and structures with lower density (density = 1.137 g/cm^3) found in this patient's cultured skin fibroblasts, although lacking catalase, contained functional peroxisomal enzymes. This distinguishes this disorder from other disorders of peroxisome biogenesis. (*Pediatr Res* 34: 270–276, 1993)

Abbreviations

n-ALD, neonatal adrenoleukodystrophy
β-ketothiolase or thiolase, 3-ketoacyl-CoA thiolase
bifunctional enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
VLCFA, very-long-chain fatty acid
DAB, diaminobenzidine
DHAP-AT, dihydroxyacetone phosphate acyltransferase
RCDP, rhizomelic chondrodysplasia punctata
IRD, infantile Refsum's disease

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Peroxisomes are subcellular organelles that participate in the β-oxidation of long-chain fatty acids and VLCFA (1–3), the synthesis of plasmalogens (4), the oxidation of L-pipecolic acid (5, 6), and bile acid synthesis (7). In addition, peroxisomes contain catalase that degrades the H_2O_2 produced by various oxidases (8). Peroxisomes, like mitochondria, arise by growth and division of preexisting peroxisomes (9). All peroxisomal proteins studied so far are synthesized on free polyribosomes, then translocated into preexisting peroxisomes. Peroxisomal proteins are generally synthesized in their final size with two exceptions. The β-ketothiolase is synthesized as a 44-kD precursor converted into a 41-kD mature enzyme, and the acyl-CoA oxidase is synthesized as a 72-kD precursor converted into two subunits of 52 and 20 kD. In both cases, the proteolytic cleavage occurs inside peroxisomes (10).

The term "disorders of peroxisome biogenesis" refers to a group of inherited neurodegenerative disorders in which the structure and metabolic function(s) of peroxisomes are defective (reviewed in 9). Tissues from infants with cerebrohepatorenal syndrome (Zellweger syndrome), n-ALD, or IRD contain none or few catalase-containing peroxisomes with a normal morphology (11, 12). In these disorders, peroxisomes fail to be formed normally, leading to a generalized defect of the peroxisomal enzymes (9). RCDP differs from these disorders because peroxisomes are grossly normal in this disease and contain catalase. Moreover, only three peroxisomal functions are impaired (plasmalogen synthesis, oxidation of phytanic acid, and processing of β-ketothiolase) (13–15).

This article reports a study of peroxisomal enzyme activities in a patient with clinical features of IRD and absence of catalase-containing peroxisomes in liver and fibroblasts. Western blot analysis demonstrated the presence of peroxisomal β-oxidation enzymes in the liver. The activities for oxidation of VLCFA and DHAP-AT were partially defective in fibroblasts. The peroxisomes from cultured skin fibroblasts had a bimodal distribution in the Nycodenz gradient: a small population with normal density (1.17 g/cm^3) and a major population of peroxisome-like structures with lighter density (1.137 g/cm^3). These two peroxisomal populations showed varying degrees of peroxisomal enzyme activities but lacked catalase. These lighter density peroxisome-like structures are physically and functionally distinct from the recently described peroxisomal membrane "ghosts" (16, 17) and w-particles (18). The disorder reported here is therefore different from other disorders of peroxisomal biogenesis.

MATERIALS AND METHODS

Case report. The patient was a female infant born after an uneventful 42 wk of gestation. The parents were not related. She weighed 3620 g at birth and no facial dysmorphism was noted. Cholestasis was present during the neonatal period. Bilateral hearing loss was noted at 2 y. The patient walked at 2½ y and could speak several words at 3 y. At 3½ y, clinical evaluation showed cerebellar ataxia, retinitis pigmentosa with extinguished electroretinogram, and peripheral neuropathy. Brain-stem auditory evoked responses showed severe dysfunction of central auditory pathways. Hepatomegaly was detected. Between 3½ and 8 y, she deteriorated slowly and lost most of her motor and language abilities. At 8½ y, weight was 30 kg and head circumference 54 cm (98th percentile). The patient had few spontaneous movements, brief visual fixation, and could still respond to her surroundings. She was not able to walk or to sit up. Other findings included quadriparesis with Babinski signs and ankle clonus, generalized hypotonia, severe pigmentary retinopathy, and hepatomegaly. A skeletal survey was normal. Aspartate transaminase and alanine transaminase were 0.55 and 0.43 μkat/L, respectively (normal <0.60 μkat/L). A morning ACTH concentration was 18 nmol/L (normal <20). Visual and brain-stem auditory evoked responses were normal. A computed tomography scan showed moderate cortical atrophy without demyelination. At 15 y of age, her neurologic status has remained unchanged.

Materials and general methods. The sources of reagents were as follows: cell culture reagents were from Gibco Laboratories, Grand Island, NY; [1-^{14}C]phytanic acids (55 mCi/mmol), [^{125}I] sodium iodide (100 mCi/mL), and [U-^{14}C]glycerol 3-phosphate (159 mCi/mmol) were from Amersham Corp., Arlington Heights, IL; perdeuterated DL-pipecolic acid was from MSD Isotopes Div., Montreal, Quebec, Canada; molecular weight markers for electrophoresis and protein A were from Sigma Chemical Co., St Louis, MO; Nycodenz was from Accurate Chemical and Scientific Corp., Westbury, NY; and rabbit antisera against purified rat liver peroxisomal acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (bifunctional enzyme), and 3-ketoacyl-CoA thiolase (19–21) were generously donated by Dr. Takashi Hashimoto, Shinshu University, Matsumoto, Japan. [1-^{14}C]lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with K^{14}CN (15). All other reagents were of analytical grade and obtained from commercial sources.

A liver sample was obtained by needle biopsy at 8½ y. Electron microscopy of liver tissue was performed as reported previously (22), and catalase activity was stained cytochemically by the DAB method as described by Roels and Goldfischer (23). Catalase activity was assayed by the method of Peters *et al.* (24). The subcellular distribution of catalase (cytosolic or organelle-associated) was measured by disruption of fibroblasts with digitonin (25) and quantitation of catalase in gradient fractions (15).

Biochemical assays of peroxisomal function. Levels of VLCFA were measured in the lipid fraction of plasma and cultured fibroblasts by gas-liquid chromatography–mass spectrometry (26). The capacity of the fibroblasts to oxidize [1-^{14}C]lignoceric acid to acetate (water-soluble products) was measured as described previously (27). Briefly, the fatty acid substrate was solubilized with α-cyclodextrine and added to the assay medium. The enzyme reaction was stopped with 1.25 mL of 1 M potassium hydroxide in methanol, and the denatured protein was removed by centrifugation. The supernatant was incubated at 60°C for 1 h, neutralized with acid, and partitioned with chloroform and methanol. The amount of radioactivity in the upper phase is an index of the amount of 1-^{14}C–labeled fatty acid oxidized to acetate. For solubilization of the fatty acid substrate with α-cyclodextrine, the fatty acid (20×10^6 dpm) was first dried in a tube under nitrogen and then resuspended in 3.5 mL (20 mg/mL) of α-cyclodextrine by sonication for 1 h at 4°C. The sp act of [1-^{14}C]lignoceric acid used in fibroblast subcellular fractions and homogenates was 52 and 57 mCi/mmol, respectively.

The peroxisomal steps of plasmalogen biosynthesis were assayed in fibroblasts by the double label, double substrate method (28), and the activity of DHAP-AT was measured according to the procedure described previously (29, 30). For substrate (dihydroxyacetone phosphate) synthesis, the reaction mixture contained 10 μCi [U-^{14}C]glycerol 3-phosphate (sp act 156 mCi/mmol), 0.6 mM glycerol-3-phosphate, 5 mM pyruvate, 1 mM NAD^+, lactate dehydrogenase (10 units), α-glycerol-3-phosphate dehydrogenase (10 units), and 50 mM triethanolamine buffer, pH 7.6. After 60 min of incubation at 25°C, the reaction was stopped by the addition of an equal volume of chloroform and, after mixing vigorously, the upper phase containing [U-^{14}C]dihydroxyacetone phosphate was transferred to another set of tubes. Under these conditions, the conversion of [U-^{14}C]glycerol 3-phosphate to [U-^{14}C]dihydroxyacetone phosphate was quantitative (30). For assay of DHAP-AT activity, a reaction mixture containing 0.1 mM [U-^{14}C]dihydroxyacetone phosphate, 8 mM $MgCl_2$, 8 mM sodium fluoride, 0.4 mg albumin, 0.15 mM palmitoyl-CoA, and 75 mM acetate buffer, pH 5.4, in 0.12 mL was incubated at 37°C for 2 h. The reaction was stopped with 0.45 mL of chloroform:methanol (1:2), 150 μL of chloroform, and 150 μL 2 M KCl/0.2 M H_3PO_4. The lower phase (200 μL) was applied to filter papers (Whatman 3MM), which were dried at room temperature and then washed with 20 mL of 10%, 10 mL of 5%, and 10 mL of 1% trichloroacetic acid, respectively. Filter papers were dried overnight and the radioactivity was counted.

Plasma levels of phytanic acid were measured by gas-liquid chromatography–mass spectrometry (26) and α-oxidation of phytanic acid by fibroblasts was determined by the method of Poulos *et al.* (31). The concentrations of the bile acid intermediates, trihydroxycoprostanoic acid and dihydroxycoprostanoic acid, were determined by the method of Bjorkhem and Falk (32). L-Pipecolic acid was measured in serum using gas chromatography-mass spectrometry by a stable isotope dilution method (33).

Subcellular fractionation of cultured fibroblasts. Skin fibroblasts were grown to confluency in 75-cm^2 dishes, and 30 or more confluent flasks were harvested by mild trypsinization and incubated for 1 h as a suspension in Dulbecco's modified Eagle medium supplemented with 15% FCS at 37°C. After centrifugation, cell pellets were washed with homogenizing medium (0.25 M sucrose, 1 mM EDTA, 1 μg/mL antipain, 0.7 μg/mL leupeptin, 0.2 mM phenyl methylsulfonylfluoride, 0.1% ethanol, and 3 mM imidazole buffer, pH 7.4) and subfractionated by differential and isopyknic density gradient using Nycodenz as described previously (27). Subcellular fractions containing different organelles were identified by appropriate marker enzymes: catalase for peroxisomes (34); cytochrome *c* oxidase for mitochondria (35) and NADPH-cytochrome *c* reductase for microsomes (36). The protein concentrations were measured by the procedure of Bradford (37).

Immunoblot analysis of peroxisomal proteins. Liver homogenates were analyzed by SDS-PAGE and immunoblotting as previously described, using antisera against the peroxisomal fatty acid β-oxidation enzymes (38).

RESULTS

Electron microscopy of liver biopsy and fibroblasts. Bile canaliculi were dilated with membranous deposits filling the lumen. The cytoplasm of mesenchymal cells contained inclusions with spikelike protrusions. The light or dense matrix of these cells contained osmiophilic globules with abundant rectilinear trilamellar structures (not shown). The hepatocytes contained numerous dark bodies, generally located near bile canaliculi, and rare trilamellar structures were sometimes present. Normal catalase-positive peroxisomes were not demonstrable with DAB staining technique in liver and fibroblasts (not shown).

Biochemical studies of peroxisome structure and function. The results of these assays are presented in Table 1. VLCFA levels were elevated in plasma but much less in cultured fibroblasts compared with Zellweger syndrome, n-ALD, or IRD (Table 1) (39–41). β-Oxidation of VLCFA in cultured fibroblasts was decreased (64% of control values) but less than in Zellweger, n-ALD or IRD cells (Table 1) (39–41). Bile acid intermediates (trihydroxycoprostanoic acid and dihydroxycoprostanoic acid), which are not normally detectable in urine, were found in the patient's urine but not in the plasma. Phytanic acid oxidation was markedly decreased in fibroblasts, and plasma levels of phytanic acid were elevated. The pipecolic acid level in serum was largely increased. In contrast to Zellweger syndrome and n-ALD, plasmalogen biosynthesis was normal in fibroblasts (Table 1), although DHAP-AT activity was decreased (42% of control values). This decrease was less severe than in patients with Zellweger syndrome, n-ALD, or IRD (Table 1). Catalase was found in normal amount in fibroblasts from this patient. Total sp act of catalase was 10.7 ± 1.4 mU/mg compared with 8.5 ± 1.6 mU/mg in control fibroblasts. However, the subcellular distribution of catalase in the patient's fibroblasts was abnormal. After digitonin-disruption of control fibroblasts, more than 90% of the catalase was sedimentable at 12 000 × *g* for 10 min, indicating that it was contained in the peroxisomes. In contrast, the majority (94%) of catalase in the patient's fibroblasts was not sedimentable, indicating that it was free in the cytoplasm.

Immunoblot analysis of peroxisomal β-oxidation enzymes in liver. Immunoblotting showed the presence of all three peroxisomal β-oxidation enzymes in the patient's liver (Fig. 1). The bifunctional enzyme was present in amounts similar to those in the control, as shown by the similar intensity of staining of the band in the immunoblot. The 52 kD and 21 kD subunits of acyl-CoA oxidase were present in decreased amounts and the 72-kD subunit was not detected. The β-ketothiolase immunoreacted as a 41-kD molecular size protein, indicating that the enzyme was present in the mature processed form, in amounts similar to those in the control. These results demonstrate that the distribution of β-oxidation enzymes in the liver of the patient is completely different from that of patients with disorders of the biogenesis of peroxisomes (Fig. 1, *lanes 2* and *5*) (38). Thus, despite the absence of catalase-containing peroxisomes in the patient's tissues, peroxisomal β-oxidation enzymes were detectable immunologically in the liver.

Enzyme activities in different subcellular fractions from cultured skin fibroblasts. Subcellular organelles from control and patient cultured skin fibroblasts were prepared in Nycodenz gradients by a procedure described previously by our laboratory (27). The distribution of marker enzymes for different organelles and the enzyme activities of DHAP-AT, acyl-CoA oxidase, and oxidation of lignoceric acid are shown in Figure 2. As judged by the marker enzymes for different organelles in the gradient (catalase for peroxisomes, NADPH-cytochrome *c* reductase for microsomes, and cytochrome *c* oxidase for mitochondria), these organelles were easily distinguished from each other in the gradient from control cultured skin fibroblasts. Although the distribution of cytochrome *c* oxidase (mitochondria) and NADPH-cytochrome *c* reductase (microsomes) was normal in this patient, catalase was found only in the soluble fraction at the top of gradient instead of the peroxisomal fraction density (1.17 g/cm^3) at the bottom of the gradient (Fig. 2*A*). These results confirm the findings of digitonin treatment (Table 1) that almost all of the catalase in this patient was confined to the subcellular compartment, cytosol, a pattern observed in other disorders of peroxisomal biogenesis (*e.g.* Zellweger syndrome, n-ALD, and IRD). In agreement with our previous studies (27), the enzyme activities for DHAP-AT, acyl-CoA oxidase, and oxidation of lignoceric acid in a gradient from control fibroblasts were only found in peroxisomal peak (density = 1.17 g/cm^3) (Fig. 2*B*). In the gradient from this patient, these enzyme activities were observed in the peroxisomal peak with normal density (1.17 g/cm^3) and in the mitochondrial peak (density = 1.137 g/cm^3), suggesting that the peroxisomal peak and the mitochondrial peak of this patient, although lacking catalase, contained peroxisomal enzyme activities (Fig. 2*B*). The specific activities for DHAP-AT, acyl-CoA oxidase, and the oxidation of lignoceric acid in the peroxisomal peak and mitochondrial peak containing peroxisomal activities are shown in Table 2. The specific activity of DHAP-AT in the normal peroxisomal peaks (density = 1.17 g/cm^3) from control

Table 1. *Biochemical assessment of peroxisome function**

	Patient	Control	n-ALD	Zellweger syndrome	IRD
Plasma C26:0 (μmol/L)	2.85	0.35 ± 0.19 (400)	3.71 ± 1.66 (10)†	5.88 ± 1.59 (15)†	2.39 ± 0.85 (4)†
Fibroblast C26:0/C22:0	0.148 ± 0.027	0.08 ± 0.04 (60)	0.87 ± 0.35 (10)†	1.16 ± 0.51 (15)†	0.62 ± 0.38 (4)†
Plasma phytanic acid (μmol/L)	49	2.05 ± 0.65 (400)	7/10†‡	8/15†‡	35 ± 16 (4)†
Bile acid intermediates					
Urine THCA (μmol/L)	1.05	<0.1 (20)	1.45 ± 0.65 (5)†	0.65 ± 0.30 (5)†	0.80 ± 0.35 (2)†
Urine DHCA (μmol/L)	1.25	<0.1 (20)	2.8 ± 2.2 (5)†	2.9 ± 2.3 (5)†	1.2 ± 0.7 (2)†
Serum pipecolic acid (μmol/L)	45.2	1.5 ± 0.6 (20)	55.0 ± 26.6 (10)†	25.4 ± 13.9 (15)†	27.3 ± 12.7 (4)†
Fibroblast phytanic acid oxidation (pmol/h/mg protein)	5 ± 2	101 ± 30 (10)	4.4 ± 4.0 (5)†	4.5 ± 3.5 (5)†	6.5 ± 4.3 (4)†
Fibroblast plasmalogen synthesis ($^3H/^{14}C$)	0.76 ± 0.10	0.62 ± 0.16 (28)	4.66 ± 1.63 (10)§	10.40 ± 2.85 (6)§	ND§
Fibroblast DHAP-AT (pmol/h/mg protein)	292 ± 66	699 ± 50 (60)	67 ± 31 (10)†	30.2 ± 18 (15)†	106 ± 43 (4)†
Fibroblast C24:0 oxidation (pmol/h/mg protein)	57 ± 12	89 ± 32 (58)	14.7 ± 4.0 (10)†	3.4 ± 1.8 (12)†	17.2 ± 4.7 (4)†
Fibroblast catalase distribution (% sedimentable)	6 ± 4	90 ± 3 (5)‖	13.0 ± 8.0 (16)‖	10.0 ± 7.0 (21)‖	5 (2)¶

* The results for patient's fibroblasts are mean ± SD of duplicate tests; values are given as mean ± SD for controls; number of subjects in parentheses. THCA, trihydroxycoprostanoic acid; DHCA, dihydroxycoprostanoic acid; $^3H/^{14}C$, ratio of [9′,10′-^{3}H]hexadecylglycerol to [1-^{14}C] hexadecanol incorporation in plasmalogen biosynthesis; ND, no data.

† Data from F. Rocchiccioli and M. O. Rolland (unpublished results).

‡ Patients with plasma phytanic acid level >4 μmol/L.

§ Data from Watkins *et al.* (41).

‖ Data from Hoefler *et al.* (39).

¶ Data from Poll-The *et al.* (40).

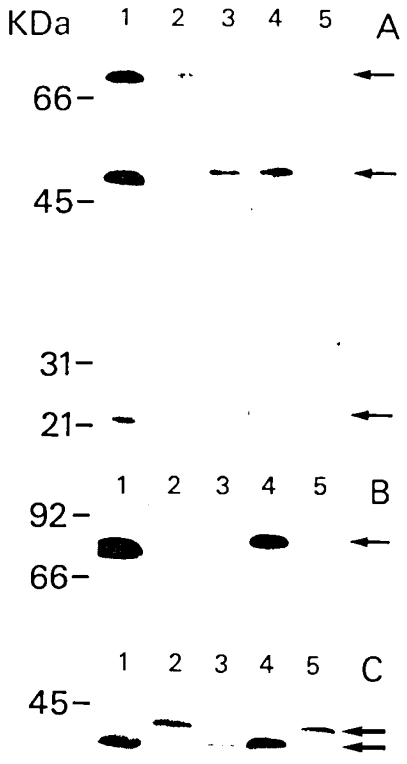

Fig. 1. Immunoblot analysis of peroxisomal β-oxidation enzymes in liver sample. Liver samples, stored at −70°C, were homogenized. The homogenates (about 5 μg of protein) were analyzed for the presence of the peroxisomal β-oxidation enzymes acyl-CoA oxidase (*A*), bifunctional enzyme (*B*), and β-ketothiolase (*C*) using antibodies against the purified rat liver enzymes. *Lane 1*, postmortem liver control; *lane 2*, liver sample from an n-ALD patient; *lane 3*, fetal tissue control; *lane 4*, liver biopsy from this patient; and *lane 5*, liver sample from a patient with Zellweger syndrome. The positions of molecular markers are at left. The positions of the 72; 52; and 20-kD acyl-CoA oxidase (*A*), the 78-kD bifunctional enzyme (*B*), and the 44-kD β-ketothiolase precursor and the 41-kD mature thiolase are indicated by *arrows* at the right.

fibroblasts was higher (18.6 ± 3.8 nmol/h/mg protein) than that from the patient (4.1 ± 1.3 nmol/h/mg protein). However, the mitochondrial peak containing peroxisomal activities from the patient's fibroblasts had higher activity (1.08 ± 0.51 nmol/h/mg protein) of DHAP-AT than the control mitochondria (0.34 ± 0.14 nmol/h/mg protein) (Table 2). Similarly, the enzyme activities of acyl-CoA oxidase and the oxidation of lignoceric acid had lower activity in the normal peroxisomal peak (density = 1.17 g/cm^3) from the patient than in the peroxisomal peak (density = 1.17 g/cm^3) from control fibroblasts and the mitochondrial peak containing peroxisomal activities from the patient had higher activity than the mitochondrial peak from control fibroblasts (Table 2). Specific activity values of peroxisomal enzymes in the mitochondrial peaks were lower because of the relatively high mitochondrial and other protein content compared with peroxisomes in these fractions. These results suggest that, in this patient's fibroblasts, the majority of the peroxisomal enzyme activities (DHAP-AT, acyl-CoA oxidase, and the oxidation of lignoceric acid) are present in structures (density = 1.137 g/cm^3) that are lighter than normal peroxisomes and that these structures lack catalase.

DISCUSSION

The deficiency of peroxisomal functions observed in patients with disorders of peroxisomal biogenesis correlates with a lack of or a marked reduction in the number of peroxisomes in tissues and cultured skin fibroblast (9). Complementation analysis using somatic cell fusion showed that these disorders segregate into at least six different complementation groups (42, 43). This indicates that several gene products play a role in peroxisome biogenesis.

Although catalase-containing peroxisomes of normal appearance are absent in liver samples from patients with Zellweger syndrome, recent studies involving cell fractionation, immunofluorescence, and electron microscopy have provided evidence for the presence of aberrant empty peroxisomal membrane vesicles called peroxisomal membrane "ghosts," in which the peroxisomal integral membrane proteins (22, 36, and 70 kD) have been identified (16, 17, 25, 44, 45). The catalase in fibroblasts from patients with Zellweger syndrome was considered to be a cytosolic constituent. Recently, however, catalase has been localized in w-particles, membrane structures distinct from peroxisomal ghosts (18).

Our patient's liver lacks normal catalase-positive peroxisomes on the basis of DAB staining and electron microscopy. Catalase was found only in the cytosol of fibroblasts, and abnormalities in the metabolism of phytanic, pipecolic, and bile acids were clearly demonstrated (Table 1). The oxidation of VLCFA in fibroblasts was only mildly defective (64% of control). This reduction may, however, be sufficient to impair VLCFA catabolism, as reflected by their accumulation in the patient's plasma. A comparable situation is observed in carriers of X-linked adrenoleukodystrophy (46, 47). DHAP-AT was moderately decreased without detectable impairment of plasmalogen synthesis in the fibroblasts from this patient.

The activities for the oxidation of VLCFA and DHAP-AT were associated with membrane fractions with density similar to normal peroxisomes (1.17 g/cm^3) and membrane fractions of lower density (1.137 g/cm^3). Although it is possible that peroxisomal proteins can be mistargeted to mitochondria, as in the case of alanine glyoxylate transferase (48), it seems unlikely that at least five proteins involved in two major metabolic pathways (synthesis of plasmalogens and oxidation of VLCFA) are incorporated in structures other than peroxisomes. The structures with lower density found in this patient's fibroblasts contained the majority of peroxisomal enzyme activities and are therefore likely peroxisome-like structures. Although we did not perform immunocytochemistry to characterize these particles, we can assume that they are different from the peroxisomal particles described in other disorders of peroxisome biogenesis. They had higher density (1.137 g/cm^3) than the membrane ghosts observed in Zellweger syndrome (1.10 g/cm^3) (16, 17), and RCDP (1.1–1.12 g/cm^3) (13, 15), and than w-particles (1.13 g/cm^3) (18). Another difference is that membrane ghost structures and w-particles in Zellweger syndrome or RCDP do not contain enzyme activities for DHAP-AT and oxidation of VLCFA (15, 18), whereas the peroxisome-like structures seen in our patient had significant activity for these enzymes. This indicates that the defect of peroxisome biogenesis observed in this patient is likely to be different from that reported in Zellweger syndrome, n-ALD, IRD, and RCDP.

Recent studies showed that the tripeptide sequence of Ser-Lys-Leu (PTS1) located near the C-terminal is an essential targeting signal for the import of proteins into peroxisomes (49). Immunoelectron microscopy and Western blot analysis revealed that anti-PTS1 antibody crossreacts only with proteins of the peroxisomal matrix, not with proteins of the peroxisomal membrane (50). This suggests that other topogenic signals are responsible for the incorporation of membrane proteins into peroxisomes. Recently, a second microbody targeting signal (PTS2) was identified in the first 11 amino acids of the precursor β-ketothiolase that encodes sufficient information to target this protein to peroxisomal matrix (51). Peroxisomal ghosts are able to import the unprocessed form of β-ketothiolase (13, 45), suggesting that the defect of peroxisomal protein import in Zellweger does not involve the PTS2 machinery. The proteolytic processing of this

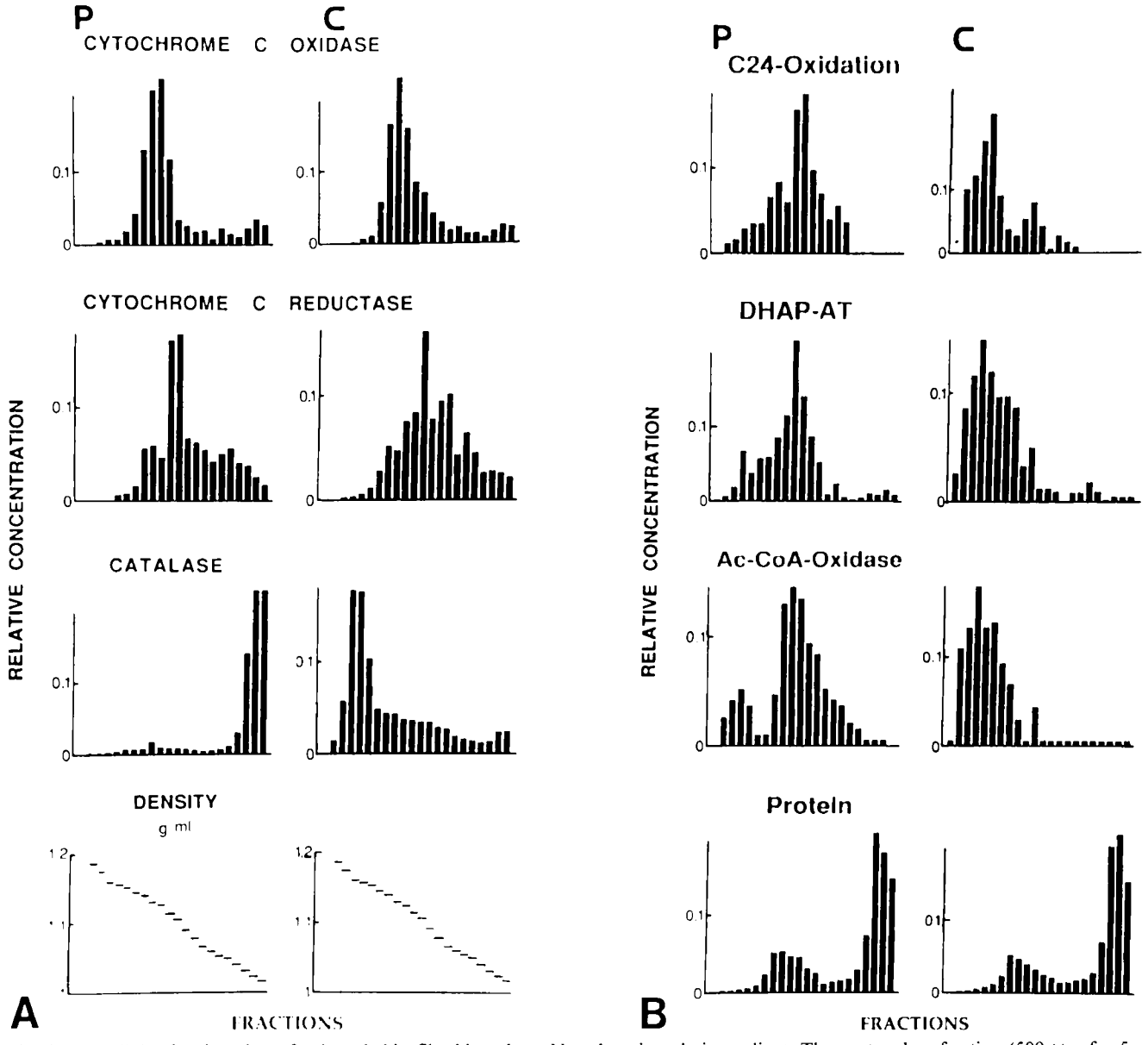

Fig. 2. Subcellular fractionation of cultured skin fibroblasts by a Nycodenz isopyknic gradient. The postnuclear fraction (500 × *g* for 5 min supernatant) from cultured skin fibroblasts of the control and from the patient were further fractionated with an isopyknic continuous gradient as described in the text. The distribution pattern of marker enzymes for different subcellular organelles (*A*) and enzyme activities of DHAP-AT, acyl-CoA oxidase, and fatty acid oxidation (*B*) are shown. *P* represents the gradient profile of various enzyme activities of cultured skin fibroblasts from the patient and *C* represents a similar distribution from cultured skin fibroblasts from the control.

Table 2. *Specific activities (Nycodenz gradient) in peroxisomal peak and in mitochondrial peak containing peroxisome-like structures from patient and control fibroblasts**

	Acyl-CoA oxidase (μmol/h/mg protein)	Lignoceric acid oxidation (pmol/h/mg protein)	DHAP-AT (nmol/h/mg protein)
Peroxisomes (density = 1.170 g/cm^3)			
Control	15.2 ± 5.8	96 ± 35	18.6 ± 3.8
Patient	4.08 ± 1.32	37 ± 12	4.1 ± 1.3
Peroxisome-like structures (density = 1.137 g/cm^3)			
Control	0.25 ± 0.14	2.6 ± 1.2	0.34 ± 0.14
Patient	1.20 ± 0.25	7.2 ± 2.1	1.08 ± 0.51

* The results are mean ± SD of duplicate tests on patient and control cell lines.

enzyme protein is, however, deficient in these particles. In our patient, the enzymes of peroxisomal matrix (β-oxidation enzymes) and of peroxisomal membrane (DHAP-AT) were found active. This indicates that their import into peroxisomal structures did occur. The fact that β-ketothiolase was normally processed is consistent with the hypothesis that the corresponding protease is imported by a microbody-targeting signal pathway (51), possibly the PTS1 pathway. Because several peroxisomal proteins using the PTS1 and PTS2 signals were imported in the peroxisome-like structures of our patient, we hypothesize that the defect of peroxisomal protein import involves neither of these signal machineries.

In summary, this study demonstrates the presence of DHAP-AT, acyl-CoA oxidase, and other β-oxidation system enzymes in catalase-negative peroxisome-like structures of decreased density. We therefore propose that these particles represent immature forms of peroxisomes, intermediate between normal peroxisomes and peroxisomal ghosts. In addition, the marked defect in the metabolism of phytanic, pipecolic, and bile acids, compared with the partial deficiency of activities for DHAP-AT and VLCFA oxidation, distinguishes this patient's disorder from other peroxisomal disorders with multiple enzyme deficiencies: Zellweger syndrome, n-ALD, IRD, RCDP, and Zellweger-like syndrome. This disorder may therefore be a new peroxisomal disease.

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