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Peroxisomal Defects in Neonatal-Onset and X-Linked Adrenoleukodystrophies

Abstract. *Accumulation of very long chain fatty acids in X-linked and neonatal forms of adrenoleukodystrophy (ALD) appears to be a consequence of deficient peroxisomal oxidation of very long chain fatty acids. Peroxisomes were readily identified in liver biopsies taken from a patient having the X-linked disorder. However, in liver biopsies from a patient having neonatal-onset ALD, hepatocellular peroxisomes were greatly reduced in size and number, and sedimentable catalase was markedly diminished. The presence of increased concentrations of serum pipecolic acid and the bile acid intermediate, trihydroxycoprostanic acid, in the neonatal ALD patient are associated with a generalized diminution of peroxisomal activities that was not observed in the patient with X-linked ALD.*

Two distinct types of adrenoleukodystrophy (ALD) that differ in their age of onset and mode of inheritance have been identified. An X-linked form of ALD (Schilder's disease) usually appears in prepubertal boys and is characterized by progressive destruction of cerebral white matter and adrenal cortex. Death occurs during adolescence in most cases (*1*). A neonatal-onset (neonatal) form of ALD that is not X-linked has also been described (*2*). Affected children suffer from severe hypotonia and seizures and usually do not live longer than six years. Accumulations of very long chain fatty acids (C_{26} and C_{24}) are characteristic of neonatal and X-linked types of ALD, and fibroblasts from patients with both forms of ALD are deficient in their ability to oxidize very long chain fatty acids (*2–5*).

The oxidation of some very long chain fatty acids appears to occur preferentially in peroxisomes (*6*). Therefore, we examined the morphological and biochemical properties of peroxisomes in liver biopsies from a 12-year-old boy with X-linked ALD and a 3-year-old girl with neonatal ALD (*7*). Abnormalities of cholic and chenodeoxycholic acid synthesis and pipecolic acid excretion were also investigated in these patients because bile acid intermediates (*8*) and pipecolic acid (*9*) accumulate in the serum and urine of infants with Zellweger's cerebro-hepato-renal syndrome (*10*), in which there are no detectable hepatic peroxisomes.

The hepatic architecture was normal in the biopsies from both patients; however, portal connective tissue was increased in the liver of the patient having the neonatal disease. There were striking differences in the size, number, and configuration of hepatocellular peroxisomes in these two diseases. In the child with X-linked ALD, peroxisomes were abundant and normal in size, ranging from 0.25 to 0.75 μm in diameter in 39 electron micrographs photographed at a magnification of ×19,500 to ×37,500 (*11*). The peroxisomes had a characteristic coarsely fibrillar, moderately electron-opaque matrix and were often found in clusters (Fig. 1A). In the patient with neonatal ALD, peroxisomes were sparse, scattered, and small and, when measured in 62 electron micrographs, had diameters that ranged from 0.1 to 0.25 μm (*12*) (Fig. 1B). Sometimes their matrices were denser than normal and separated from the membranes.

There was a tenfold decrease in the number and mean volume of hepatocellular peroxisomes in the patient having neonatal ALD as compared to the patient having X-linked ALD (*13*). The mean peroxisomal volume in the X-linked ALD child was 0.070 μm^3. In the neonatal ALD patient, total peroxisomal volume per hepatocyte was 1 percent of that observed in the liver of the X-linked disease patient. Anucleoid peroxisomes (0.07 to 0.20 μm in diameter) were also present in the intestinal epithelium of the patient with neonatal ALD (Fig. 1C); these resemble normal intestinal peroxisomes in size and shape (*11, 12*).

In the neonatal ALD patient, lysosomal and cytoplasmic inclusions composed of parallel lamellae (2.5 to 5 nm in thickness) were common in Kupffer cells (Fig. 1D). These lamellae are similar to those described as containing cholesterol esters with very long fatty acid chains after extraction in propylene oxide (*3*). Plasma concentrations of the very long chain fatty acid hexacosanoic acid (C26:0) were elevated and the ratios of C26:0 to docosanoic acid (C22:0) were abnormal in both patients (Table 1).

Hepatic catalase activity (*14*) in the X-linked ALD patient was normal. Moreover, as in two controls, half of the catalase could be sedimented under centrifugation conditions where peroxisomes, but not soluble catalase, are pelleted (Table 2). This indicates that at least half of the catalase was in peroxisomes; the balance may have been located in the cytosol or may have been released from peroxisomes damaged during homogenization. In contrast, the neonatal ALD patient had a low liver catalase activity of which only 12 percent was sedimentable (Table 2). In absolute terms, sedimentable catalase was 4 percent of that of the X-linked ALD patient. The specific activity of catalase in the intestinal mucosa of the neonatal ALD patient was 68 milliunits (mU) per milligram of protein. In two controls, the catalase-specific activities were 44 mU per milligram of protein.

The peroxisomal β-oxidation capacity of the X-linked ALD patient's liver, assayed with palmitoyl–coenzyme A (palmitoyl CoA) as substrate (*15*), was 1.4 nmol/min per milligram of protein. This may be compared with a value of 0.96 nmol/min per milligram of protein that we observed on an adult control, and with a published normal human value of approximately 1 nmol/min per milligram of protein (*16*).

Increased concentrations of pipecolic acid (*17*) were found in serum samples of the neonatal ALD patient at 2 and 3 years of age (87 nmol/ml; control, 5

Table 1. Plasma hexacosanoic acid (C26:0) and ratio of C26:0 to docosanoic acid (C22:0). For ALD patients, the means of two determinations are shown (*32*). Mean ± standard deviation in controls were derived from assays of 65 normal individuals. Quantitation of fatty acids was as described in (*4*).

Subject	C26:0 (μg/ml)	C26:0/ C22:0
X-linked ALD	1.278	0.075
Neonatal ALD	1.783	0.215
Control	0.33 ± 0.18	0.014 ± 0.076

Table 2. Hepatic catalase activity and sedimentability in ALD patients and controls. Duplicate determinations agree within 5 percent. (A) Male child evaluated for hepatosplenomegaly; no evidence of liver disease; (B) 6-year-old boy with terminal cystinosis and cystic fibrosis; (C) 2½-year-old male who died without history of liver disease; (D) adult male who had normal portion of liver removed due to a tumor. For measurement of sedimentability, a volume of 150 μl was centrifuged in a 1.5-ml microfuge tube for 30 minutes at 10,000 rev/min in a Sorvall HB-4 swinging bucket rotor with a rubber adapter (r_{max} = 8.7 cm).

Item	ALD		Controls			
	Neonatal	X-linked	A	B	C	D
Total homogenate activity*	23	151	112	43	173	87
Sedimentability†						
Homogenate	(100)	(100)	(100)	(100)	–	–
Pellet	12	49	45	61	–	–
Supernatant	80	49	34	21	–	–
Recovery	92	98	79	82	–	–

*Milliunits per milligram of protein. †Percent.

nmol/ml). In the child with X-linked ALD the concentration of pipecolic acid was 10.3 nmol/ml. Sera from mothers of both patients were indistinguishable from those of the controls.

Total serum bile acids (*18*) were normal in both patients. However, in the neonatal ALD patient, trihydroxycoprostanic acid (THCA), which is not present in normal serum, represented 20 percent of the total serum bile acids in two determinations (1.1 and 1.2 μ*M* THCA in 5.4 and 5.6 μ*M* total serum bile acids, respectively). No THCA was detected in the serum of the patient with X-linked ALD.

Fibroblasts cultured from patients with both forms of ALD have been shown to be deficient in their ability to oxidize lignoceric acid (C_{24}) (*4*), and studies of rat liver have suggested that this oxidation is, at least partially, a peroxisomal function (*5*). In neonatal ALD, the depression of very long chain fatty acid oxidation activity may simply reflect the deficiency of peroxisomes. In X-linked ALD, if the defect is peroxisomal, it would appear to be a consequence of an X-linked gene involved in very long chain fatty acid oxidation because peroxisomes are structurally normal, have normal levels of sedimentable particulate catalase, and have normal peroxisomal β-oxidation activity toward palmitoyl CoA.

In our patient with neonatal ALD, peroxisomes were so small and so sparse that they could not be detected (*19*) until we used cytochemical staining for cata-

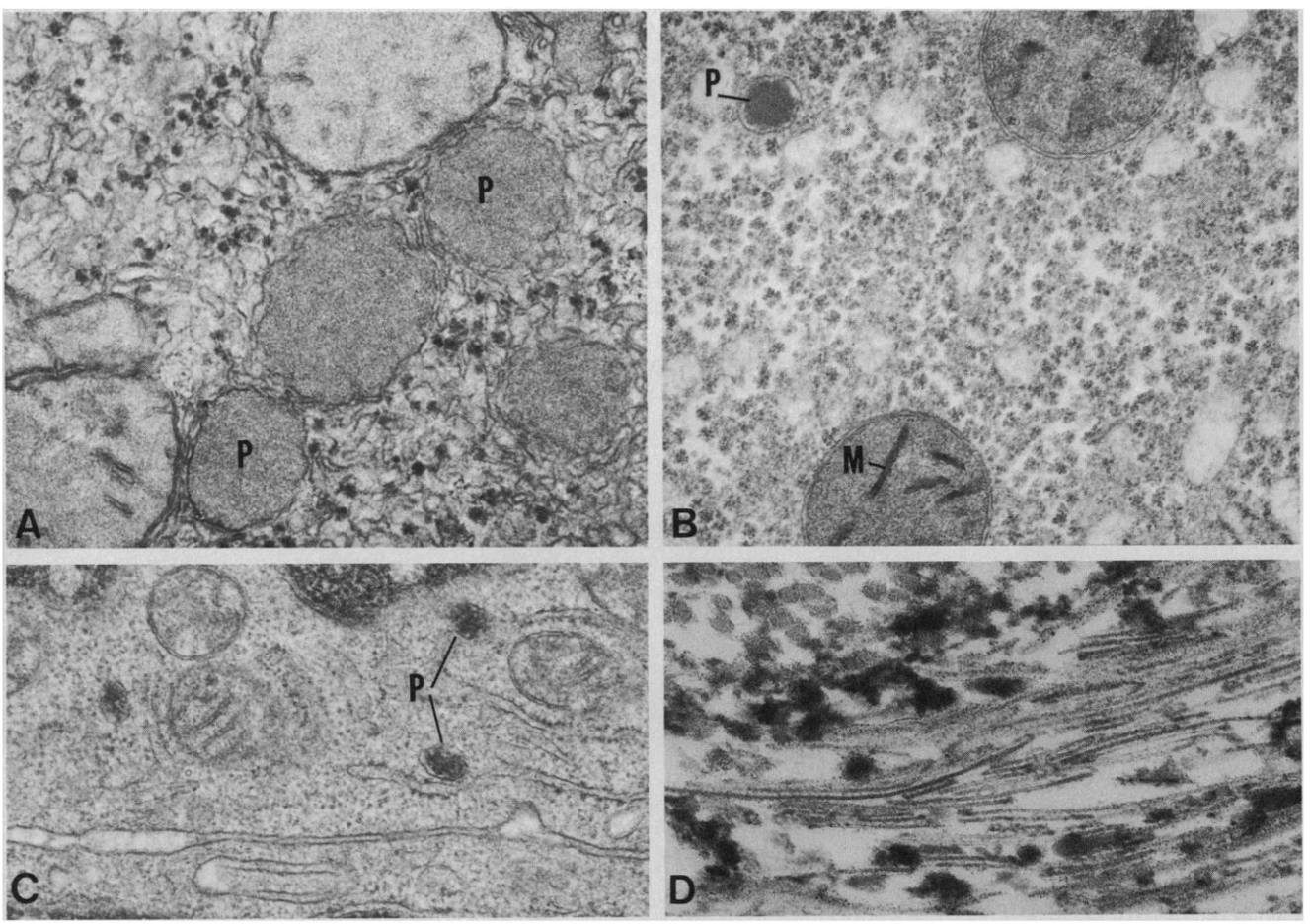

Fig. 1. (A) Hepatocytes in X-linked ALD; P, peroxisomes. Final magnification, ×37,500. (B) Hepatocytes in neonatal ALD. Peroxisomes were identified by the presence of reaction product in sections incubated to demonstrate catalase, a marker enzyme for hepatocellular peroxisomes. Mitochondrial cristae (M) are also stained because cytochrome oxidase is reactive in H_2O_2-diaminobenzidine medium. Final magnification, ×37.500. (C) Intestinal epithelial cells in neonatal ALD. Peroxisomes stained intensely with diaminobenzidine. Final magnification, ×42,000. (D) Kupffer cells in neonatal ALD with lamellar inclusions. Final magnification, ×49,000.

lase. Hepatic and renal peroxisomes appear to be absent in another neonatal autosomal-recessive disease, the cerebro-hepato-renal syndrome of Zellweger (CHRS) (*10, 20–22*). Demyelination, severe hypotonia, and increased tissue concentrations of very long chain fatty acids are present in CHRS (*23, 24*), as well as in neonatal ALD. If the relative abundance of peroxisomes in developing neurons and in oligodendrocytes plays a role in myelin formation (*25*), the peroxisomal deficiencies in very long chain fatty acid oxidation may contribute to the profound degeneration of myelin that occurs in both forms of ALD and in CHRS. However, children with CHRS do not have adrenal atrophy and usually die by 1 year; patients with neonatal ALD (*2*) do not have the characteristic facial dysmorphism, neuronal heterotopia, renal cortical cysts, iron storage, or skeletal abnormalities associated with CHRS. Whether CHRS and neonatal ALD are distinct entities, or reflect different degrees of expression of a single defect that regulates peroxisomal biogenesis, remains to be determined.

Several of the oxidative reactions in the formation of bile acids occur in peroxisomes (*26*). The detection of an abnormal bile acid intermediate in serum from neonatal ALD, but not in X-linked ALD, is consistent with earlier data demonstrating increased concentrations of C_{27} bile acid intermediates in bile and serum of CHRS patients (*8*), and supports the view that the bile acid defect is a consequence of a profound decrease in hepatic peroxisomes (*26, 27*).

Pipecolic acid concentrations are elevated in serum and urine in patients with CHRS (*9*), and in our patient with neonatal ALD (*28*), but not significantly in the patient with X-linked ALD. Pipecolic acid is a product of lysine metabolism; lysine degradation proceeds via glutaryl-CoA, which can be oxidized either by a mitochondrial dehydrogenase or a peroxisomal oxidase (*29*). The increased pipecolic acid concentrations in CHRS and neonatal ALD may reflect a deficiency of the oxidase.

In rat liver, peroxisomal enzymes and a major peroxisomal membrane polypeptide are synthesized on free polysomes. They are transported through the cytosol and enter peroxisomes post-translationally (*30, 31*). The multiple deficiencies in CHRS and neonatal ALD may reflect a defect in a gene that regulates synthesis of peroxisomal enzymes. Alternatively, the underlying defect could be in the synthesis of a peroxisomal membrane protein required for organelle assembly. If a defective membrane could not support the import of newly synthesized peroxisomal enzymes, these enzymes would remain in the cytosol where some of them might be subjected to more rapid degradation.

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7. A liver biopsy was obtained at the time of gastrostomy placement in a 13-year-old boy with X-linked ALD who, at age 8, had developed dementia with progressive seizures and spasticity. Computed tomography scans showed decreased density of white matter, and nerve biopsy demonstrated lamellar inclusions characteristic of ALD. A brother, 16 years of age, has elevated serum levels of very long chain fatty acids, and the patient's mother and sister have blood C26:0/C22:0 FA ratios compatible with hemizygosity. Three cousins died of ALD. Liver biopsies were also obtained at ages 1 and 3 years from a 4-year-old girl having neonatal-onset ALD and no family history of consanguinity or genetic disorders. At age 2 months, sucking and swallowing were poor, and hypotonicity with normal deep tendon reflexes as well as growth delay were noted. Minor motor seizures with generalized multifocal spikes and sharp waves appearing in electroencephalographic analyses began by age 2.5. She was blind and deaf and showed marked developmental delay.
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11. Electron microscopic studies of liver were performed on samples fixed by two different procedures: (i) Primary fixation in glutaraldehyde (3 percent) in 0.1*M* cacodylate buffer, *p*H 7.3 for 3 hours followed by post fixation for 2 hours in osmium tetroxide (2 percent) in 0.1*M* phosphate buffer, *p*H 7.3; and (ii) primary fixation in a solution of osmium tetroxide (2 percent) in 0.1*M* phosphate buffer, *p*H 7.3, and 0.36*M* $K_4Fe(CN)_6$. Intestinal biopsies (patient No. 2) and a biopsy of normal intestinal epithelium, were fixed in glutaraldehyde as in (*1*) for 90 minutes and postfixed in osmium tetroxide for 1 hour.
12. Unfrozen chopper sections (Smith-Farquhar; Sorvall), 40 μm in thickness, of glutaraldehyde and formaldehyde-fixed livers were incubated for 90 minutes by a procedure that yields optimal staining for the peroxidatic activity of catalase in human tissues [F. Roels and S. Goldfischer, *J. Histochem. Cytochem.* **27**, 1471 (1979)]. Sections of intestinal tissue were similarly incubated, but at 37°C. After incubation the sections were postfixed in osmium tetroxide and embedded in Epon.
13. Sections were examined from blocks that had been processed for routine electron microscopy, and from samples incubated in a cytochemical medium to demonstrate peroxisomal catalase in situ (*12*). Morphometric measurements were made with the negatives of electron micrographs (magnification ×6500) that were projected on an epidiascope screen. Thirteen plates of neonatal ALD liver and 15 of the liver of the X-linked patient were measured. The image analysis apparatus was a Cambridge/Imanco Quantimet 720 equipped with an image editor module and function analyzer for perimeter and area measurements. Data were interfaced to a Hewlett-Packard 8930 microcomputer for collection and analysis.
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33. Supported in part by grants from the National Institutes of Health (AM-17702, N5-03356, AG-01468, BRSG-507, RR-05399-22, AM-19394), National Science Foundation (PCM82-08315), and the Gail I. Zuckerman Foundation, and by an Established Fellowship from the New York Heart Association (P.B.L.). B. Bangaru and J. Dancis of New York University and N. F. LaRusso of the Mayo Clinic generously supplied control samples used for determining hepatic catalase. We are grateful to J. Dancis for pipecolic acid determinations.

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Abrupt Induction of a Membrane Digestive Enzyme by Its Intraintestinal Substrate

Abstract. *The regulation of amino-oligopeptidase (AOP), an intestinal brush border hydrolase essential for the surface digestion of peptide nutrients, was examined in rats in vivo. Short-term (30-minute) intraintestinal perfusion of a tetrapeptide substrate, Gly-Leu-Gly-Gly, or a synthetic substrate, leucyl-β-naphthylamide, induced a doubling in the incorporation of [^{3}H]leucine into the AOP in association with intracellular membranes. The subsequent conversion of AOP from nascent to mature enzyme and its membrane-associated transport to the brush border occurred at normal rates.*

Amino-oligopeptidase (AOP), an integral digestive enzyme of the intestinal brush border surface (*1*), hydrolyzes the oligopeptides produced by luminal pancreatic proteases at the intestinal membrane surface in preparation for the intestinal transport of the final dipeptide and amino acid products. This hydrolase plays an essential role in the assimilation of dietary protein in mammals (*2*).

Studies of the intact rat have shown that rapid synthesis of AOP as a nascent protein occurs within 15 minutes after an intraintestinal amino acid "pulse-chase" (short-term perfusions with labeled and unlabeled substrate) in association with the endoplasmic reticulum (ER). Within 30 to 45 minutes, apparent post-translational glycosylation and transfer to Golgi membranes (the site of fucosylation) takes place. Final vectorial transfer to the luminal brush border (BB) surface is not achieved until 3 hours after the intraluminal amino acid pulse (*3, 4*). Although the activity of the BB aminopeptidase in rats is decreased by starvation and increased by a high protein diet (*5*), the mechanism of dietary protein or peptide action on regulation of the aminopeptidase is unknown. We examined the regulatory role of high-affinity specific substrates of the AOP, the tetrapeptide Gly-Leu-Gly-Gly, and leucyl-β-naphthylamide (LeuNA), in the synthesis, intracellular assembly, and transport of AOP to the BB surface.

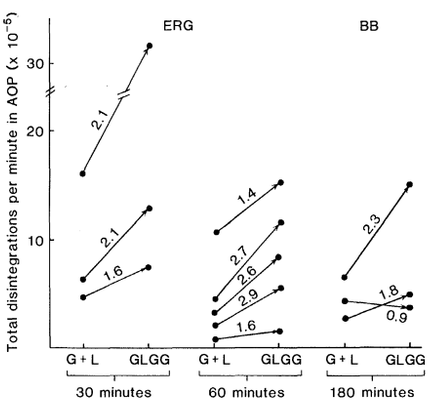

Fig. 1. Comparison of the incorporation of [^{3}H]leucine into AOP in ERG and BB fractions of paired rats receiving intraintestinal 2 m*M* Gly-Leu-Gly-Gly (GLGG) or its constituent amino acids (G+L). Rats were perfused with tetrapeptide or amino acid solution for 30 minutes and then pulse-labeled with [^{3}H]leucine intraluminally and chased with the peptide or amino acid solution for 30, 60 or 180 minutes. AOP was specifically immunoprecipitated from the Triton-solubilized ERG and BB fractions, and the total amount of label in the enzyme was determined by allowing for the fractional solubilization by Triton X-100 and the yield of the marker enzyme (*3*). The arrows connect paired animals at times after the [^{3}H]leucine pulse; ratios of the amount of label in AOP from rats perfused with tetrapeptide to that from rats perfused with amino acid (control) are given over each arrow. Perfusion with Gly-Leu-Gly-Gly significantly increased incorporation of label into AOP in the ERG (see text). Similarly, in two of the three experiments, the total amount of label in AOP in the BB from the substrate-perfused rats at 180 minutes was twice that in control, and the specific amount of label in AOP (disintegrations per minute per milliunit) in the BB fraction was increased significantly (see text). Statistical analysis was by the paired *t*-test; data are reported as the mean ± standard error.

Male Sprague-Dawley rats, paired by age and weight (200 to 300 g) and fed a protein-deficient diet (*6*) for 12 days, were anesthetized, and jejunal loops (25 cm long) were isolated and cannulated as described (*2*). The intestinal loop of the paired rats was simultaneously perfused for 30 minutes with 150 m*M* NaCl containing either 2 m*M* L-Gly-Leu-Gly-Gly or the equivalent amino acid solution (6 m*M* glycine and 2 m*M* L-leucine; control). After a 5-minute intraluminal "pulse" with L-[^{3}H]leucine (2.5 mCi) and a 30-, 60-, or 180-minute "chase" with the substrate or amino acid solution, BB and ER-Golgi (ERG) membranes were prepared (*3*) and their purity assessed by use of sucrase (as a marker for the BB) and aryl esterase (for the ERG). The average purification factors were 13 for the BB and 4.1 for the ERG; the fractional yields were 0.40 for the BB and 0.08 for the ERG. Leucyl-β-naphthylamide hydrolase, sucrase, aryl esterase, and protein were assayed as described (*3*). After solubilization of the BB and ERG membranes with 0.5 percent Triton X-100, AOP and sucrase were isolated by specific immunoprecipitation with monospecific rabbit antibodies to AOP, and the total and specific amount of label in the immune precipitates obtained by centrifugation was determined by scintillation counting (*3, 4*). Purification factors, fractional yields, and percent solubilization of the membrane fractions were similar to those reported (*3, 4*) and were not different for animals perfused with either substrate or amino acid. Several experiments were done at each time after the pulse (see Fig. 1).

Although the rats' dietary protein intake was restricted, the AOP activity in the BB (1260 ± 120 mU per milligram of protein) was similar to that for animals fed a regular diet (1290 ± 90 mU per milligram). The total amount of label incorporated into AOP in the ERG fractions from rats perfused with Gly-Leu-