Multiple Peroxisomal Enzymatic Deficiency Disorders

A Comparative Biochemical and Morphologic Study of Zellweger Cerebrohepatorenal Syndrome and Neonatal Adrenoleukodystrophy

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Biologic, morphologic, and biochemical investigations performed in 2 patients demonstrate multiple peroxisomal deficiencies in the cerebrohepatorenal syndrome of Zellweger (CHRS) and neonatal adrenoleukodystrophy (NALD). Very long chain fatty acids, abnormal bile acids, including bile acid precursors (di- and trihydroxycoprostanoic acids), and C_{29}-dicarboxylic acid accumulated in plasma in both patients. Generalized hyperaminoaciduria was also present. Peroxisomes could not be detected in CHRS liver and kidney; however, in the NALD patient, small and sparse cytoplasmic bodies resembling altered peroxisomes were found in hepatocytes. Hepatocellular and Kupffer cell lysosomes were engorged with ferritin and contained clefts and trilaminar structures believed to represent very long chain fatty acids. Enzymatic deficiencies reflected the peroxisomal defects. Hepatic glycolate oxidase and palmitoyl-CoA oxidase activities were deficient. No particle-bound catalase was found in cultured fibroblasts, and ether glycerolipid (plasmalogen) biosynthesis was markedly reduced. Administration of phenobarbital and clofibrate, an agent that induces peroxisomal proliferation and enzymatic activities, to the NALD patient did not bring about any changes in plasma metabolites, liver peroxisome population, or oxidizing activities. (Am J Pathol 1986, 125:524–535)

THE ABSENCE of recognizable peroxisomes in liver and kidney from patients with the cerebrohepatorenal syndrome of Zellweger (CHRS)[1] and the establishment of a major role of peroxisomes in the oxidation of very long chain fatty acids,[2] which consequently accumulate in blood and tissues from patients with peroxisomal dysfunction,[3–5] have resulted in the characterization of a new group of metabolic diseases, the peroxisomal disorders.[6–9] Although various discrete features of these disorders have been described, we now report the integrated biologic, biochemical, and morphologic findings in 2 patients with peroxisomal deficiency syndromes. Clinically and pathologically, 1 patient with CHRS was indistinguishable from children described by Bowen, Lee, Zellweger, and Lindenberg.[10] Our second patient has had a different clinical course with prolonged survival that is more suggestive of neonatal adrenoleukodystrophy (NALD).[11–13] Peroxisomal functions were investigated *in vivo* by measuring plasma me-

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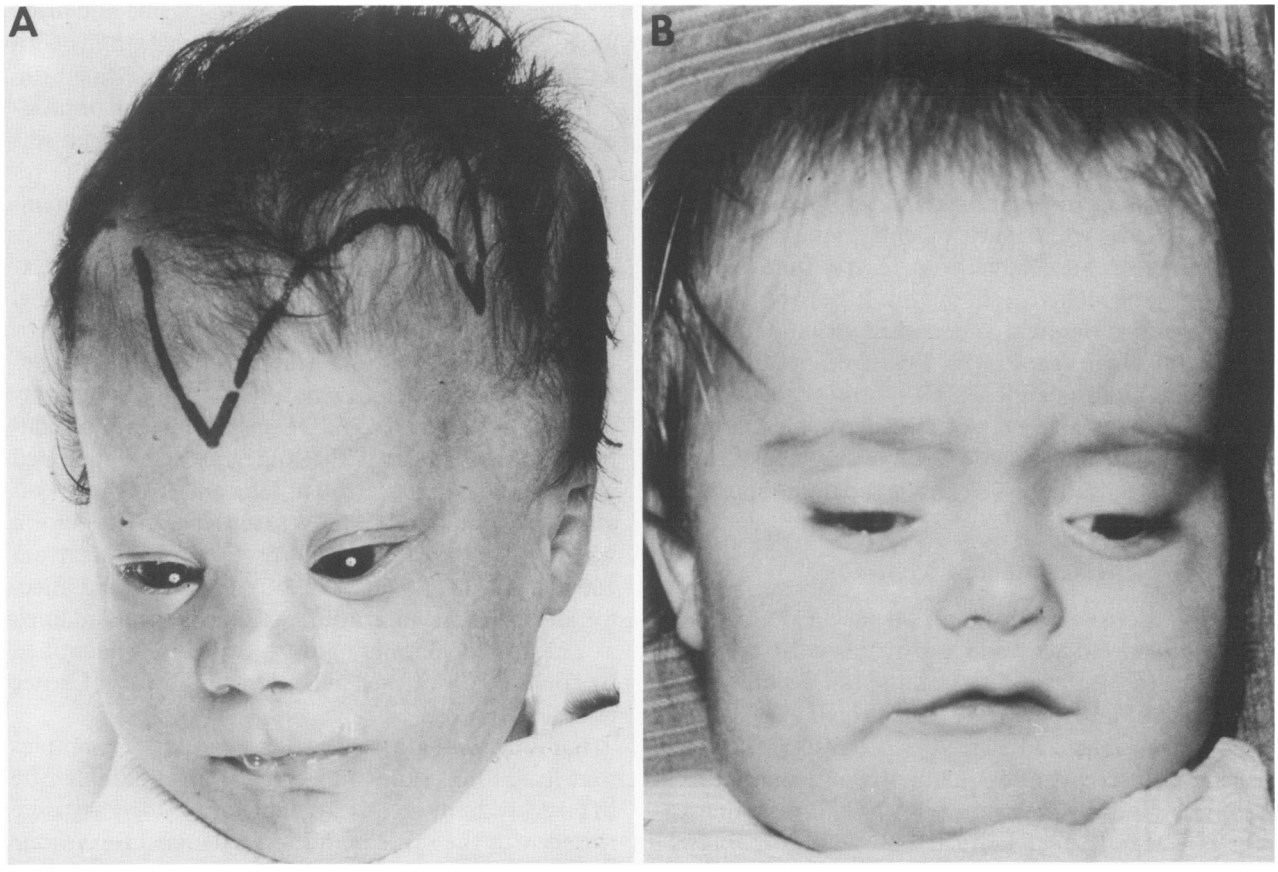

Figure 1—Patient 1. Girl with CHRS who died at 7 weeks. Note the enlargement of the fontonelle. **B**—Patient 2. Boy with NALD, alive at 3 years.

tabolites, including bile acids, very long chain fatty acids, and pipecolic acid and, *in vitro*, by enzymatic assays of liver samples and cultured skin fibroblasts. Four peroxisomal matrix enzymes, fatty acyl-CoA oxidase, D-amino acid oxidase, L-α-hydroxyacid oxidase, and catalase, as well as a membrane-associated enzyme that is the first step in plasmalogen synthesis, dihydroxyacetone phosphate acyltransferase (DHAP-AT), were assayed.

Case Reports

Zellweger CHRS (Patient 1)

This patient (Figure 1A) displayed severe hypotonia and peripheral neuropathy and was unresponsive to visual and auditory stimuli from birth. She had a high forehead, epicanthal folds, and an arched palate. Feeding was poor and weight gain was minimal before death at the age of 7 weeks. Seizures that were poorly responsive to phenobarbital appeared after 4 weeks. Hepatomegaly followed by icterus became evident from the fifth week of life. X-ray examination revealed typical patellar stippled calcifications. Ultrasonography showed hyperechogenicity of the subcapsular region of both kidneys, and postmortem examination showed this to correspond to multiple small cortical cysts that are characteristic of CHRS. CT scan disclosed hypodense zones at the level of cerebral white matter and abnormal gyrations, mainly in the temporal region. Abnormal laboratory data included elevated serum transaminases, lactate dehydrogenase, and alkaline phosphatase, global hyperaminoaciduria, and elevated cerebrospinal fluid protein concentrations. Significant elevations in pipecolic acid were not detected in blood or urine.

NALD (Patient 2)

This patient (Figure 1B) was considered normal until the age of 3 months, except for blindness. Progressive severe hypotonia, severe psychomotor retardation, and peripheral neuropathy developed. Facial abnormalities became striking only after the first year. At the age of 2 he had moderate hepatomegaly and the same blood, urine, and cerebrospinal fluid abnormalities as Patient 1. Other abnormalities included an elevated iron

binding capacity, increased pipecolic acid concentration (300 M; control, 5M) in serum and lowered plasma levels of vitamins D and E. He suffered from numerous spontaneous bone fractures. Patellar calcifications and echographic signs of renal cysts have not been detected. Computerized tomographic scan and nuclear magnetic resonance demonstrated extensive lesions in the hemispheric white matter. Ocular impairment was similar in both children, with a pale, gray fundus, and, in Patient 1, pigmentary retinopathy. In both cases, electroretinograms were flat and evoked visual potentials were highly disturbed. Patient 2 received clofibrate (25 mg/kg/day) and phenobarbital (7 mg/kg/day) for 6 months without any evident clinical or biochemical effect. He is still alive at the age of 3 years. More detailed clinical data have been given in a separate report.[14]

Materials and Methods

Peroxisomal enzymes, including fatty acyl-CoA oxidase, D-amino acid oxidase, and L-α-hydroxyacid oxidase, were assayed by their H_2O_2 production according to Vamecq and Van Hoof,[15] with 0.5 mM palmitoyl-CoA, 100 mM D-proline, and 20 mM glycolate as substrates, respectively. Activity of mitochondrial monoamine oxidase was similarly measured with 20 mM tyramine as substrate. Catalase latency was assayed on fibroblasts as previously described.[16,17] As controls we utilized biopsy samples obtained for diagnostic purposes from children of both sexes under the age of 4. All controls were examined by electron microscopy; and hepatic peroxisomes were normal in size, number, and distribution. *"De novo" plasmalogen biosynthesis* (^{14}C-hexadecanol incorporation test), activity of acyl-CoA, DHAP-AT, and plasmalogen levels in fibroblasts were assayed as previously described.[16-19]

Amino acids and *pipecolic acid* in plasma and urine were separated by ion-exchange chromatography with a Liquimat III Kontron apparatus, using a Mitsubishi cation-exchange resin (Type K10F). Detection of products was achieved with ninhydrin. Pipecolic acid was quantified as described previously.[20]

Bile acid and *very long chain fatty acid* determinations were performed as described elsewhere.[21,22]

Phytanic acid concentrations were measured by extracting plasma organic acids at pH 1.0 with diethyl ether and ethyl acetate, successively. They were trimethylsilylated with bis(trismethylsilyl)trifluoroacetamide, separated and identified by gas chromatography using an Intersmat IGC-121DL gas chromatograph coupled to an Intersmat ICR-1B integrator and equipped with a capillary column 50 m long and 0.32 mm in diameter (Chrompack CP Sil 5 fused silica). The elution was obtained by raising the oven temperature from 160 C to 220 C at the rate of 2 C/min. The solid injector and the flame ionization detector were maintained at 250 C. Gas chromatography peaks were identified by comparison of their retention time with reference retention times.

For *morphologic studies*, liver and kidney samples were fixed for 1 hour with 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. They were rinsed with the same buffer and postfixed for 1 hour in a 1% OsO_4–1% $K_4Fe(CN)_6$ mixture, dehydrated, and embedded in Spurr's plastic mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM-301 or Siemens 101 electron microscope. Extraction of lipids with acetone (twice 15 minutes) or *n*-hexane (twice 15 minutes after acetone) was performed on glutaraldehyde-fixed sections, which were subsequently osmicated.

Table 1—Assay or Metabolites Found in Abnormal Amounts in Sera

	Patient 1 (CHRS)	Patient 2 (NALD)		Controls
		Before therapy	During therapy	
Pipecolic acid (μM)	<10	300	406	<10
Very long chain fatty acids				
$\frac{C_{24}}{C_{22}}$ ratio	1.46	1.49	1.49	0.89 ± 0.18
$\frac{C_{26}}{C_{22}}$ ratio	0.69	0.28	0.26	0.02 ± 0.01
Bile acids (%)				
common bile acids (cholic and chenodeoxycholic acids)	37%	28%	12%	100
bile acid precursors (di- and trihydroxycoprostanoic acids)	40%	49%	66%	ND
C_{26}-dicarboxylic acid	23%	23%	22%	ND

The concentration of various bile acids is expressed as the percentage of the total bile acid content. ND, not detectable.

Results

Plasma, Bile, and Urine Metabolites

Very long chain fatty acids and bile acids were assayed in plasma for estimation of the *in vivo* ability of peroxisomes to shorten very long chain substrates and to oxidize hydroxycoprostanoic acids. As enumerated in Table 1, all these metabolites accumulated in plasma. In both patients, the ratio between the concentration of the very long chain fatty acids, hexacosanoic acid (C_{26}) and behenoic acid (C_{22}) in plasma was at least 20-fold higher than normal, resulting from the large excess of hexacosanoic acid.

Bile acid analysis revealed the presence of a high concentration of the bile acid precursors, di- and trihydroxycoprostanic acids, and the C_{29}-dicarboxylic acid, metabolites that are not normally detected in plasma. These unusual bile acid precursors represent more than 70% of the total serum bile acid content in both patients. The bile acid patterns were also studied in urine and bile (Table 2). In CHRS bile trihydroxycoprostanic acid accumulations were greater than that of dihydroxycoprostanic acid (Table 2A). C_{29}-dicarboxylic acid was not detected in bile, but was found in CHRS urine (Table 2B). The total amount of bile acids in plasma was abnormally high in the CHRS patient (mean of two samples 35 μg/ml), whereas it was normal in the NALD patient (mean of three samples, 8 μg/ml). In both patients the concentrations of common bile acids (cholic and chenodeoxycholic acids) were low, consistent with their decreased formation and the high concentrations of their precursors. In NALD, dihydroxycoprostanic acid was always more abundant than trihydroxycoprostanic acid, whereas in CHRS their ratio was reversed. In CHRS plasma the trihydroxycoprostanic/dihydroxycoprostanic acid ratio suggests a more severe impairment in the formation of cholic acid than chenodeoxycholic acid.[23]

Table 2—Details of Bile Acid Composition in the Various Biologic Fluids

	Patient 1 (CHRS)	Patient 2 (NALD)
A) In bile	(n = 2)	
Chenodeoxycholic acid	27	Not examined
Cholic acid	22	
Dihydroxycoprostanic acid	6	
Trihydroxycoprostanic acid	43	
Unknown	2	
C_{29}-dicarboxylic acid	0	
B) In urine	(n = 1)	(n = 2)
Deoxycholic acid	1	2
Chenodeoxycholic acid	21	25
Cholic acid	17	33
Ursodeoxycholic acid	—	22
Hyocholic acid	8	14
Dihydroxycoprostanic acid	4	1
Trihydroxycoprostanic acid	41	3
C_{29}-Dicarboxylic acid	8	0
C) In plasma	(n = 1)	(n = 3)
3-β-OH-Δ5-Cholenic acid	2	—
Deoxycholic acid	4	—
Lithocholic acid	—	1
Chenodeoxycholic acid	10	13
Cholic acid	2	7
Hyocholic acid	2	2
Cholic acid (epimer)	17	—
Dihydroxycoprostanic acid	13	45
Trihydroxycoprostanic acid	27	10
C_{29}-Dicarboxylic acid	23	22

Values are expressed as the percentage of the total bile acid content.

Table 3—The Concentration of Phytanic Acid, Expressed as Micrograms per Milliliter of Plasma, in the NALD Patient and in 4 Controls Compared with Values Reported in the Literature for Control and IRD Patients

	Phytanic acid concentration (μg/ml plasma)
NALD patient	
Control	1.79
1	0.88
2	0.99
3	1.26
4	1.15
Poulos et al[25]	
Controls	<5
IRD	15 to 125
Scotto et al[24]	
Controls	Around 1
IRD	50 to 100

Plasma amino acids were normal in both patients except for L-pipecolic acid, which was increased in NALD. A general, but mild, hyperaminoaciduria was recorded in both cases. Plasma concentrations of phytanic acid were measured in NALD and found to be slightly increased (Table 3). In infantile Refsum's disease (IRD), another condition in which hepatocellular peroxisomes appear to be absent, plasma phytanic acid is increased 20-fold.[24,25]

Morphology

Despite an extensive search in which over 10,000 sq μm of sections were examined, no recognizable peroxisomes could be detected in CHRS liver and kidney samples which were obtained soon after death. In the liver, lysosomes were of normal size but more numerous than usual, especially in Kupffer cells. Most lysosomes contained ferritin; many displayed needlelike electronlucent clefts, sometimes associated with lamellas or leaflets (Figure 2). Intralysosomal clefts and lamellas were also present in the kidney proximal tubular cells.

Neuropathologic findings were consistent with the diagnosis of Zellweger CHRS. Anatomic study of the brain (580 g) displayed zones of disturbances of gyra-

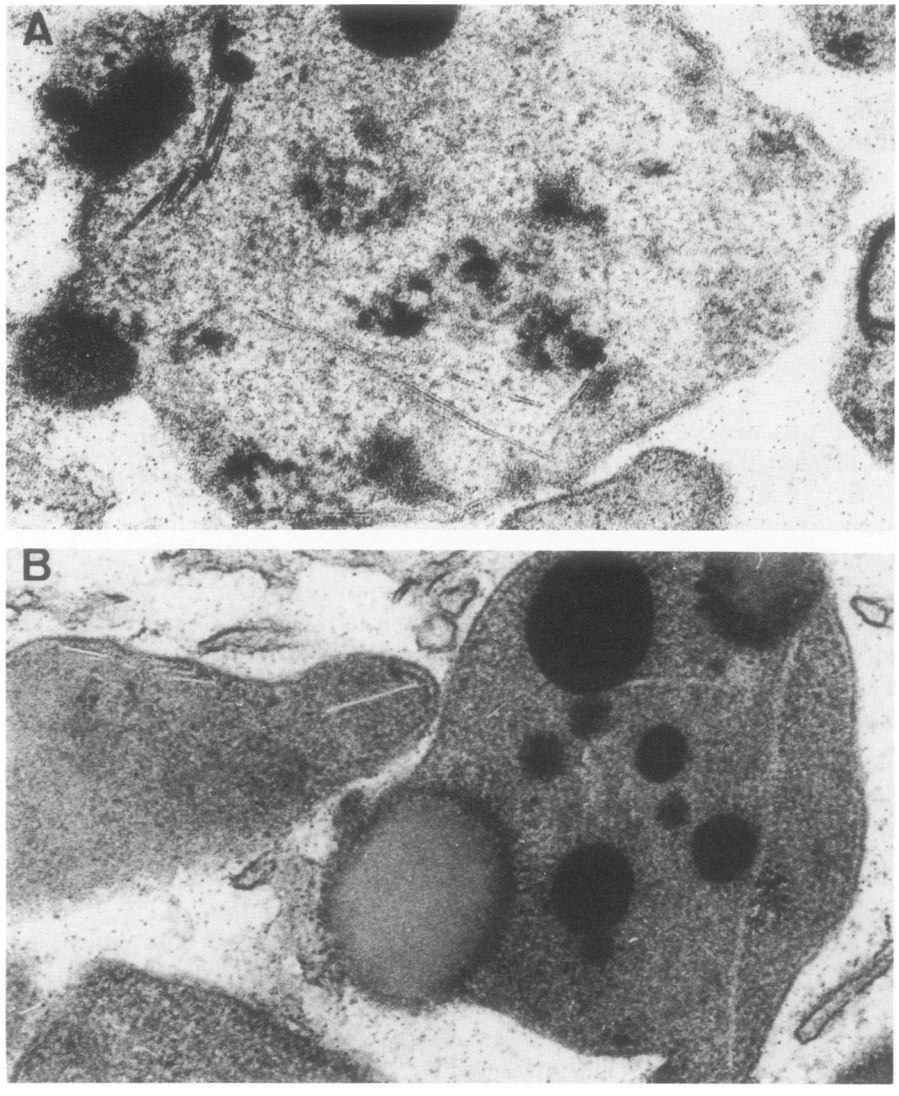

Figure 2—Intralysosomal very long chain fatty acid and ferritin deposition in hepatocytes from Patient 1 (CHRS). The presence of ferritin in lysosomes often facilitates the detection of very long chain substrates in the form of trilaminar structures (**A**) or clefts (**B**). (**A**, ×68,400; **B**, ×63,000)

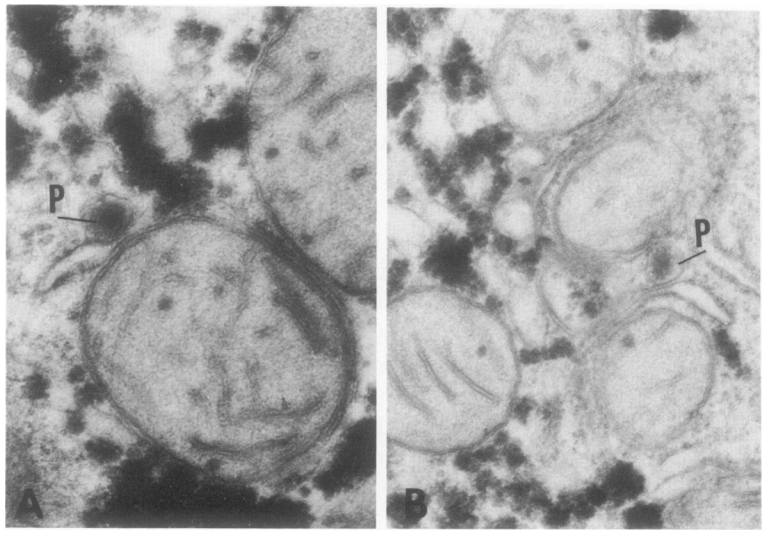

Figure 3—Single membrane-bound organelles (P) in hepatocytes from Patient 2 (NALD) that are often close to mitochondria and are similar to altered peroxisomes observed in another patient with NALD,[13] but staining for catalase, an important reaction for the microscopic identification of peroxisomes, was not performed on this patient. The matrix of these atypical peroxisomes generally appears dense and retracted. (**A**, ×36,000; **B**, ×34,500)

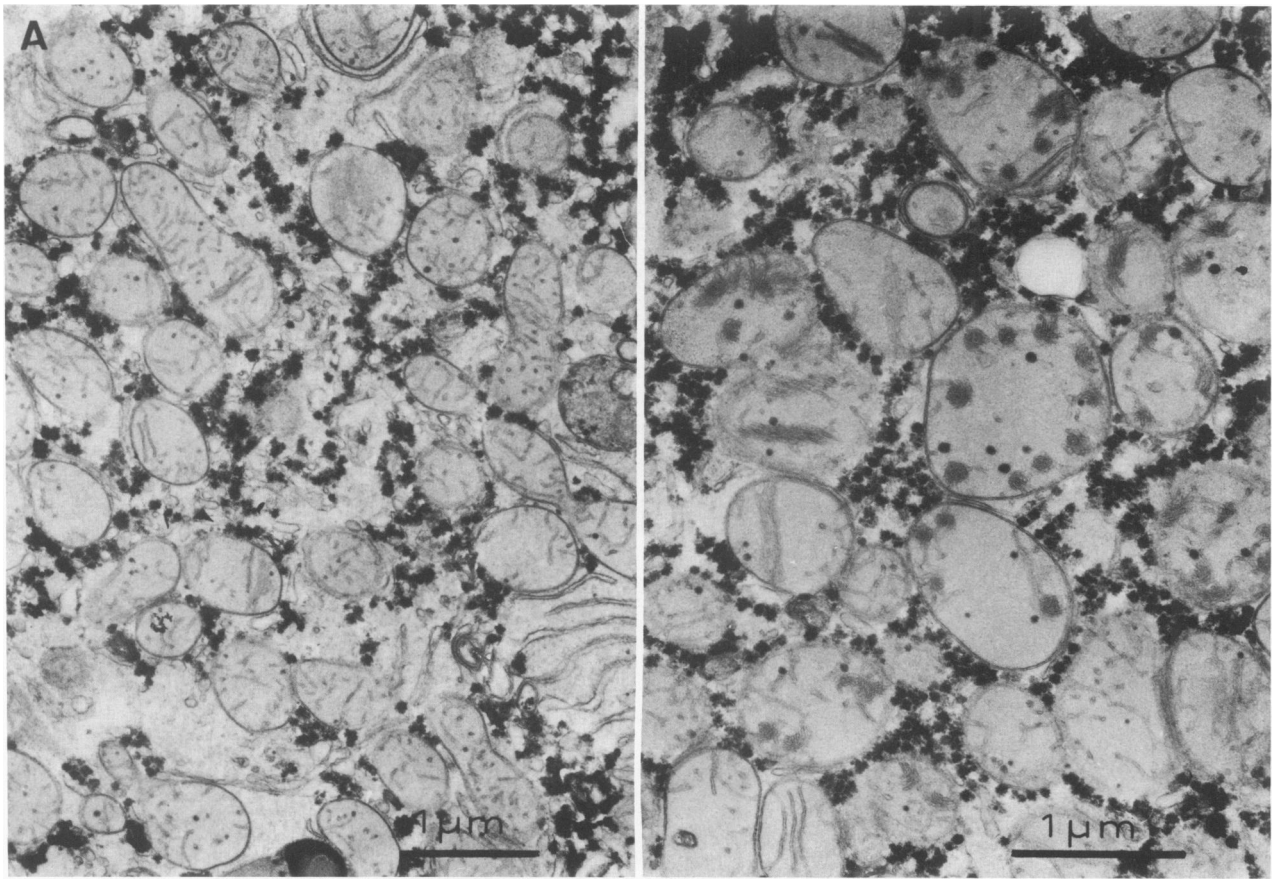

Figure 4—Ultrastructure of liver mitochondria from Patient 2 before treatment with clofibrate and phenobarbital (**A**) and during drug therapy (**B**). The swelling of mitochondria, increased size and number of matrix granules, and an apparent redistribution of mitochondrial cristae was observed on the second liver biopsy (**B**).

tion, mostly at the level of the parietal cortex, and partial agenesis of the corpus callosum. Light microscopy revealed an inversed cytoarchitectonic pattern of the layers of the cerebral cortex, neuronal intracortical and subcortical heterotopic fields in brain, subcortical heterotopias in cerebellum, and areas of demyelination.

Two needle biopsies of liver were examined from the NALD patient, the first before treatment and the second after treatment with clofibrate and phenobarbital for 6 months. Typical peroxisomes were not found; however, cytoplasmic bodies, often very close to mitochondria, with a moderately dense matrix shrunken away from the membrane, were observed (Figure 3). Their structure was similar to altered, catalase-positive hepatic peroxisomes previously described in a patient with NALD.[13] The only changes induced by therapy were an apparent redistribution of mitochondrial cristae, enlarged intramitochondrial granules (Figure 4), and a somewhat greater occurrence of intralysosomal clefts. Ferritin deposition in lysosomes was not excessive. The lipid nature of the trilamellar structures present in lysosomal matrix was studied by classical lipid extraction procedures (Figure 5); however, treatment of liver slices with acetone and *n*-hexane prior to plastic embeddment failed to dissolve these peculiar structures.

Biochemistry

Palmitoyl-CoA and glycolate oxidase activities could not be detected in either patient (Table 4). However, D-amino acid oxidase activity, measured with D-proline as substrate, was low in CHRS and higher than normal in NALD. In the latter, combined therapy with clofibrate and phenobarbital did not restore palmitoyl-CoA oxidase and glycolate oxidase activities. Tyramine oxidase, a mitochondrial H_2O_2-generating enzyme, was normally active, demonstrating good preservation of the tissue samples and the absence of artifactual interference in our assay system, which measures H_2O_2 production.

DHAP-AT activity in NALD fibroblasts was reduced to about 8% of normal (Table 5). *De novo* plasmalo-

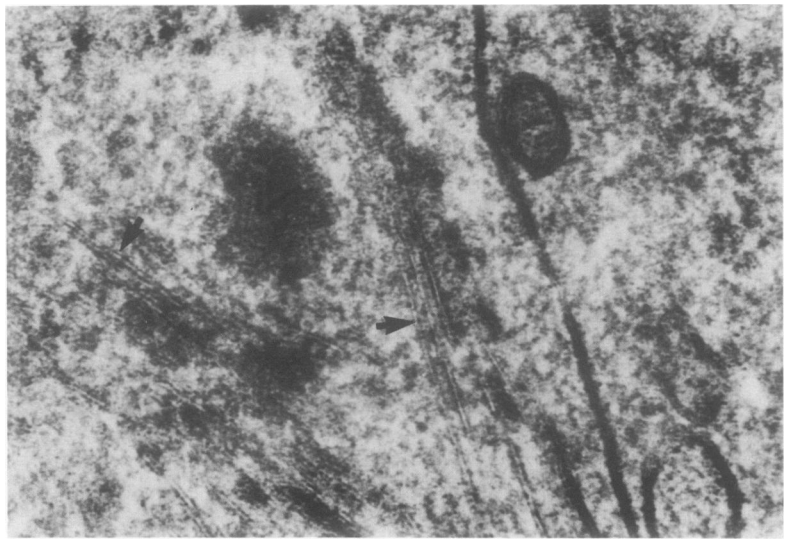

Figure 5—Aspect of lysosomal matrix in Kupffer cells from Patient 2 in liver slices treated with acetone and hexane. The electron-opaque parts of the trilamellar structures are resistant to the lipid extraction fixed tissues. ×23,000

gen biosynthesis was estimated by measuring the incorporation of ^{14}C-hexadecanol into newly synthesized phospholipids in an experiment in which fibroblasts from both patients were continuously cultured in the presence of the radioactive precursor. In these conditions, alkenyl-phosphatidylethanolamine (pPE) accounted for 3.6% and 5.3% of the radioactivity incorporated in the phospholipids, as compared with 52.4% in controls. The proportion of plasmalogens among the radioactive phospholipids extracted from fibroblasts was lower than normal in both patients. In fibroblasts from the patients, radioactive hexadecanol is incorporated differently in the various classes of phospholipids (Figure 6).

Catalase activity was normal in fibroblasts from both patients, but unlike control cells, no particle-bound catalase activity could be detected in the mutant fibroblasts. This indicates that the enzyme is not linked to peroxisomal structures in sufficient quantities to be measured biochemically.

Table 4—Oxidase Activities in Human Liver

	Patient 1 (CHRS)	Patient 2 (NALD)		Controls
		Before therapy	During therapy	
Palmitoyl-CoA oxidase	ND	ND	ND	150 ± 40 (n = 4)
Glycolate oxidase	ND	ND	ND	160 ± 20 (n = 10)
D-aminoacid oxidase	203	1620	2442	900 ± 280 (n = 4)
Tyramine oxidase	162	360	NA	260 ± 50 (n = 10)

The enzyme activities are expressed as nanomoles of H_2O_2 produced per minute and per gram of liver ND, not detectable; NA, not assayed.

Table 5—Peroxisomal Enzyme Investigations on Human Cultured Skin Fibroblasts : ^{14}C-Hexadecanol Incorporation Into Newly Synthesized Plasmalogens

	Patient 1 (CHRS)	Patient 2 (NALD)	Zellweger patients	Controls
Dihydroxyacetone phosphate acyltransferase activity (nmol/2 hr/mg protein)	NA	0.67	0.66 ± 0.50 (n = 9)	8.80 ± 2.10 (n = 30)
^{14}C-Hexadecanol incorporation test				
% dpm in P.E.	13.2	11.5	14.8 ± 3.0 (n = 15)	57.3 ± 9.9 (n = 11)
% pPE in P.E.	27.6	46.2	48.9 ± 15.8 (n = 15)	91.4 ± 4.1 (n = 11)
% dpm in P.C.	61.6	57.0	63.1 ± 4.4 (n = 15)	31.9 ± 4.9 (n = 11)
% pPC in P.C.	1.0	1.3	1.4 ± 0.9 (n = 15)	20.3 ± 7.1 (n = 11)
Catalase : % particle bound	<5	<5	< 5 (n = 9)	65 ± 8 (n = 11)

PE, total phosphatidylethanolamine; pPE, plasmalogen phosphatidylethanolamine; PC, total phosphatidylcholine; pPC, plasmalogen phosphatidylcholine; NA, not assayed.

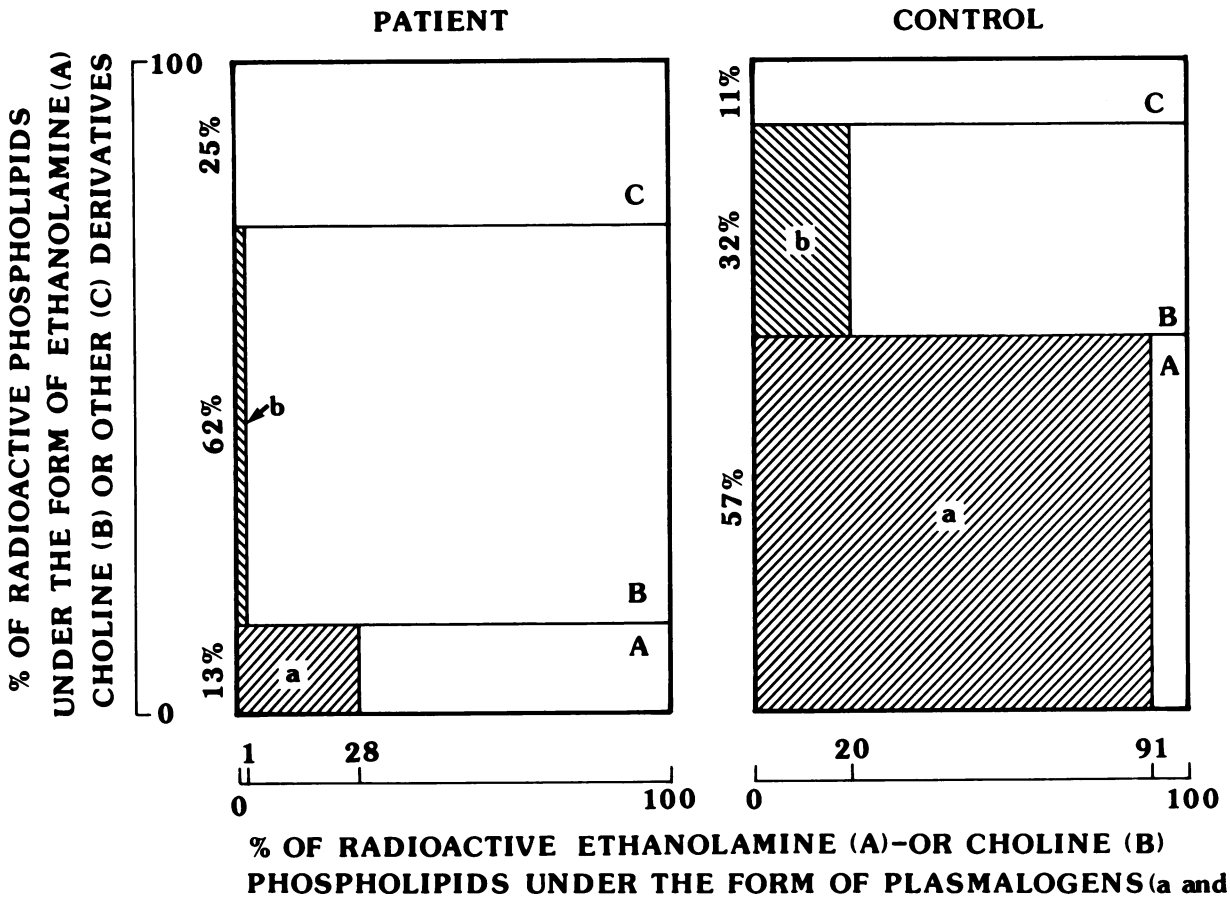

Figure 6—The plasmalogen deficiency in fibroblasts from Patient 1 (CHRS). Comparable results for the NALD patient are evident in the data from Table 5. Total areas in both pathologic and control conditions correspond to the total radioactive phospholipids and represent around 1% of the total radioactive hexadecanol added to the culture medium of the human skin fibroblasts. *Hatched areas* illustrate the content of total labeled phospholipids in (**a**) ethanolamine plasmalogens, and (**b**) choline plasmalogens. When these results are compared with control values and are expressed as the portion of radioactivity incorporated in ethanolamine or choline phospholipids in the form of ethanolamine or choline plasmalogens (% pPE in PE and % pPC in PC on Table 5), the synthesis of choline plasmalogens (1% versus 20%) appears to be affected more than that of ethanolamine plasmalogens (28% versus 91%.) However, this difference is only apparent, and the interpretation of results thus expressed is complicated by the oxidation of a part of the radioactive hexadecanol in palmitate with consecutive labeling of various kinds of lipids, including ether and ester forms. When choline and ethanolamine plasmalogen contents are expressed as a function of total radioactive phospholipids (*hatched areas*) and compared with the corresponding control values, the distinction disappears between anabolism of choline and ethanolamine plasmalogens. Thus, it may be calculated that in our patients the residual capacity of the latter pathway supplied by exogenous hexadecanol is between 6.9% and 11.6% of that of controls.

Discussion

Common clinical features in both patients consist of severe hypotonia, visual and auditory impairment, poor sucking, facial dysmorphism (high forehead, arched palate, epicanthal folds) hepatomegaly, and failure to thrive. These are characteristic findings in children affected by neonatal peroxisomal diseases such as CHRS[1,10,26-28] and NALD.[3,11-13] Clinical similarities and analogous biologic findings in both pathologic entities suggest that in these diseases essentially the same enzyme activities are affected by the basic peroxisomal defect. A remarkable but crucial feature is the absence of recognizable peroxisomes in liver and kidney in the patient with Zellweger's CHRS. In NALD we observed small cytoplasmic bodies resembling the altered peroxisomes that have been described in this disorder.[13] Absence of peroxisomes has also been reported in infantile Refsum's disease (IRD).[29,30] Whether the elevated, but relatively low, concentration of phytanic acid in CHRS and NALD is a reflection of a metabolic defect that is different from IRD, or a function of the younger ages of our patients, is unknown.

Peroxisomal proteins are synthesized by free ribosomes and subsequently incorporated into the membrane or the matrix of preexisting peroxisomes.[31] Catalase, a marker enzyme of peroxisomes, is present but not particle-bound in fibroblasts (see also Wanders et al[16]). This can best be explained by a normal biosynthesis of the enzyme and a lack of its incorporation into peroxisomes. This view is supported by the finding that

in isotonic liver homogenates from Zellweger CHRS or NALD patients, unlike controls, catalase is not sedimentable.[13,32,33] Other peroxisomal enzymes, including D-amino acid oxidase and L-α-hyroxyacid oxidase, are synthesized in CHRS, but not sequestered within peroxisomes.[33]

Peroxisomal β-oxidation is severely affected in these patients. This is evident from 1) the *in vitro* deficiency of palmitoyl-CoA oxidase in liver; 2) the accumulation of very long chain fatty acids in serum; and 3) the markedly reduced production of common bile acids, which reflects a defect in the peroxisomal cleavage of the cholesterol side chain.[34] The latter deficiency leads to the abnormal presence in plasma of di- and trihydroxycoprostanoic acids, bile acid precursors which are normally subjected to shortening in peroxisomes. Another product of sterol metabolism, the C_{29}-dicarboxylic acid, also accumulates in plasma and urine, but not in bile.[20,22] Involvement of a peroxisomal enzyme in the further metabolism of this compound has not been reported, but it is known that peroxisomes can oxidase long-, medium-, and short-chain dicarboxylic acids.[15,35-37] The C_{29}-dicarboxylic acid could be a metabolite formed from the C_{27}-trihydroxycoprostanoic acid when peroxisomal β-oxidation is impaired.[23,38,39] Impaired catabolism of cholesterol to common bile acids as well as deficient oxidation of very long chain fatty acyl-CoA esters could give rise to increased formation of cholesteryl esters of very long chain fatty acids. Trilaminated structures and clefts within lysosomes are believed to represent free or cholesteryl very long chain fatty acids,[40] but our extraction experiments do not confirm this.

Other peroxisomal H_2O_2-generating oxidases are affected differently. Hepatic oxidation of glycolate is impaired in the patients, but activity of D-amino acid oxidase is normal. Similar results have been reported by Wanders et al[16,33] for D-amino acid oxidase. A decreased capacity for plasmalogen synthesis, a characteristic feature in CHRS patients,[16-19,41] is present in both children. The first two enzymes of this pathway are associated with the peroxisomal membrane.[42-44] Activity of the first enzyme, DHAP-AT, was considerably reduced in NALD fibroblasts. Phosphoether lipid biosynthesis was also investigated in CHRS and NALD cultured skin fibroblasts by measuring the incorporation of ^{14}C-hexadecanol into plasmalogens. The deficiency of this enzymatic process (6.0–11.6% of control values) is of the same order of magnitude as that of the first enzyme of plasmalogen biosynthesis (7.6% of residual activity).

Increased amounts of pipecolic acid were found in urine and plasma of the NALD patient. The normal levels of pipecolic acid in our CHRS patient do not rule out disturbances of the metabolism of the N-alkylaminoacid in this patient, as interpretation of these results in the absence of pipecolic acid loading test is hazardous.[45] Relationships between peroxisomes and this imino acid rest on 1) increased serum and urine concentrations of pipecolic acid in many patients with peroxisomal disorders, 2) stimulation of pipecolic acid metabolism in animals treated by clofibrate,[46] a known peroxisome proliferator in rodents, 3) the *in situ* oxidation of pipecolic acid by peroxisomes in histochemical preparations,[47] and 4) the oxidation of pipecolic acid by peroxisome-enriched fractions.[47]

Several mechanisms may be postulated by which the absence or deficiency of membrane-bounding of peroxisomal proteins can lead to multiple enzymatic deficiencies (Figure 7). The absence of a surrounding membrane could result in the exposure of active peroxisomal enzymes to rapid proteolysis and denaturation in the cytosol. Peroxisomes contain their own pool of coenzyme A.[48-50] In the absence of a peroxisomal membrane, dilution of peroxisomal cofactors in the cytosol should lead to decreased efficiency of related enzymatic reactions. Peroxisomes also contain their pool of flavin cofactors in the form of flavorproteins, including D-amino acid oxidase, glycolate oxidase, and fatty acyl-CoA oxidase. One molecule of FAD (or of FMN) is associated with one protein subunit.[51] In peroxisomes, the equilibrium favors the holoenzyme form, since flavins are confined in a limited space. Dilution of the holoenzyme and flavins in the cytosol could increase the proportion of the labile apoenzyme.

Peroxisomes represent a privileged and confined region in which the concentration of the enzyme activity catalyzing the first step of sequence reactions leads to the formation *in loco* of products in concentrations which allow their further optimal utilization as substrates for subsequent enzymatic steps. This occurs during peroxisomal β-oxidation and in the initial steps of the phosphoether lipid biosynthesis. Thus, dilution in cytosol of peroxisomal enzymes would seriously affect the efficiency of peroxisomal sequence reactions.

If the transport of all peroxisomal enzymes into the organelle were blocked, it would be impossible to distinguish peroxisomes from other cytoplasmic vacuoles by morphology or enzyme cytochemistry. Many of the clinical and biochemical consequences of such a defect would be identical to those present in the disorders in which the formation of the peroxisomal membrane was inhibited or degradation of the organelle was enhanced.

The failure to find peroxisomes in electron micrographs and the absence of demonstrable latency of peroxisomal enzymatic activity must be interpreted with

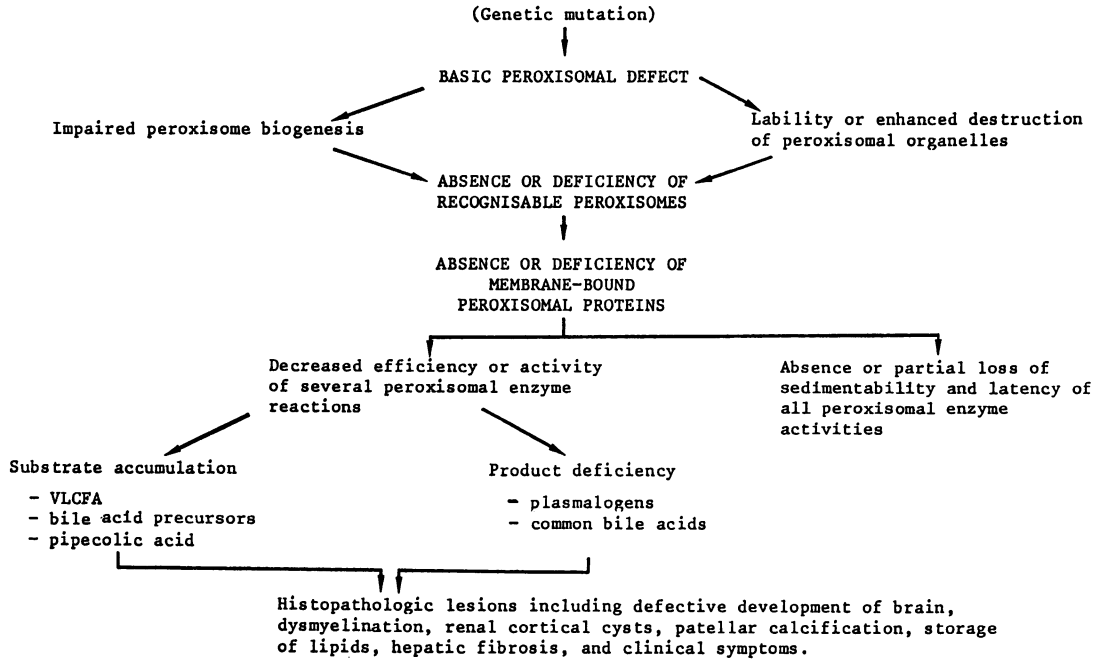

Figure 7—Proposed mechanisms for the pathogenesis of peroxisomal deficiency syndromes. Absence or deficiency of recognizable peroxisomes, which may result from different basic peroxisomal defects, is presented as the central event in these disorders. Similar morphologic, biochemical, biologic, and anatomic and clinical alterations may be found in different diseases.

caution. Such results do not permit the conclusions that peroxisomes are entirely absent. Sparse and altered peroxisomes can escape detection, even in sections stained for catalase; low levels of latent activity cannot be recorded by current assay systems. The small quantity of plasmalogens present in CHRS and NALD patients is more consistent with a marked reduction of peroxisomes than their total absence. The extent to which peroxisomes are reduced in various tissues other than liver and kidney, eg, fibroblasts[53] and intestine,[13,54] may explain the differences in survival, severity of lesions[55,56] and symptoms in CHRS, NALD, and related syndromes such as IRD and rhizomelic chondroplasia punctata.[57]

The fundamental defect (or defects) in CHRS and NALD remains unknown. Whether these are different diseases reflecting distinct underlying pathogenic phenomena or varying degrees of expression of a single entity is uncertain. Another factor that must be considered in the peroxisomal deficiency syndromes is the mitochondrial electron transfer defect in CHRS[1,6,27,35,58-60]; mitochondrial respiration has not been studied in NALD. Whether this phenomenon, which has been ignored in recent studies that have focused entirely on peroxisomes, is of primary significance or secondary to the peroxisomal abnormality is unknown. The metabolic interdependence of mitochondria and peroxisomes has been defined in plants and protozoa; their functional relationship in mammalian systems is still obscure.

Although clofibrate and other hypolipidemic agents induce peroxisomal proliferation and enzymatic activities in rodents, they do not affect monkeys and humans.[61-64] A recently described hypolipidemic agent, 4-([(1,3-benzodioxal)-5-yl]methyl)amino-benzoic acid (DL-040), which induces peroxisome proliferation and increased peroxisomal fatty acid oxidation in primate liver (rhesus monkey),[65] may be of therapeutic value in human peroxisomal disorders.

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