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Zellweger Syndrome: Biochemical and Morphological Studies on Two Patients Treated with Clofibrate

PAUL B. LAZAROW, VIRGINIA BLACK, HELEN SHIO, YUKIO FUJIKI, AMIYA K. HAJRA, NABANITA S. DATTA, BABU S. BANGARU, AND JOSEPH DANCIS

The Rockefeller University, 1230 York Avenue, New York, New York 10021 [P.B.L., H.S., Y.F.] Departments of Cell Biology [V.B.] and Pediatrics [B.S.B., J.D.], New York University School of Medicine, 550 First Avenue, New York, NY 10016; and Neuroscience Laboratory, The University of Michigan, Ann Arbor, Michigan 48109 [A.K.H., N.S.D.]

ABSTRACT. Two infants with Zellweger syndrome (cerebro-hepato-renal syndrome) have been studied biochemically and morphologically. Peroxisomal enzymes involved in respiration, fatty acid β-oxidation, and plasmalogen biosynthesis were assessed. In liver, catalase was present in normal amounts but was located in the cell cytosol. Dihydroxyacetone phosphate acyltransferase activity was less than one-tenth of normal. The amount of the bifunctional protein catalyzing two β-oxidation reactions was found by immunoblotting to be greatly reduced. Catalase activity was normal in intestine. D-Amino acid oxidase was subnormal in kidney. The observed enzyme deficiencies may plausibly explain many of the metabolite imbalances observed clinically. Morphologically, peroxisomes were absent from liver. In intestine, normal peroxisomes were also missing, but some rare, smaller (0.04–0.13 μm) bodies were seen with a slight positive cytochemical reaction for catalase. These results, together with current concepts of peroxisome biogenesis, suggest but do not prove, that the primary defect in Zellweger syndrome may be in peroxisome assembly. The infants were treated with clofibrate, but it was ineffectual as assessed biochemically, morphologically, and clinically. (*Pediatr Res* 19: 1356–1364, 1985)

Cerebro-hepato-renal syndrome (Zellweger syndrome) is a fatal autosomal recessive disease with multiple congenital abnormalities. Prominent findings are distinctive facies, severe hypotonia, microcysts of the kidney, hepatic dysfunction, and disorganization of the structure of the central and peripheral nervous systems (1–3). The usual prognosis is death within weeks or months, although some patients survive into the second year. Electron microscopy has revealed an absence of peroxisomes in the liver and kidney and structurally abnormal mitochondria (4). Biochemical abnormalities include an accumulation of very long-chain fatty acids in plasma, brain and fibroblasts (5, 6), a reduction in tissue plasmalogens (7), hyperpipecolic acidemia (8, 9), dicarboxylic aciduria (10), and defective bile acid synthesis (11).

How might these diverse symptoms be caused by a single genetic change, which is implied by the observed autosomal recessive mode of inheritance (2)? The functions of peroxisomes include respiration (forming H_2O_2) (12), fatty acid catabolism (13, 14), and the initial steps in plasmalogen biosynthesis (15). The peroxisomal fatty acid β-oxidation system acts on saturated and unsaturated fatty acyl-CoAs with chainlengths from 6 to 26 (14, 16, 17), the sidechain of cholesterol (18), dicarboxylic fatty acids (19), and glutaryl-CoA (20). Thus a generalized deficiency in peroxisomal enzymes could plausibly account for many of the observed defects.

The morphologically observed absence of peroxisomes does not necessarily imply a deficiency of peroxisomal enzymes. These enzymes are synthesized on free polysomes and enter preexisting peroxisomes posttranslationally (21–26). Therefore a defect in peroxisome assembly could result in the peroxisomal enzymes being left in the cell cytosol, where they might or might not be active.

We have had the opportunity to investigate Zellweger syndrome defects biochemically and morphologically in two infants. Enzymes belonging to each of the major functional pathways mentioned above were selected for study. In addition, the patients were treated with clofibrate. This hypolipidemic drug causes a striking proliferation of hepatic peroxisomes in rats (27) together with an increase of 10-fold or more in the activity of the peroxisomal system for the β-oxidation of fatty acids (13, 28). Thus it was hoped that clofibrate might overcome the genetic defect in peroxisome formation. Some of the results have been presented in abstract form (29).

CASE HISTORIES

Patient 1 was admitted to the Clinical Research Unit at 3 months of age for special study, with the diagnosis of Zellweger syndrome. She was the fourth child of parents who are first cousins. The first child had died at 2 months of age of Zellweger syndrome. The middle two children are normal.

The mother had noted less intrauterine activity during this pregnancy than with her normal children. Patient 1 was born at term and was noted in the nursery to be "floppy" and a poor feeder. Jaundice developed briefly at 4 wk of age, following which the course was progressively downhill.

Physical examination on admission to our unit revealed a small irritable white female infant with the facies characteristic of Zellweger syndrome. The weight (2.9 kg) was slightly below birth weight. Subcutaneous fat was wasted. The musculature was poorly developed and atonic. There was little voluntary movement and no social contact. Response to light was retained. The liver was large and firm. There was a single palmar crease. X-rays of the knees revealed the typical patellar calcifications.

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Send reprint requests to Professor Joseph Dancis, Department of Pediatrics, The New York University School of Medicine, 550 First Avenue, New York, NY 10016.
Send correspondence to Professor Paul B. Lazarow, The Rockefeller University,1230 York Avenue, New York, NY 10021.
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The usual laboratory studies added no significant information. A complete blood count and urinalysis revealed no abnormalities. Serum creatinine, urea, and electrolytes were within normal limits. The plasma amino acid chromatogram was normal. Gas phase chromatography of plasma revealed no excesses of short chain monocarboxylic acids (up to 5 carbons). Urine analysis also excluded a gross excess of glutaric acid. The serum bile acids, determined by gas-phase chromatography, revealed an excess of trihydroxycoprostanic acid. Serum pipecolic acid was slightly elevated at 7 wk of age (24 μmol/liter) and reached a level of 116 μmol/liter prior to death.

The patient was admitted to the Clinical Research Unit on two occasions during the next 3 months for several special studies. Clofibrate therapy was begun on patient 1 at the age of 4 months with 50 mg twice daily by mouth (34 mg/kg/day). Blood levels 3 days later were 23 μg/ml 2 h postclofibrate administration (presumed time of maximal concentration) and 17 μg/ml (11 h postclofibrate). Two weeks later, the dose was increased in two steps to 100 mg twice daily and the respective blood levels increased to 56 and 26 μg/ml. These clofibrate concentrations are within the range reported for studies on adults (30). The patient was maintained on clofibrate for 24 days. On discharge, there was little noticeable change in her general condition. She was readmitted at the age of 6½ months, moribund with pneumonia and dehydration, and died. Autopsy was refused.

Patient 2 is the third child born to unrelated parents. The first child had died of Zellweger syndrome. The second child was the product of artificial insemination and is normal. The present child at 6 wk of age (3.1 kg) had the characteristic facies, hypotonia, abnormal bile acid pattern, and hyperpipecolatemia. Clofibrate, 50 mg twice daily, was begun at the age of 6 wk and maintained for 6 wk. At 8 months of age she was still alive but doing poorly. She was admitted to the Clinical Research Unit on two occasions for special studies.

Patient 3 was referred to the unit as a Zellweger patient at 15 months of age with hypotonia and characteristic facies. The serum pipecolic acid concentration was normal but there was a prolonged elevation of serum pipecolic acid following an oral load of the L-isomer of 50 mg/kg. This patient differed from classical Zellweger patients in that she was older and in that she proved to have hepatic peroxisomes which were biochemically normal to the extent that we investigated them. This "pseudo-Zellweger" patient is included as a control for the purposes of this study.

METHODS

Biopsies. Percutaneous liver and kidney biopsies were performed with a Mengheni needle. An intestinal biopsy was obtained by aspiration with a steerable biopsy instrument (Medi-Tech) introduced into the jejunum under fluoroscopy. Immediately on biopsy, liver and intestine samples were taken to a room adjoining that of the patient and divided into portions for biochemical and morphological analyses. The kidney biopsy sample was used to determine D-amino acid oxidase.

Morphological studies. Approximately 20% of the liver sample was immersed in ice-cold 2.5% glutaraldehyde/0.1 M cacodylate buffer, pH 7.4. After1–2 h of fixation, half of the sample was cut into small cubes with a razor blade and fixed further for morphological studies. The other half was hand cut into very thin slices with a razor blade for catalase cytochemistry. The slices were incubated for 45 min to 1 h in the alkaline 3,3′-diaminobenzidine medium of Novikoff *et al.* (31) at 37° C (patient 1) or, as recommended for human tissue (32), 45° C (patient 2). Both morphological and cytochemical samples were postfixed in 1% OsO_4 and processed for electron microscopy as described previously (33).

The intestinal samples were prepared for morphological examination by spreading them flat and fixing them immediately in cold 3% glutaraldehyde in 0.1 M sodium cadocylate buffer, pH 7.4, for 90 min. They were then placed in cold 0.1 M sodium cacodylate buffer for at least 12 h. For cytochemical demonstration of peroxisomes the tissue was chopped into 25–50 μm thick slices on a Porter-Blum TC2 tissue chopper. These were incubated in diaminobenzidine-containing medium under the conditions found by Novikoff and Novikoff (34) to be optimal for adult human intestine. However, KCN was omitted. Incubations were performed at 37 and 45° C. The slices were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, stained *en bloc* with 1% aqueous uranyl acetate, dehydrated, and flat embedded in Epon in such a way as to insure that every section would traverse the entire thickness of the mucosa. Gold to silver sections were cut on a MTII ultramicrotome and examined in a Philips 301 electron microscope.

Biochemical studies. Approximately 80% of each liver and intestinal biopsy sample was homogenized with a Potter-Elvehjem homogenizer in 0.5 ml of 0.25 M sucrose/5 mM imidazole buffer, pH 7/0.1% ethanol. Considerable effort was required to homogenize some of the liver biopsies, which appeared to be quite fibrotic. Samples of the homogenates (150 μl) were centrifuged for 30 min at 10,000 rpm in 1.5-ml microcentrifuge tubes in a Sorvall HB-4 rotor (8,900 × *g*) in order to separate soluble and particulate components. The pellets were resuspended in 150 μl of the above medium. Homogenates, resuspended particulates, and soluble fractions were assayed immediately or stored frozen in aliquots at −20° C for later analysis.

Catalase was assayed as described by Baudhuin *et al.* (35) after treatment of the samples with 1% Triton X-100. The enzyme has first order reaction kinetics: one unit of activity decreases the H_2O_2 concentration 10-fold/min at 0° C in a reaction volume of 50 ml. Ethanol is included in the homogenization medium in order to protect catalase from inactivation (36) and the enzyme is stable frozen. Protein was measured according to Lowry *et al.* (37) with bovine serum albumin as standard. Dihydroxyacetone phosphate acyltransferase and glycerophosphate acyltransferase were assayed using ^{32}P-labeled substrates and palmitoyl-CoA (38). Dihydroxyacetone phosphate acyltransferase is fairly stable but does lose activity upon repeated (more than three to four times) freezing and thawing whereas glycerophosphate acyltransferase is more labile and loses activity during long storage at 4° C or by freezing and thawing more than once. The results presented are for those biopsy samples from which a frozen aliquot of homogenate was immediately shipped to Michigan for analysis. D-Amino acid oxidase was measured by incubation with D-[3-^{14}C]phenylalanine (Research Products International Corp., 0.2 μmol, 0.1 μCi) for 20 min at 37° C as described by Wellner and Lictenberg (39) except that the final volume was 0.2 ml. Following incubation 0.2 μmol of phenylpyruvic acid was added, protein was precipitated with 0.1 ml of 5% metaphosphoric acid, and the 2,4-dinitrophenylhydrazone was made, extracted, and counted as previously described (40). Oxidation of [1-^{14}C]valine to $^{14}CO_2$ was as described (40).

Peroxisomal β-oxidation was assayed as the palmitoyl-CoA-dependent reduction of NAD to NADH in the presence of cyanide to inhibit mitochondria (41). Unlike rat liver homogenates, in which peroxisomal β-oxidation is stable when frozen, these more dilute human liver homogenates (controls) lost activity upon freezing, although not consistently. Blood clofibrate concentrations were determined by Mitchell N. Cayen (Ayerst Research Laboratories, Montreal). Dr. Norman Javitt, NYU Medical Center, performed the bile acid analysis.

Immunoblot analyses. Samples of liver biopsy homogenates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 7–15% gradient slab gels (42). The proteins were transferred electrophoretically to nitrocellulose paper (43) which was then reacted as described (26, 43) with rabbit antiserum raised against highly purified rat liver peroxisomal bifunctional enoyl-CoA hydratase-hydroxyacyl-CoA dehydrogenase

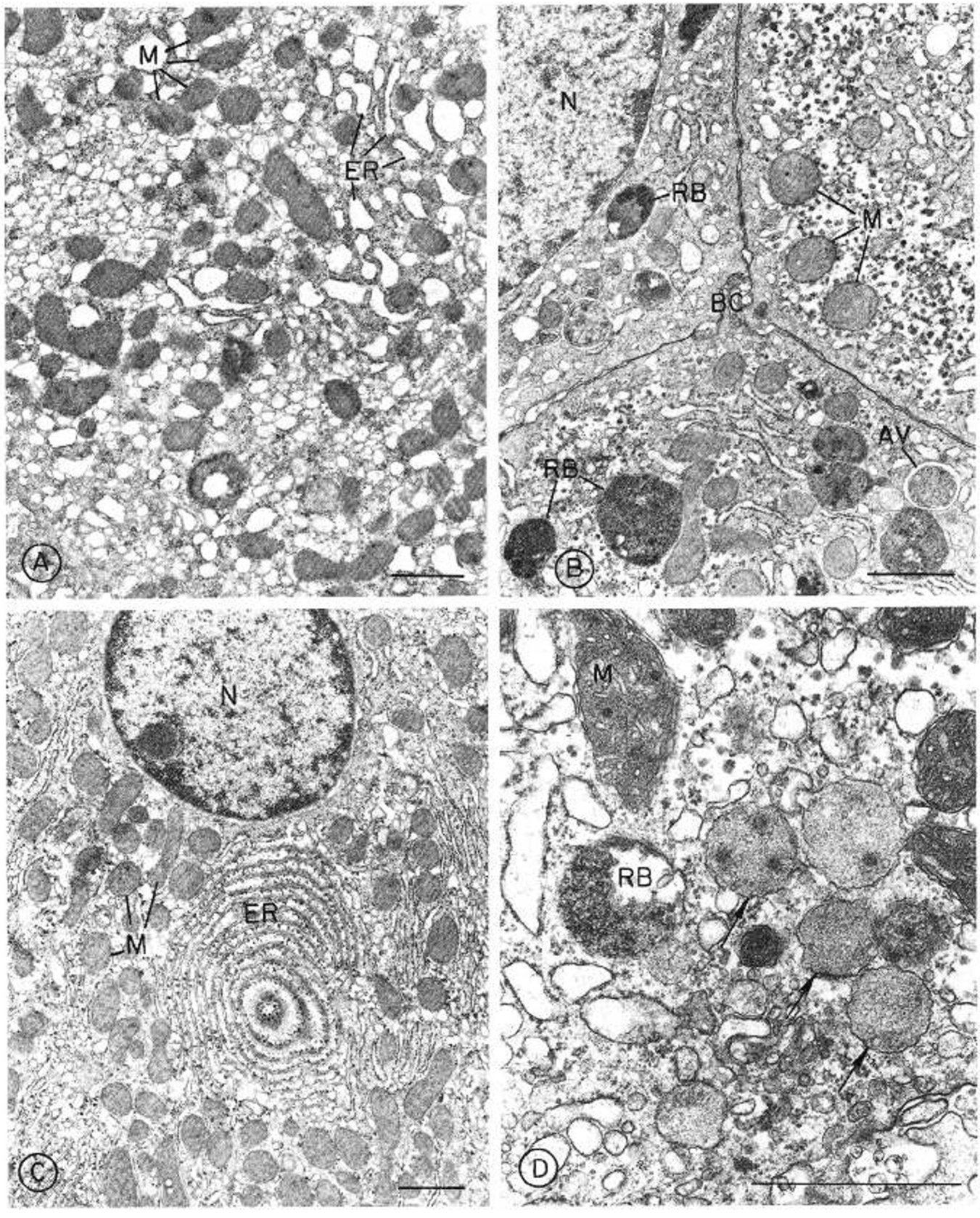

Fig. 1. Electron microscopy of the liver of the first Zellweger patient. *A*, morphology before clofibrate treatment. *B–D*, after clofibrate treatment: *B*, morphology; *C*, *D*, cytochemical reaction for catalase—no diaminobenzidine-positive bodies are seen. *D*, rare diaminobenzidine-negative membrane-bounded bodies with small vesicular inclusions (*arrows*). *M*, mitochondria; *ER*, vesiculated endoplasmic reticulum; *N*, nucleus; *BC*, bile canaliculus; *AV*, autophagic vacuole; *RB*, residual body. Magnifications: *A*, 13,000; *B*, 16,000; *C*, 12,000; *D*, 38,000. *Bar* = 1 μm.

(44). Antigen-antibody complexes were detected with [^{125}I]protein A and autoradiography (26).

These investigations were approved by the Institutional Review Boards of the New York University School of Medicine and The Rockefeller University.

RESULTS

Ultrastructure. The liver from 3-month-old patient 1 was fibrotic, while that from 6-wk-old patient 2 was not. Within hepatocytes, the striking abnormality in both patients was the absence of peroxisomes (Fig. 1 *A* and *B*), in agreement with the report of Goldfischer *et al.* (4). The diaminobenzidine cytochemical reaction was carried out in an effort to detect peroxisomes that might have been missed morphologically but none were found in either patient before (not illustrated) or after clofibrate treatment (Fig. 1 *C*). Normal human liver peroxisomes are seen in Figure 2.

Large residual bodies were more numerous than in normal hepatocytes (Fig. 1*B*). They ranged in size between 0.5 and 4 μm, and appear to be even more abundant after clofibrate treatment. The endoplasmic reticulum was often dilated and frequently appeared to be vesicular (Fig. 1*A*). In both postclofibrate biopsies, occasional sworls of cisternal endoplasmic reticulum were observed (Fig. 1*C*). Mitochondria appear to be normal in appearance in these patients, in contrast to the patient of Goldfischer *et al.* (4).

During the course of a thorough search for peroxisomes in these liver samples, occasional 0.2–0.5 μm membrane-bounded bodies were observed as illustrated in Figure 1*D* (*arrows*). These are in the size range of peroxisomes, but they contain small vesicular inclusions which have not been reported in human peroxisomes (45–48), and they are negative in the cytochemical test for catalase. Our interpretation is that these are most likely lysosomal in nature, but we cannot exclude the possibility that they might be rare, abnormal diaminobenzidine-negative peroxisomes. As with residual bodies, the abundance of these structures is somewhat greater in the postclofibrate biopsies.

In the intestinal epithelium from a normal child, peroxisomes were easily detected as diaminobenzidine-positive, elongated, or spherical structures, 0.12–0.30 μm in diameter (Fig. 3*A*). In intestinal epithelium from the patients with Zellweger syndrome, peroxisomes appeared to be missing (Fig. 3*B*). However, extensive searching revealed rare bodies in a smaller size range, 0.04–0.13 μm in diameter, which appear to be diaminobenzidine-positive, although the intensity of the reaction product is much less than in the control tissue (Fig. 3*B*). The mitochondria of the intestinal cells from normal and Zellweger syndrome appeared similar in size, shape, number of cristae, and matrix density.

Biochemistry. The total liver catalase activities in the Zellweger patients were comparable to those of six controls (Table 1). In the Zellweger samples, (either before or after clofibrate treatment), however, the catalase could not be sedimented by centrifugation at 10,000 rpm for 30 min whereas half of the catalase in the controls was sedimentable under these conditions (Table 1). Thus in the absence of peroxisomes, hepatic catalase is present in normal amounts, but it is located in the cell sap.

Intestinal catalase activity in the two patients was 54 and 42 mU/mg protein, which may be compared to values of 44 and 44 in two control biopsies. In kidney, the D-amino acid oxidase activity of the second patient at 11 wk (postclofibrate) was 22–36% of four controls, after normalizing for valine catabolism (Table 2).

Dihydroxyacetone phosphate acyltransferase, the first enzyme in the pathway for the biosynthesis of ether-linked glycerolipids, including plasmalogens, was markedly reduced in the second patient's liver, and clofibrate treatment did not restore it to normal (Table 3). The activity of glycerophosphate acyltransfer-

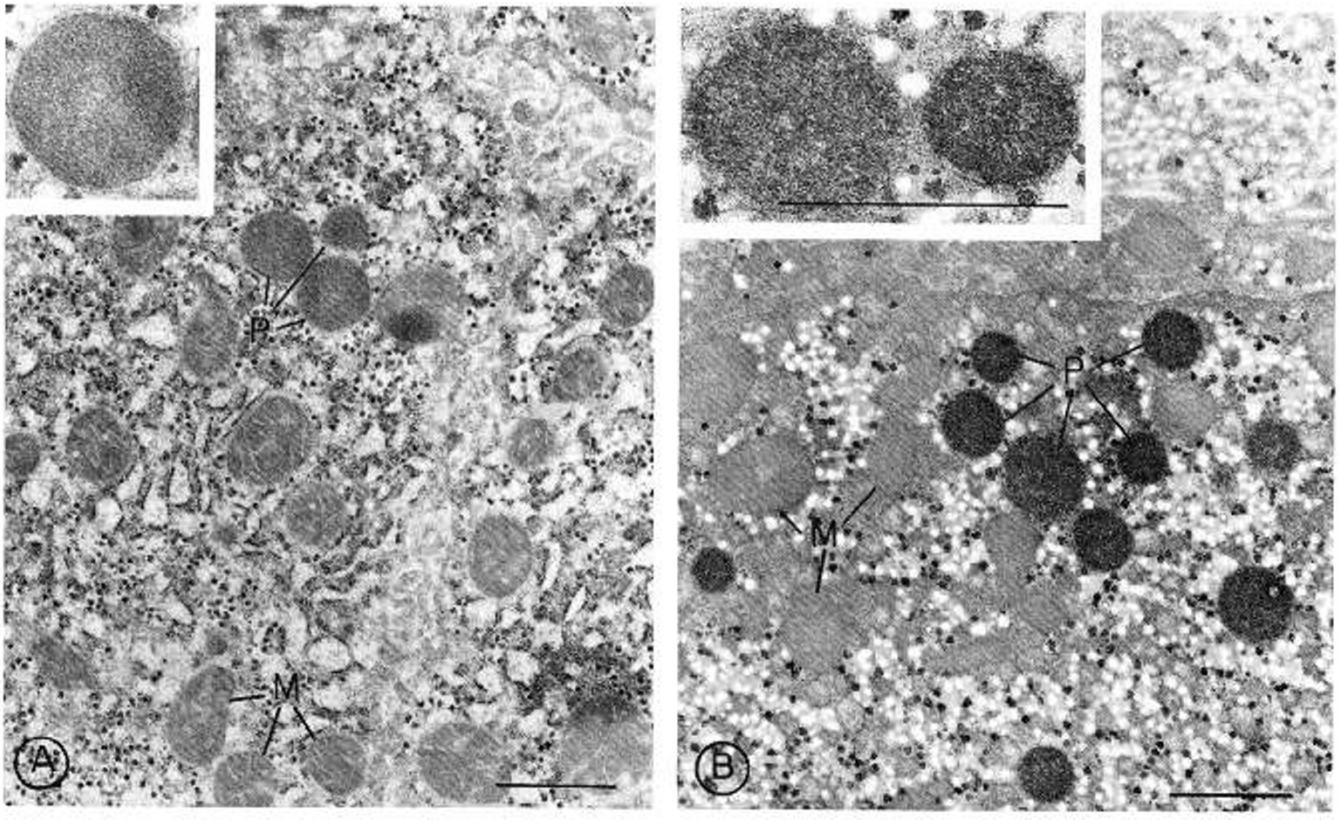

Fig. 2. Electron microscopy of liver of control "pseudo-Zellweger" patient (case 3). *A*, morphology. *B*, catalase cytochemistry showing diaminobenzidine-positive peroxisomes (*P*). Magnification = 16,000. *Bar* = 1 μm. *Insets* show peroxisomes at higher magnification (38,000); *bar* = 1 μm.

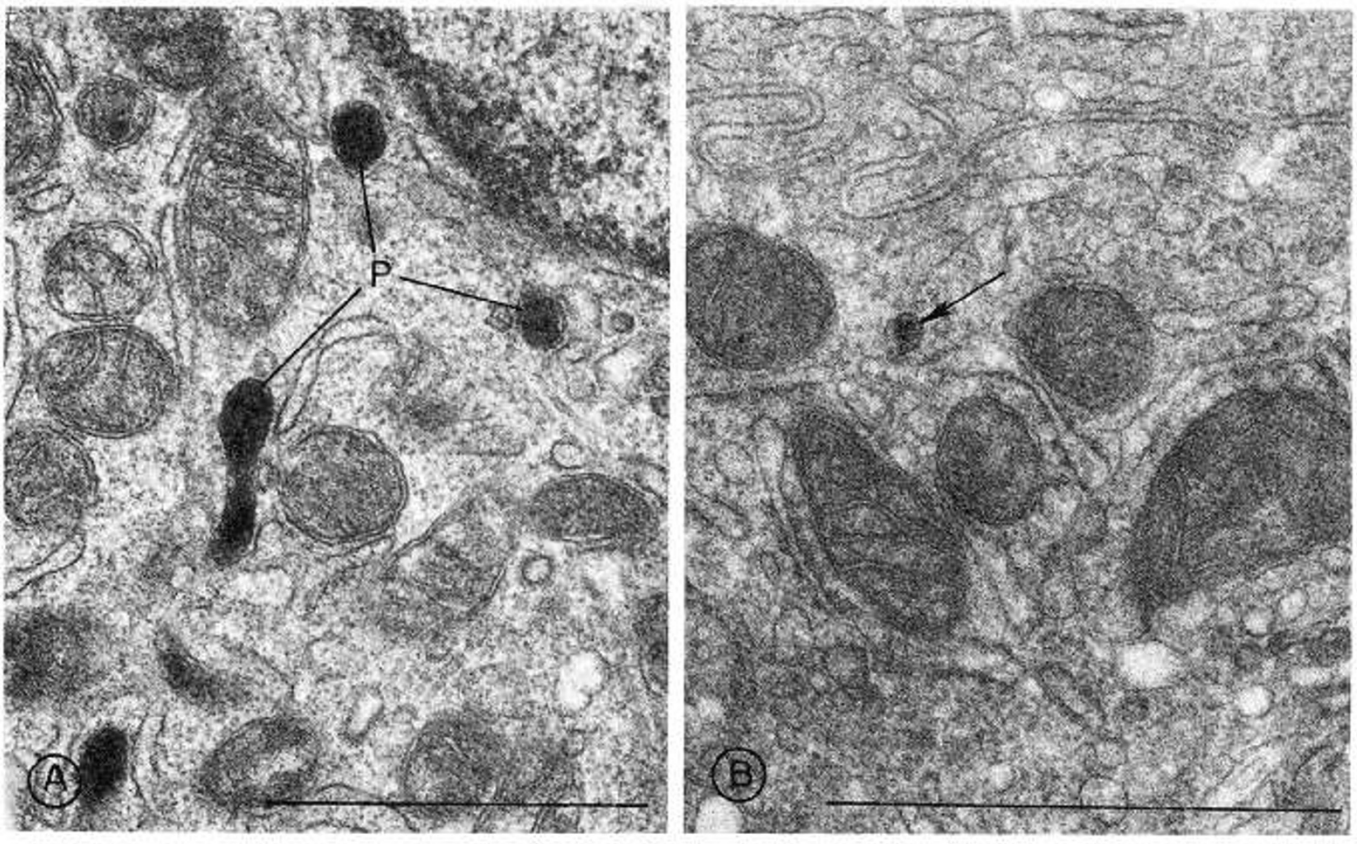

Fig. 3. Electron microscopy of intestinal epithelium with catalase cytochemistry. *A*, human control. *B*, Zellweger patient before clofibrate. No normal-sized peroxisomes (*P*) like those in *A* are seen in *B*. *Arrow* in *B* indicates a rare, small body with a little diaminobenzidine reaction product. Magnifications = 50,000 (*A*) and 68,000 (*B*). *Bar* = 1 μm.

Table 1. *Hepatic catalase activity*

	Total (mU/mg protein)	Pellet (%)	Supernatant (%)	Recovery (%)
Zellweger—before clofibrate				
Patient 1	117	4	85	89
Patient 2	181	4	96	100
Zellweger—after clofibrate				
Patient 1	152	7	93	100
Patient 2	113	4	89	93
Controls*				
A	112	45	34	79
B	43	61	21	82
C	173	ND†	ND	ND
D	87	ND	ND	ND
E	151	49	49	98
F	155	41	50	91

* A, male child evaluated for hepatosplenomegaly; no evidence of liver disease found; B, 6-yr-old male with terminal cystinosis and cystic fibrosis; C, male who died at 2½ yr with no history of liver disease; D, adult male—normal portion of liver removed due to a tumor; E, childhood adrenoleukodystrophy patient described in Reference 49; F, patient 3 with undiagnosed disorder with hypotonia and abnormal facies. Peroxisomes are present and those peroxisomal enzymes that have been assayed are in the normal range.

† Not determined.

ase, which initiates the biosynthesis of the nonether-linked glycerolipids, might be reduced in these biopsy samples. However, as mentioned above, this enzyme is somewhat labile to handling and it is possible that it was partially inactivated before the assays.

Table 2. *Kidney D-amino acid oxidase*

	Age	D-Amino acid oxidase (nmol/min/mg protein)	Valine catabolism* (nmol/min/mg protein)	Amino acid oxidase Valine catabolism (nmol/min/mg protein)
Patient 2	11 wk	0.72	8.99	0.08
Controls				
1	4 wk	4.86	21.8	0.22
2	2 yr	17.9	49.6	0.36
3	59 yr	7.19	ND†	ND†
4	70 yr	6.24	17.5	0.36

* Oxidation of [1-^{14}C]valine to $^{14}CO_2$ was assayed as a nonspecific index of metabolic activity.

† Not determined.

Table 3. *Hepatic acyltransferases*

	Dihydroxyacetone phosphate acyltransferase (nmol/min/mg protein)	Glycerol phosphate acyltransferase (nmol/min/mg protein)
Zellweger—before clofibrate		
Patient 2	0.02	0.19
Zellweger—after clofibrate		
Patient 2	0.04	0.10
Controls		
C	0.45	0.56
D	0.36	0.08
F	0.26	0.56

Assay of the peroxisomal β-oxidation activity in liver biopsies gave variable results. The second (postclofibrate) biopsy of patient 2, assayed fresh, showed no detectable activity, whereas the first biopsy, after some months in the freezer, gave an apparent activity of 0.6 nmol NADH/min/mg protein. This may be compared with values of 0.96 for one control biopsy (*C* in Table 1), 1.4 for a patient with childhood adrenoleukodystrophy (*E* in Table 1) (49), 0.76 for patient 3 (*F* in Table 1, see case histories) and a published human control value of ~1 (50). Purified rat liver peroxisomes exhibited good β-oxidation activity when mixed with the second patient's biopsy sample. Both of the first patient's liver biopsies, assayed after storage at −20° C, were inactive. As noted in "Methods," control human biopsies sometimes (irreproducibly) lost β-oxidation activity upon storage in the freezer.

Because of these serious uncertainties with the β-oxidation

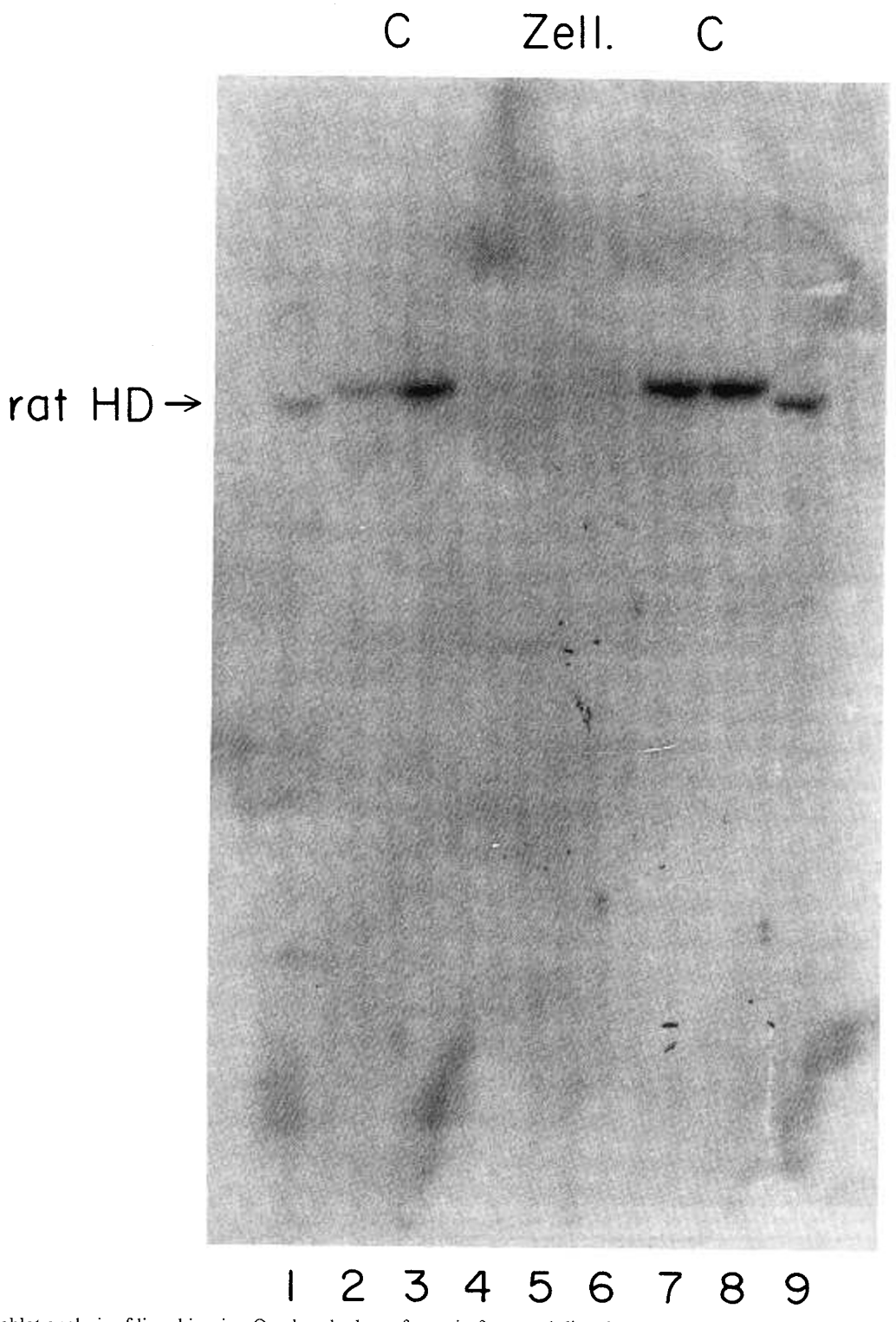

Fig. 4. Immunoblot analysis of liver biopsies. One hundred μg of protein from each liver homogenate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with antiserum against rat liver peroxisomal bifunctional hydratase-dehydrogenase. Autoradiograph shows [^{125}I]protein A—antibody-antigen complex. *Lanes 1 and 9*—normal rat; *lanes 2 and 3*—two human controls; *lanes 4-6*—Zellweger: *4*, patient 1 after clofibrate; *5*, patient 2 before clofibrate; *6*, patient 2 after clofibrate; *lane 7*—patient 3, pseudo-Zellweger; *lane 8*, X-linked adrenoleukodystrophy patient (49). *Arrow* indicates rat liver hydratase-dehydrogenase (*HD*).

assay, we turned to an immunochemical analysis of the amount of the bifunctional protein carrying two of the peroxisomal β-oxidation enzymes, enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase. As shown in Figure 4, *lanes 1 and 9*, rabbit antiserum raised against purified rat liver hydratase-dehydrogenase recognizes just this 77kDa antigen in a rat liver homogenate. The antiserum cross-reacts with a single, slightly larger protein in normal human liver (*lanes 2 and 3*). This protein is also present in livers of two non-Zellweger patients (*lanes 7 and 8*), but is greatly reduced in Zellweger liver biopsies (*lanes 4 to 6*), even after clofibrate treatment (*lanes 4 and 6*).

Clinical observations. Clofibrate treatment for 3–6 wk caused no obvious change in the clinical status of the patients or in the progression of their disease.

DISCUSSION

These results demonstrate that enzymes involved in three major peroxisomal functions, respiration, fatty acid catabolism, and plasmalogen biosynthesis, are affected in Zellweger syndrome. However, the extent of the effect varies with the enzyme examined. In liver, catalase activity is normal whereas the bifunctional β-oxidation protein and dihydroxyacetone phosphate acyltransferase activity are markedly reduced. The deficient liver enzymes include both a matrix enzyme [bifunctional hydratase-dehydrogenase (51, 52)] and a membrane enzyme [dihydroxyacetone phosphate acyltransferase (53)]. In other tissues, catalase activity is normal in intestine whereas D-amino acid oxidase is reduced in kidney. Thus whatever the nature of the primary defect in this disease, the synthesis of peroxisomal proteins is not abolished.

In this investigation, enzyme assays have been performed on fresh patient biopsy samples. While this report was being written, several communications appeared with enzyme analyses on autopsy samples or cultured fibroblasts which are consistent with and extend the present observations. Catalase and D-amino acid oxidase activities were normal and L-α-hydroxyacid oxidase activity was 50% of normal in Zellweger autopsy liver (54). Dihydroxyacetone phosphate acyltransferase was one-tenth of normal or less in fibroblasts, leukocytes, liver, and brain (54–56). Oxidation of [1-^{14}C]tetracosanoic acid (C24:0) to $^{14}CO_2$ was depressed in Zellweger fibroblasts and amniocytes (6). Thus peroxisomal defects have been found in all tissues examined so far. These include tissues in which peroxisomes are abundant and large (~0.5 μm diameter) (liver and kidney), as well as cell types in which peroxisomes are smaller (0.1–0.3 μm diameter) [intestine (34), developing brain (57)] and rare [fibroblasts (58), amniocytes].

These peroxisomal enzyme defects can plausibly account for many of the observed metabolic abnormalities seen in Zellweger syndrome. The marked deficiency of the bifunctional protein catalyzing two β-oxidation reactions suggests a generalized deficiency in peroxisomal β-oxidation. In view of the broad substrate specificity of this system, which includes not only medium- and long-chain fatty acids (C10–C20) (14, 16), but also very long-chain fatty acids (C24:0 and C26:0) (17), cholesterol (18), dicarboxylic fatty acids (19), and glutaryl-CoA (20), this deficiency could explain the clinically observed accumulations of very long-chain fatty acids (5), bile acid intermediates (11), dicarboxylic fatty acids (10), and (because pipecolic acid catabolism proceeds via glutaryl-CoA) perhaps also pipecolic acid (8). The reduction in activity of dihydroxyacetone phosphate acyltransferase, which is the first step in the synthesis of ether-linked glycerolipids including plasmalogens, explains the observed tissue plasmalogen deficiency. Since plasmalogens normally constitute 25% or more of brain phospholipids, it is tempting to speculate that this deficiency might be the cause of the profound neurological problems of Zellweger syndrome.

Other biochemical alterations and clinical symptoms of this disease cannot be explained at present on the basis of the known peroxisomal deficiencies. Mitochondria appeared normal ultrastructurally in our patients, but this does not preclude biochemical defects such as those reported by Goldfischer *et al.* (4) and by Kelley and Corkey (59). It is not easy to explain mitochondrial abnormalities in terms of peroxisomal dysfunction unless perhaps mitochondria require plasmalogens. Thus, the present results support the possibility that Zellweger syndrome is a primary defect affecting peroxisomal enzymes, but much further work remains to establish the chain of causality in this disease.

The present finding that catalase is located in the cytosol of Zellweger liver fits well with our current concepts of peroxisome biogenesis (60). Catalase (21, 22) as well as other rat liver peroxisomal proteins (23–25, 61, 62), including a major integral membrane protein (26), are synthesized on free polyribosomes (mostly at their final sizes) and are imported posttranslationally into preexisting peroxisomes. Thus, if peroxisomes are absent (or unable to import newly made proteins), these proteins would be left in the cytosol. In order to become enzymatically active, many of these proteins must bind prosthetic groups and/or oligomerize. Failure to do so in the cytosol could result in the presence of normal amounts of an inactive protein. The fact that catalase is enzymatically active indicates that heme addition and tetramerization has taken place in Zellweger liver cytosol. This contrasts with the situation in normal rat liver, where heme addition and tetramerization occur mainly within peroxisomes (21), but is not too surprising in view of the fact that abundant active catalase is found normally in liver cytosol of certain animals, *e.g.* sheep (63). In Zellweger fibroblasts, catalase is similarly found in the cytosol instead of its normal peroxisomal location (54, 64).

The amount of the various peroxisomal enzymes found in the cytosol in Zellweger syndrome would be affected by the rate at which these proteins are degraded in a cellular compartment in which they are not normally found. Some peroxisomal proteins might be degraded much more rapidly than others [in contrast to the normal situation, where peroxisomal proteins appear to turn over approximately synchronously (65)]. The immunoblot analysis of the bifunctional hydratase-dehydrogenase (Fig. 4) clearly demonstrates that at least one peroxisomal protein is physically mostly missing, and not just inactive, in Zellweger syndrome.

The present results suggest several mechanisms by which a single genetic change could cause the various peroxisomal defects observed in Zellweger syndrome: one possibility is defective membrane assembly; another is defective import of matrix proteins through the peroxisomal membrane. In the latter case (nearly) empty peroxisomal membranes probably could not be recognized as such morphologically. The first alternative appears to be the simplest one at present, but the ultrastructural observation of rare, small particles containing a little diaminobenzidine cytochemical reaction product (abortive peroxisomes??) in intestine (Fig. 3*B*) lends some support to the second possibility. The properties of the peroxisomal membrane are reviewed elsewhere (66).

Clofibrate treatment had no discernible biochemical or morphological effect on these two patients, and it did not affect the clinical course. Its use cannot be recommended for other Zellweger babies.

Patient 3 physically resembled our two Zellweger patients, but she proved to have normal hepatic peroxisomes (Fig. 2) which contained normal activities of catalase (Table 1), β-oxidation enzymes (text), and dihydroxyacetone phosphate acyltransferase (Table 3). In addition, she was older than classical Zellweger cases. In view of the accumulating evidence that Zellweger syndrome is a peroxisome deficiency disease, these results indicate that patient 3 has a different genetic basis for her pathology. We refer to her as a "pseudo-Zellweger" patient at present, for lack of a more specific term. The case raises interesting questions for future research.

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