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Lignoceric acid is oxidized in the peroxisome: Implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy

(β oxidation/hexacosanoic acid/catalase/rat liver subcellular fractionation/potassium cyanide/mitochondria)

INDERJIT SINGH*, ANN E. MOSER*, SIDNEY GOLDFISCHER†, AND HUGO W. MOSER*

*John F. Kennedy Institute and Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; and †Department of Pathology and Liver Research Center, Albert Einstein College of Medicine, Bronx, NY 10461

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ABSTRACT **The deficient oxidation and accumulation of very-long-chain fatty acids in the Zellweger cerebro-hepato-renal syndrome (CHRS) and X chromosome-linked adrenoleukodystrophy (ALD), coupled with the observation that peroxisomes are lacking in CHRS, prompted us to investigate the subcellular localization of the catabolism of lignoceric acid (C24:0). Peroxisomal and mitochondrial-rich fractions were separated from rat liver crude mitochondria by sucrose density gradient centrifugation. Enzyme activity for the oxidation of [1-^{14}C]palmitic acid to water-soluble acetate was 2- to 3-fold higher in the mitochondrial than in the peroxisomal-rich fraction whereas [1-^{14}C]lignoceric acid was oxidized at a 2- to 3-fold higher rate in the peroxisomal than in the mitochondrial fraction. Moreover, unlike palmitic acid oxidation, lignoceric acid oxidation was not inhibited by potassium cyanide in either rat liver fractions or human skin cultured fibroblasts, showing that lignoceric acid is mainly and possibly exclusively oxidized in peroxisomes. We also conducted studies to clarify the striking phenotypic differences between CHRS and the childhood form of ALD. In contrast to CHRS, we found normal hepatocellular peroxisomes in the liver biopsy of a childhood ALD patient. In addition, in the presence of potassium cyanide, the oxidation of palmitic acid in cultured skin fibroblasts was inhibited by 62% in control and X chromosome-linked ALD patients compared with 88% in CHRS and neonatal ALD. This differential effect may be related to differences in peroxisomal morphology in those disorders.**

Peroxisomes are now known to have a variety of physiological functions in addition to their previously recognized role in the formation and reduction of hydrogen peroxide. More than 40 enzymes have now been localized to this organelle (1). These include those playing a major role in the β oxidation of fatty acids, particularly long-chain-length fatty acids (2, 3). It has been estimated that 30% of palmitate (2) and 50–67% of erucic acid (C22:1) (3) oxidation takes place in the peroxisomes. Among other peroxisomal enzymes are those involved in certain steps in the biosynthesis of glycerolipids and glycerol/ether lipids (4) and of bile acids (5), D-amino acid oxidases, the enzymes of the glyoxylate cycle, and those concerned with ethanoxanthine and urate (6) metabolism. Recently, 3-hydroxy-3-methylglutaryl-Co A reductase has also been localized in the peroxisome (7). The recognition that peroxisomes have a wide range of physiological functions, coupled with the demonstration that they are absent or diminished in certain disease states, has led to the concept of a newly recognized category of diseases, namely, disorders of the peroxisome (8, 9).

The Zellweger cerebro-hepato-renal syndrome (CHRS) is the most well-known example of the peroxisomal disorders. CHRS is an autosomal recessive disorder characterized by profound abnormalities of neuronal migration (10, 11), enlarged and malfunctioning liver, renal cortical cysts, abnormal calcification, retinal degeneration, and multiple congenital anomalies (12). Repeated attempts to demonstrate peroxisomes in the liver and kidney of CHRS patients by cytochemical techniques have failed (13–15), and this feature is now a required diagnostic criterion (16). In addition, CHRS patients show certain biochemical abnormalities, such as greatly diminished levels of plasmalogens (17) and defects in bile acid synthesis (18), that have been attributed to defective function of peroxisomal enzymes.

The present studies of lignoceric acid (*n*-tetracosanoic acid, C24:0) metabolism were prompted by our observation that striking elevation of plasma and tissue levels of very-long-chain (>22) fatty acids—particularly hexacosanoic acid—are present in all patients with CHRS (19). These acids are normal constituents of myelin sphingolipids, and elevated levels are also observed in all patients with a somewhat more common group of disorders, the adrenoleukodystrophies (ALD) (20, 21). Unlike CHRS, the childhood form of ALD and an adult form referred to as adrenomyeloneuropathy (AMN) (22) are X chromosome-linked (23) and defects are confined to nervous system white matter, adrenal cortex, and testis. The rarer neonatal form of ALD shows an autosomal recessive mode of inheritance and resembles CHRS (24, 25). Table 1 lists the main points of distinction between these disorders.

We have shown that the elevated very-long-chain fatty acid levels in patients with CHRS and all forms of ALD are due to defects in the degradation of these acids (29, 30), whereas palmitate degradation proceeds normally. Since previous studies of the subcellular localization of fatty acid oxidation had focused on fatty acids with a carbon chain length of 22 or fewer (2, 3), we undertook the present study to ascertain the subcellular localization of very-long-chain fatty acid oxidation and to determine its relationship to the pathogenesis of these disorders. In view of the striking difference between the tissue-restricted abnormalities in childhood ALD and AMN, and the multiple defects in CHRS and neonatal ALD, we also examined the morphology and histochemical properties of peroxisomes in a liver biopsy specimen of a patient with childhood ALD.

This report shows that in rat liver and cultured human skin fibroblasts lignoceric acid, unlike palmitic (*n*-hexadecanoic) acid, is oxidized mainly, and possibly exclusively, in peroxisomes and that it is this system that is defective in ALD and CHRS. In sharp contrast to CHRS, liver peroxisomes in a childhood ALD patient were normal in size and number

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Abbreviations: CHRS, cerebro-hepato-renal syndrome; ALD, adrenoleukodystrophy(ies); AMN, adrenomyeloneuropathy.

Table 1. Main clinical features of the three forms of ALD and the Zellweger syndrome

Clinical finding	Childhood ALD	AMN	Neonatal ALD	Zellweger syndrome
Most likely age for first symptoms, yr	4–8	20–30	0–1	0
Neurological symptoms	Dementia, impaired vision and hearing, paralysis	Progressive leg stiffness and paralysis	Seizures, severe retardation, hypotonia	Hypotonia, severe retardation, seizures
Mode of inheritance	Sex-linked recessive	Sex-linked recessive	Autosomal recessive	Autosomal recessive
Number and appearance of liver peroxisomes	Normal (Fig. 3)	—	Diminished or absent (26, 27)	Absent (13)
Pipecolic acid level	Normal*	Normal*	Increased*	Increased (28)
Plasmalogen synthesis	—	—	—	Impaired (17)
Bile acid synthesis	—	—	—	Impaired (18)

*R. I. Kelley, personal communication.

when studied by cytochemical techniques. We postulate that in CHRS the primary defect involves the assembly of peroxisomes whereas in childhood ALD and AMN defective function is confined to peroxisomal degradation of very-long-chain fatty acids.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitic acid (53 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. [1-^{14}C]Lignoceric acid (51.1 mCi/mmol) was synthesized according to Hoshi and Kishimoto (31). ATP and CoASH were obtained from P-L Biochemicals. Malate, α-cyclodextrin, FAD, KCN, NAD, and L-carnitine were purchased from Sigma.

Fractionation of Liver Homogenate. Rats (Sprague–Dawley) were killed by decapitation and about 3 g of liver was homogenized in 30 ml of 0.25 M sucrose/0.1% ethanol in a glass homogenizer fitted with a Teflon pestle. A single up-and-down stroke was used for homogenization of the minced tissue, and the homogenate was centrifuged at 800 × *g* for 10 min to remove cell debris and nuclei. The supernatant was centrifuged at 12,500 × *g* for 20 min to sediment the crude mitochondrial fraction and then at 20,000 × *g* for 30 min and at 105,000 × *g* for 60 min to obtain heavy and light microsomal fractions, respectively. The crude mitochondrial fraction was suspended in 0.25 M sucrose/0.1% ethanol by a single up-and-down homogenization and further fractionated by discontinuous gradient centrifugation in which the following sucrose gradients were used (from bottom to top): 8 ml of 54.6%, 8 ml of 50%, 8 ml of 45.8%, 6 ml of 38.6%, and 6 ml of 23.2% in 5 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer, pH 7.4/0.1% ethanol. Centrifugation was carried out for 90 min at 25,000 × *g* at 4°C with a Beckman SW-27 rotor. Eight fractions were collected, one for the solution of each density (except 23.2%) and one for each interface between solutions of different densities. The fractions were numbered from top to bottom and diluted with cold water before centrifugation at 20,000 × *g* for 30 min, and the residue was then suspended in 0.25 M sucrose. Each fraction was examined for the following marker enzymes: cytochrome *c* oxidase and glutamate dehydrogenase for mitochondria, catalase and urate oxidase for peroxisomes, cytochrome reductase for endoplasmic reticulum, and arylsulfatase A for lysosomes.

Fatty Acid Oxidation to Acetate (Water-Soluble Products). The reaction mixture (total vol, 0.5 ml) contained 12 μM [1-^{14}C]fatty acid coated on Celite (32), 20 mM Mops buffer, pH 7.0/30 mM KCl/1 mM $MgCl_2$/8.5 mM ATP/0.25 mM NAD/0.17 mM FAD/2.5 mM L-carnitine/0.08 mM CoASH, and 1 mg of α-cyclodextrin. The reaction was started by the addition of 200–400 μg of protein and stopped with 0.5 ml of ice-cold 0.6 M perchloric acid. After centrifugation, the supernatant was transferred to another tube and partitioned by the procedure of Folch *et al.* (33), and the radioactivity in the upper layer was measured. In this report, these values are used as an index of the amount of fatty acid oxidized to acetate.

Fatty Acid Oxidation to CO_2. The assay conditions were identical to those reported previously (29, 30). The reaction was started by addition of the enzyme and stopped by the transfer of 0.25 ml of 1.5 M H_2SO_4 from the side-arm tube. The radioactive CO_2 was collected on a glass fiber filter paper soaked in alkali, suspended from the top of the tube equipped with a side arm and a stopper.

Marker Enzymes. Catalase was measured according to Bergmeyer (34); urate oxidase and glutamate dehydrogenase, according to the procedure of Leighton *et al.* (35); cytochrome *c* oxidase, according to the procedure of Omura and Takesue (36); and proteins, by the method of Miller (37).

Patients and Tissue Samples. Patients in the various disease categories included in this study have been described (19, 20, 24). Liver biopsies were carried out by the percutaneous punch technique. Approval of the Human Investigations Committees and informed parental consent had been obtained. For the cytochemical demonstration of peroxisomes, we used the method of Roels and Goldfischer (38) to process a portion of the biopsy specimen for cytochemistry and electron microscopy. Fixation was for 1 hr at 4°C and overnight at room temperature in 4% Formol/calcium in 0.1 M cacodylate buffer (pH 7.4). For the demonstration of catalase, a peroxisomal marker enzyme, 40-μm nonfrozen sections were incubated for 60 and 90 min at 42°C in a 3,3'-diaminobenzidine medium (38) and postfixed for 1 hr with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) and potassium ferrocyanide. The specimen was dehydrated in graded ethanol and propylene oxide and embedded in Epon/Araldite. Thin sections were counterstained with uranyl and lead acetates and examined in a Zeiss 109R electron microscope. Fibroblasts were grown in a 5% carbon dioxide atmosphere at 37°C in Eagle's minimum essential medium plus Earle's salts and 13% fetal calf serum. The cells were harvested 3 to 4 days after confluence by treatment with 0.2% trypsin. The cells were homogenized by 15 passes with a hand-driven Teflon-coated glass homogenizer in 64 mM sodium chloride.

RESULTS

Subcellular Distribution of Fatty Acid Oxidation Activity in Rat Liver. Studies of the lignoceric acid oxidation in different subcellular fractions prepared by differential centrifugation show that recovery of enzyme activity in nuclear, mitochondrial/peroxisomal, heavy microsomal, light microsomal, and cytosolic fractions was 12%, 57%, 0.4%, 0.3%, and 0.7%, respectively. The mitochondrial fraction was used as the starting material for the more detailed studies described in Table 2, which shows a comparison of the oxidation of [1-^{14}C]palmitic and [1-^{14}C]lignoceric acids in eight subcellular subfractions obtained by discontinuous sucrose density gra-

Table 2. Subcellular localization of fatty acid oxidation activity in rat liver

Fraction	Activity*, units/mg per min				Oxidation, pmol/mg per hr			
	Peroxisomal enzyme		Mitochondrial enzyme		Palmitic acid		Lignoceric acid	
	Catalase	Urate oxidase	Glutamate dehydrogenase	Cytochrome *c* oxidase	Acetate	CO_2	Acetate	CO_2
1	187	3.1	ND	0.09	230	ND	0.2	ND
2	99	4.0	0.04	0.01	860	20	8.3	0.9
3	249	2.6	0.09	0.13	830	50	8.4	1.08
4	200	7.3	0.16	0.73	2520	90	27.3	1.65
5	602	18.5	0.14	0.27	930	30	24.2	1.3
6	917	36.7	0.08	0.04	1112	10	47.3	ND
7	1384	56.1	0.05	0.02	1200	ND	41.2	ND
8	1529	51.3	ND	0.01	1200	ND	68.8	ND

ND, not detected.

*One unit corresponds to the amount of enzyme catalyzing the following reactions: catalase, degradation of 1 μmol of H_2O_2/min; urate oxidase, oxidation of 1 μmol of urate/min; glutamate dehydrogenase, reduction of 1 μmol of NAD/min; cytochrome *c* oxidase, oxidation of 1 μmol of cytochrome *c*/min.

dient centrifugation of this fraction. These subfractions were characterized with respect to their content of mitochondria and peroxisomes, based on the activity of enzyme markers. Fractions 7 and 8 are clearly enriched in peroxisomes (Table 2). Fraction 4 (and to a lesser extent fractions 3 and 5) contains the bulk of the mitochondria, but there is some overlap among fractions that is reflected in their oxidative capabilities.

Palmitic acid is known to be oxidized to acetate in both mitochondria and peroxisomes. In mitochondria, the acetate produced is further metabolized to CO_2 via the tricarboxylic acid cycle whereas, in peroxisomes, which lack tricarboxylic acid cycle enzymes, no CO_2 is produced. When [1-^{14}C]palmitic acid was incubated with each of the eight subcellular fractions, maximal rates of oxidation to acetate and CO_2 were observed in fraction 4, which is highly enriched in mitochondria. Acetate was also produced in fractions 7 and 8, which are enriched in peroxisomes, but at less than half the rate observed in fraction 4. The absence of $^{14}CO_2$ in these fractions indicates that there was little contamination with mitochondria. In contrast, the rate of acetate formation from lignoceric acid was highest in fractions 7 and 8, about twice the rate measured in fraction 4. The small amount of CO_2 detected in fractions 3–5 is presumably due to oxidation of [1-^{14}C]lignoceric acid to acetate by the peroxisomes contaminating this fraction and subsequent oxidation of the acetate to CO_2 by mitochondria.

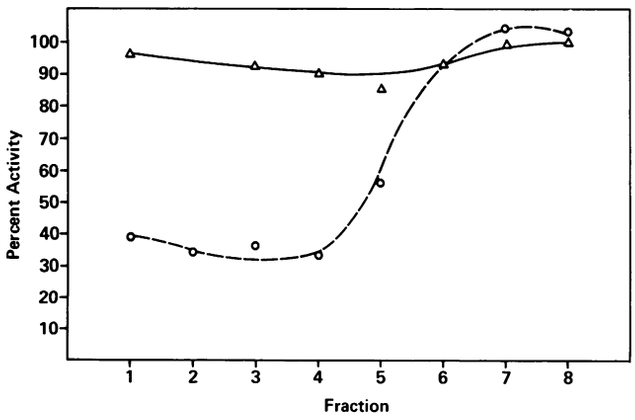

FIG. 1. Effect of KCN on fatty acid oxidation to acetate by subcellular fractions from rat liver. The oxidation of palmitic (○) and lignoceric (△) acids was compared in subcellular fractions prepared from liver crude mitochondrial fractions by sucrose density gradient centrifugation. Oxidation was measured in the absence and presence of 2 mM KCN. Results are expressed as percentage of activity in the absence of KCN.

These results suggested that lignoceric acid was oxidized mainly in peroxisomes. To verify this possibility, we examined the effect of KCN on oxidation of both lignoceric and palmitic acids in each of the subcellular fractions. KCN inhibits fatty acid oxidation in mitochondria by inactivating cytochrome *a* and cytochrome a_3 but has no effect on peroxisomal oxidation. As shown in Fig. 1, the oxidation of lignoceric acid was not affected by KCN in any fraction whereas palmitic acid oxidation was significantly inhibited in fractions enriched in mitochondria (3 and 4) and unaffected in the peroxisome-rich fractions 7 and 8. This leads to the conclusion that oxidation of lignoceric acid takes place mainly and possibly exclusively in peroxisomes.

Oxidation of Fatty Acids by Cultured Skin Fibroblasts. To extend our studies on the site of lignoceric acid oxidation, we next examined the effect of KCN (which inhibits mitochondrial fatty acid oxidation) on the oxidation of fatty acids by homogenates of cultured skin fibroblasts. In agreement with previous results (29, 30), the oxidation of lignoceric acid in fibroblasts from childhood ALD, neonatal ALD, and CHRS was reduced, respectively, to 23%, 5%, and 3% of control (Table 3). Moreover, similar to our observations on rat liver, KCN had no effect on the oxidation of lignoceric acid in any fibroblast preparation, indicating that, in fibroblasts, lignoceric acid is also oxidized in peroxisomes. As expected, however, KCN inhibited the oxidation of palmitic acid in cultured fibroblasts (Fig. 2). Interestingly, the degree of inhibition varied. In childhood ALD and control cells, 2 mM KCN reduced palmitate oxidation to 40% of the uninhibited level. In CHRS and neonatal ALD fibroblasts, the activity was reduced to 12% of the uninhibited level, suggesting that the peroxisomal defect in childhood ALD differs from that in CHRS and neonatal ALD.

Structure of Liver Peroxisomes in Childhood ALD. Liver peroxisomes in the childhood ALD patient were abundant in number and normal in size, ranging from 0.15 to 0.75 μm in diameter (Fig. 3*a*), and cytochemical catalase activity was

Table 3. Effect of KCN on oxidation of [1-^{14}C]lignoceric acid by cultured skin fibroblasts

Fibroblast sample	Lignoceric acid oxidized, cpm/hr per mg of protein	
	Without KCN	With 2 mM KCN
Control	5040 ± 2090 (5)	5710 ± 2360 (5)
Childhood ALD	1150 ± 260 (6)	1080 ± 236 (6)
Neonatal ALD	231 ± 178 (6)	264 ± 227 (6)
Zellweger	143 ± 97 (6)	130 ± 54 (6)

Numbers in parentheses refer to numbers of samples representing five control patients and three patients in each disease category.

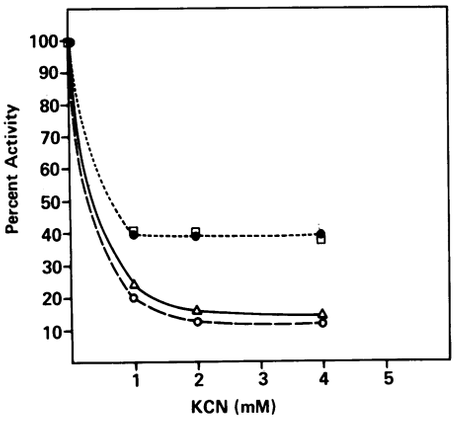

FIG. 2. Effect of KCN on palmitic acid oxidation to acetate by cultured skin fibroblasts. Oxidation was measured in homogenates of fibroblasts as described in *Materials and Methods* except that the amount of KCN was varied as indicated. ●, Control; □, childhood ALD; △, neonatal ALD; and ○, Zellweger fibroblasts. Each point represents the mean of four samples. Results are expressed as percentage of activity in the absence of KCN.

also normal (Fig. 3*b*). This is in striking contrast to what has been documented previously in CHRS and neonatal ALD, where liver peroxisomes have been reported to be either lacking or greatly reduced in size and number (13–15, 26, 27).

DISCUSSION

In contrast to the oxidation of palmitic acid, which is oxidized mainly in the mitochondrion, we find that the oxidation of lignoceric acid takes place mainly in the peroxisomes. This conclusion is based on results of subcellular fractionation studies and on the effects of adding cyanide, which inhibits mitochondrial but not peroxisomal oxidation. As shown in Table 2, the oxidation of lignoceric acid to water-soluble compounds corresponded to the distribution of peroxisomal marker enzymes. Furthermore, cyanide failed to inhibit lignoceric acid oxidation in rat liver subcellular fractions, a liver biopsy sample, and cultured human fibroblasts (Table 3). To the extent that results from these tissue samples can be generalized to other tissues, we conclude that oxidation of lignoceric acid takes place mainly, and possibly exclusively, in the peroxisomes.

In addition to increased levels of very-long-chain fatty acids, three biochemical abnormalities have now been shown to be associated with CHRS: diminished levels of alkoxyphospholipids (17), increased levels of pipecolic acid (28), an intermediate in the lysine degradative pathway, and abnormalities of bile acid composition (18). The changes in alkoxyphospholipids and of very-long-chain fatty acids have now been linked to the peroxisome and, coupled with the striking morphological and cytochemical abnormalities, provide firm evidence that CHRS is a disorder of the peroxisomes. It must be cautioned, however, that mitochondrial abnormalities have also been described in CHRS (13, 15). While a recent report concludes that the mitochondrial abnormalities may be secondary to a peroxisomal defect (39), at this time it is not fully established that the primary defect in CHRS is confined to the peroxisome.

The defect in very-long-chain fatty acid oxidation in CHRS and ALD is not complete: Residual activity is between 3% and 25% of control. Table 3 shows that this residual activity is not inhibited by cyanide, and we conclude that it is peroxisomal and not mitochondrial. It is of interest that while in both CHRS and ALD there is accumulation of fatty acids with 24 or more carbons, the levels of behenic acid

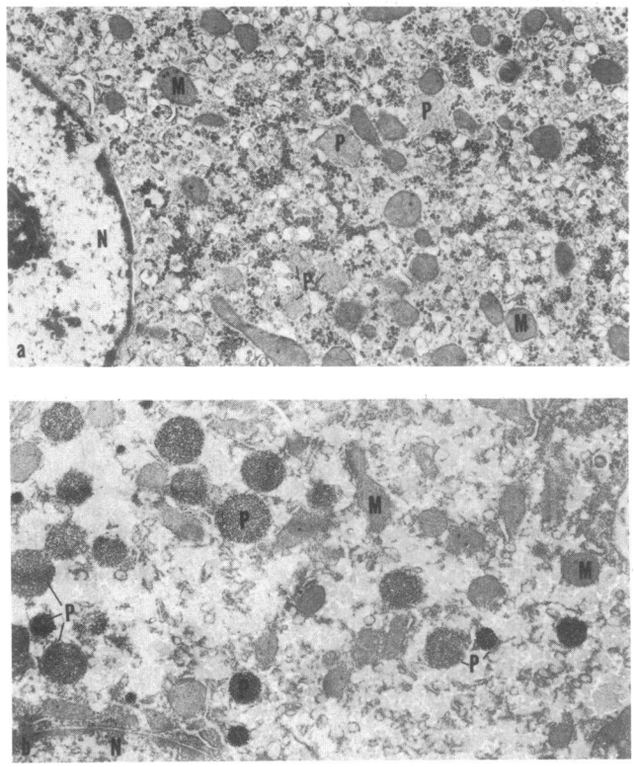

FIG. 3. Electron micrographs of a percutaneous liver biopsy sample from a patient with childhood ALD processed for morphology (*a*) and for cytochemical demonstration of catalase, a peroxisomal marker enzyme (*b*) (38). (*a*) Hepatocellular peroxisomes (P), with a characteristic flocculent matrix delimited by a single membrane, are abundant. (*b*) The contrast of peroxisomes (P) is enhanced by deposition of the osmophilic reaction product during incubation for catalase reactivity. The density of the product is inversely proportional to the size of the peroxisome. M, mitochondria; N, nucleus. Samples were fixed with formalin/cacodylate (38) and postfixed with an osmium/ferrocyanide mixture (*a*) or with osmium phosphate (*b*). (Counterstain, uranyl and lead acetates; ×11,100.)

(C22:0), erucic acid, and fatty acids with shorter chain lengths are not increased. Osmundsen (3) has shown that in rat liver the rate of peroxisomal oxidation of erucic acid ranges from 100% to 300% of that in mitochondria. These findings suggest that peroxisomal oxidation of long chain and very-long-chain fatty acids may involve more than one enzyme and that, as discussed below, these enzymes are defective to various degrees in CHRS and ALD.

There are major phenotypic differences between CHRS and X chromosome-linked forms of ALD (childhood ALD and AMN). In the latter disorders, the clinical and morphological abnormalities are confined to the adrenal cortex, testis, and nervous system white matter. We now report that, unlike CHRS, peroxisomes have a normal morphology in childhood ALD. Although CHRS and X chromosome-linked ALD are both peroxisomal disorders, we postulate that they have different disease mechanisms. We suggest that in childhood ALD a single enzyme defect leads to impaired oxidation of very-long-chain fatty acids whereas, in CHRS and probably also in neonatal ALD (27, 28), there is a defect in the formation of peroxisomes, and that this is associated with a deficiency of several peroxisomal enzymes. The results shown in Fig. 2 support this hypothesis; Cyanide inhibits palmitic acid oxidation in normal and childhood ALD fibroblasts to the same extent, whereas in CHRS and neonatal ALD it causes a more severe inhibition. We postulate that the peroxisomal enzymes that oxidize palmitate are distinct from those that oxidize lignoceric acid and that in childhood

ALD the defect is restricted to the lignocerate enzyme, whereas in CHRS and neonatal ALD both peroxisomal enzymes are involved. Additional evidence favoring a more widespread defect in CHRS is that most CHRS patients show increased levels of pipecolic acid (28) while pipecolic acid levels are normal in childhood ALD (R. I. Kelley, personal communication). The defect of very-long-chain fatty acid oxidation in CHRS also is more severe than in childhood ALD, as evidenced by significantly greater elevations of very-long-chain fatty acid levels (19) and a greater impairment in the capacity to oxidize [1-^{14}C]lignoceric acid (Table 2).

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