Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases

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Abstract The acyl-CoA ligases convert free fatty acids to acyl-CoA derivatives, and these enzymes have been shown to be present in mitochondria, peroxisomes, and endoplasmic reticulum. Because their activity is obligatory for fatty acid metabolism, it is important to identify their substrate specificities and subcellular distributions to further understand the cellular regulation of these pathways. To define the role of the enzymes and organelles involved in the metabolism of very long chain (VLC) fatty acids, we studied human genetic cell mutants impaired for the metabolism of these molecules. Fibroblast cell lines were derived from patients with X-linked adrenoleukodystrophy (X-ALD) and Zellweger's cerebro-hepato-renal syndrome (CHRS). While peroxisomes are present and morphologically normal in X-ALD, they are either greatly reduced in number or absent in CHRS. Palmitoyl-CoA ligase is known to be present in mitochondria, peroxisomes, and endoplasmic reticulum (microsomes). We found enzyme-dependent formation of lignoceroyl-CoA in these same organelles (specific activities were 0.32 ± 0.12, 0.86 ± 0.12, and 0.78 ± 0.07 nmol/h per mg protein, respectively). However, lignoceroyl-CoA synthesis was inhibited by an antibody to palmitoyl-CoA ligase in isolated mitochondria while it was not inhibited in peroxisomes or endoplasmic reticulum (ER). This suggests that palmitoyl-CoA ligase and lignoceroyl-CoA ligase are different enzymes and that mitochondria lack lignoceroyl-CoA ligase. This conclusion is further supported by data showing that oxidation of lignoceric acid was found almost exclusively in peroxisomes (0.17 nmol/h per mg protein) but was largely absent from mitochondria and the finding that monolayers of CHRS fibroblasts lacking peroxisomes showed a pronounced deficiency in lignoceric acid oxidation in situ (1.8% of control). In spite of the observation that lignoceroyl-CoA ligase activity is present on the cytoplasmic surface of ER, our data indicate that lignoceroyl-CoA synthesized by ER is not available for oxidation in mitochondria. This organelle plays no physiological role in the β-oxidation of VLC fatty acids. Furthermore, the normal peroxisomal oxidation of lignoceroyl-CoA but deficient oxidation of lignoceric acid in X-ALD cells indicates that cellular VLC fatty acid oxidation is dependent on peroxisomal lignoceroyl-CoA ligase. These studies allow us to propose a model for the subcellular localization of various acyl-CoA ligases and to describe how these enzymes control cellular fatty acid metabolism. —**Lazo, O., M. Contreras, Y. Yoshida, A. K. Singh, W. Stanley, M. Weise, and I. Singh.** Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases. *J. Lipid Res.* 1990. **31:** 583–595.

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The peroxisomal disorders are a newly recognized group of genetic diseases (1–5). These disorders can be divided into two groups: one with a generalized defect affecting peroxisomal assembly which leads to multiple enzymatic deficiencies and the other with specific enzyme defects (1–5). The oxidation of very long chain (VLC) fatty acids ($>C_{22}$) has been shown to be a peroxisomal function (6) and the accumulation of these VLC fatty acids in X-linked adrenoleukodystrophy (X-ALD) (7) is due to a defect in their oxidation (6, 8–15) resulting from impaired activity of peroxisomal lignoceroyl-CoA ligase (16–20). Adrenoleukodystrophy (X-linked) is representative of a peroxisomal disorder where accumulation of VLC fatty acids is due to a defect of a single peroxisomal enzyme, peroxisomal VLC acyl-CoA ligase. Zellweger's cerebro-hepato-renal syndrome (CHRS), is characterized by a lack of peroxisomes (21, 22) and the observed accumulation of VLC fatty acids is interpreted to be due to the absence of a peroxisomal β-oxidation pathway (8).

While studying the oxidation of different derivatives of lignoceric acid, we surprisingly found in homogenates of CHRS cultured skin fibroblasts that lignoceroyl-CoA was oxidized at an efficient rate of 55% of normal activity in contrast to the deficient oxidation of lignoceric acid (14.3% of the normal activity) (23). Although this suggests that the activated fatty acid, lignoceroyl-CoA, may be oxidized in an organelle other than the peroxisomes (23) because functional peroxisomes are absent in CHRS (8, 22–25), it does not explain the pathognomonic accumulation of VLC fatty acids in X-ALD and CHRS. To resolve this question, a better understanding of the subcellular sites for VLC fatty acid metabolism and the enzymes in the control of these pathways is necessary. In this study, therefore, we utilized cell cultures derived from X-ALD and CHRS patients for both in vitro and in situ experiments to elucidate the physiological mechanisms for the cellular metabolism of VLC fatty acids.

Abbreviations: VLC, very long chain; X-ALD, X-linked adrenoleukodystrophy; CHRS, Zellweger's cerebro-hepato-renal syndrome.

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MATERIALS AND METHODS

Reagents

Fetal calf serum, trypsin, and tissue culture media were from GIBCO Grand Island, NY. [1-^{14}C]Palmitic acid (58.7 mCi/mmol) and [K^{14}CN] (52.0 mCi/mmol) were from New England Nuclear, Boston, MA, and Nycodenz was purchased from Accurate Chemical and Scientific Corp., Westbury, NY. L-Malic acid, FAD, NAD, NADP, NADPH, L-carnitine, α-cyclodextrin, glucose-1-6-diphosphate, glucose 6-phosphate dehydrogenase, histidine, *p*-nitrophenol-2-acetamido-2-deoxy-β-D-glucopyranoside, cytochrome c, bovine serum albumin, and fatty acid-free bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. ATP and CoASH were obtained from P-L Biochemicals, Milwaukee, WI. Protein A-Sepharose CL-4B was obtained from Pharmacia LKB Biotechnology Inc., Piscataway, N. J. [1-^{14}C]Lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with [K^{14}CN] as described (26) and [1-^{14}C]lignoceroyl-CoA was synthesized as previously described (27).

Cell cultures and subcellular fractionation of skin fibroblasts

Fibroblast cell lines from X-ALD and CHRS patients and control cell lines were cultured as previously described (16). Cells from 30 or more confluent flasks (75 cm^2) were collected by centrifugation and incubated for 1 h as a suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum at 37°C. After centrifugation, cell pellets were washed with homogenization medium and subfractionated by differential centrifugation and isopycnic density gradient utilizing Nycodenz as previously described (20). The gradient fractions were analyzed for the following subcellular markers: cytochrome c oxidase for mitochondria (28), NADPH cytochrome c reductase for ER (29), catalase for peroxisomes (30), N-acetyl-β-glucosaminidase for lysosomes (31), and phosphoglucomutase for the cytosol (32). Protein concentrations were determined by the procedure of Bradford (33).

Morphological examination of subcellular organelles from fibroblast gradients

Mitochondrial, ER, and peroxisomal fractions were pelleted by centrifugation and pellets were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C. Samples were post-fixed with 1% osmium tetroxide in cacodylate buffer and then dehydrated in ethyl alcohol and propylene oxide. Samples were then infiltrated with Embed 812 and polymerized at 60°C for 36 h. Thin sections were stained with uranyl acetate and lead acetate and examined by electron microscopy.

Enzyme assay for the the activation and oxidation of [1-^{14}C]-labeled fatty acids in fibroblast homogenates and subcellular organelles

Acyl-CoA ligase activity was measured by our previously reported procedure (34). Enzyme activity for the oxidation of [1-^{14}C]-labeled fatty acid to acetate (water-soluble product) was measured by a modification (20) of the previously described procedure (6). The fatty acid substrate was solubilized with α-cyclodextrin and was added to the assay medium. The amount of radioactivity in the upper phase is an index of the amount of [1-^{14}C]-labeled fatty acid oxidized to acetate. In the case of [1-^{14}C]lignoceroyl-CoA oxidation, ATP, $MgCl_2$, and CoASH were omitted from the assay medium.

Fatty acid oxidation in fibroblast monolayers

Experiments to measure fatty acid oxidation as release of $^{14}CO_2$ from fibroblast monolayers were done as described by Rizzo et al. (12). Fibroblast monolayers were washed with Hank's balanced salt solution (HBSS) and incubated with 5 ml of DMEM containing a [1-^{14}C]-labeled fatty acids (50 μM for palmitic acid, 4 μM for lignoceric acid, and 6 μM for lignoceroyl-CoA) bound to fatty acid-free bovine serum albumin in 75-cm^2 flasks fitted with KOH-wetted cigarette filters to collect atmospheric CO_2. Flasks were slowly shaken at 37°C for 3 h and the reaction was stopped with an injection of 0.2 ml of 4.0 M H_2SO_4 into the medium. Flasks were then shaken for 1 h at 4°C. Each filter was transferred into a scintillation vial and the radioactivity was measured. Cells from parallel flasks were collected for protein determination. Lignoceric and palmitic acids and lignoceroyl-CoA were solubilized with fatty acid-free bovine serum albumin using the procedure described by Tsuji et al. (13) except that we used DMEM as the suspension solution.

Enzyme assays and the effect of antisera to palmitoyl-CoA ligase on acyl-CoA ligase and fatty acid oxidation activities

Activation (34) and oxidation (20) of fatty acid were measured according to procedures previously described. Antisera raised against purified palmitoyl-CoA ligase (35) was used to inhibit the peroxisomal palmitoyl-CoA ligase as follows. Antisera (1–2 mg of protein) were incubated with 90–120 μg of peroxisomes, mitochondria, or ER solubilized with 0.05% Triton X-100 in 20 mM MOPS-HCl buffer, pH 7.8, at 4°C in a total volume of 0.5 ml. At the end of 1 h, 100 μl of a 15% suspension of Protein-A-Sepharose was added and incubation was continued for another 40 min with constant shaking. The mixture was centrifuged for 10 min at 2,000 rpm in a microfuge, and acyl-CoA ligase and fatty acid oxidation activities were measured in the supernatant.

RESULTS

Specific activity of oxidation and activation of fatty acids in fibroblast homogenates

The specific activities of marker enzymes for different organelles were similar in homogenates of control, X-ALD, and CHRS fibroblasts (**Table 1**). The recovery of marker enzymes and the enzymes for the activation and oxidation of fatty acids in the post-nuclear fraction from control, X-ALD, and CHRS were 70 to 89% except that phosphoglucomutase was 97%. The specific enzyme activities for the activation and oxidation of palmitic acid were also similar in homogenates from control, X-ALD, and CHRS fibroblasts. The rates of oxidation of lignoceric acid were significantly lower in both CHRS (<14.3% of control) and X-ALD (40% of the control). The oxidation of lignoceroyl-CoA was normal in X-ALD because this disorder is characterized by a single enzyme deficiency affecting only the peroxisomal activation of lignoceric acid (20). However, in CHRS cells, we unexpectedly found high levels of oxidation of lignoceroyl-CoA (55% of control), suggesting that lignoceroyl-CoA may be oxidized in an organelle other than peroxisomes.

Distribution of marker enzymes for different organelles and the fatty acid activation and oxidation enzymes in isopycnic gradients of post-nuclear fractions from X-ALD, CHRS, and control fibroblasts

Subcellular organelles from cultured skin fibroblasts were prepared according to our newly developed procedure (20 and **Fig. 1A and B**). This method permits a resolution of subcellular organelles from cultured skin fibroblasts better than that of previously reported procedures (19, 36–39) and equivalent to that reported recently by Santos et al. (40) using a customized rotor. We found that the suspension of the cell pellet in tissue culture medium for 1 h prior to homogenization enhanced resolution and recovery of subcellular organelles (Fig. 1A and B). This treatment probably permits cell membranes to recover from the trypsin treatment and may promote a uniform disruption of cells during homogenization. The relative specific activities of marker enzymes for different subcellular organelles in mitochondrial, ER, and peroxisomal peaks and the percent contamination of these fractions by other organelles are shown in **Table 2**. The electron micrographs of gradient peaks containing ER, peroxisomes, or mitochondria (**Fig. 2**) support the biochemical data (Table 2) regarding the purity of these organelles.

To examine the role of different subcellular organelles in the metabolism of palmitic and lignoceric acids, we studied the activation and oxidation of these fatty acids in these organelles from control, X-ALD, and CHRS fibroblasts. Palmitic acid activation was found distributed to regions in our gradients identified by marker enzymes for peroxisomes, mitochondria, and ER (Fig. 1B). Based on peak areas in averaged gradient profiles (Fig. 1B), 12.9, 22.6, and 64.5% of the total activity recovered from the control gradient were found in the respective regions for the three organelles (**Table 3**). For palmitic acid oxidation, most of the activity was localized in gradient regions corresponding to markers for peroxisomes (45.4%) and mitochondria (41.0%) (Fig. 1B and Table 3). The distribution of palmitic acid activation and oxidation activities was similar in control and X-ALD cells. The distribution of lignoceric acid activation in control was essentially bimodal, with the major activity co-sedimenting with the

TABLE 1. Specific enzyme activities of marker enzymes and activities for oxidation and activation of palmitic and lignoceric acids in fibroblast homogenates

	Specific Activities in Homogenates		
	Control	CHRS	X-ALD
	mU/mg protein		
Catalase	5.5 ± 1.0	5.3 ± 1.1	5.1 ± 1.2
Cytochrome c oxidase	0.9 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
NADPH cytochrome c reductase	4.3 ± 0.7	4.8 ± 0.9	4.3 ± 0.9
N-Acetyl-β-glucosaminidase	144.7 ± 35.1	162.0 ± 38.4	151.6 ± 29.2
Phosphoglucomutase	103.1 ± 16.4	105.2 ± 12.9	97.1 ± 12.3
	nmol/h per mg protein		
Palmitoyl-CoA ligase	8.2 ± 1.3	8.0 ± 1.1	8.3 ± 1.2
Palmitic acid oxidation	1.6 ± 0.4	1.4 ± 0.4	1.4 ± 0.5
Lignoceroyl-CoA ligase	0.26 ± 0.02	0.24 ± 0.04	0.25 ± 0.04
Lignoceric acid oxidation	0.077 ± 0.024	0.011 ± 0.004	0.031 ± 0.010
Lignoceroyl-CoA oxidation	0.65 ± 0.03	0.36 ± 0.07	0.62 ± 0.04

Enzyme activities are expressed as the means ± SD in different cell lines (nine of controls, three CHRS, and seven of X-ALD). Significant differences between the control and CHRS and X-ALD were observed for the oxidation of lignoceric acid $P < 0.001$) and between the control and CHRS for the oxidation of lignoceroyl-CoA ($P < 0.001$).

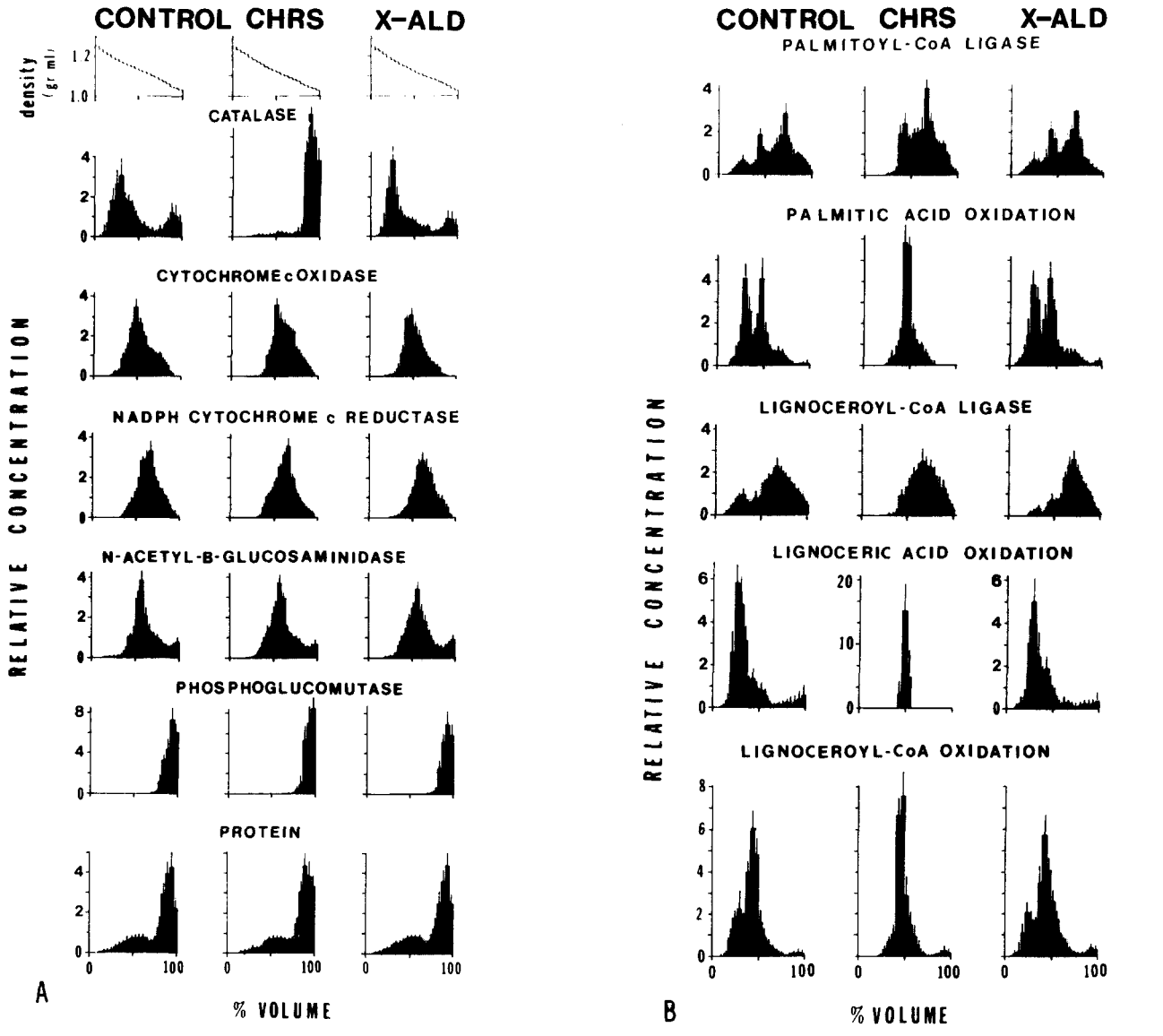


Fig. 1. Fractionation of the postnuclear fraction from cultured skin fibroblasts by a Nycodenz isopycnic gradient. The postnuclear fractions (500 *g* for 5 min-supernatant) from control, CHRS, and X-ALD fibroblasts were further fractionated by an isopycnic continuous Nycodenz gradient as described in the text. The distribution patterns of marker enzyme (1A) and enzyme activities for activation and oxidation of fatty acids (1B) are shown. The relative concentration (ordinate) is plotted against the cumulative volume as an average from nine control, three CHRS, and seven X-ALD gradients. Different cell lines were used for each experiment. The percent of recovery of different enzyme activities in the gradients of the postnuclear fraction varied from 81 to 108%. The density profile of the gradients is also shown.

ER marker (74.4%) and the peroxisomal marker (17.0%) and with only 8.6% of the activity in the mitochondrial fractions (Fig. 1B and Table 3). This bimodality was also evident in X-ALD samples, although there was a reduction in the amount of activity (5.3%) associated with peroxisomes (Fig. 1B and Table 3). The gradient distributions for the activation and oxidation of palmitic and lignoceric acids in CHRS samples were consistent with that observed for control and X-ALD cells with the exception, as expected, that peroxisomally associated activities were absent in CHRS cells. The residual activity for oxidation of lignoceric acid in CHRS was derived from mitochondria (Fig. 1B and Table 3).

The rates of oxidation of lignoceric acid in peroxisomes (0.17 ± 0.07 nmol/h per mg protein) was much higher than that observed in mitochondria (0.004 ± 0.001 nmol/h per mg protein) from control fibroblasts (**Table 4**). However, in control specimens the mitochondrial oxidation of lignoceroyl-CoA (5.97 ± 0.22 nmol/h per mg protein) was surprisingly higher than that observed in peroxisomes (4.57 ± 0.22 nmol/h per mg protein). Similar activities for the oxidation of lignoceroyl-CoA

TABLE 2. Relative specific activities of enzyme markers and percent of contamination in subcellular fractions isolated from control, CHRS, and X-ALD cultured skin fibroblasts

	Relative Specific Activity			% Contamination		
	Peroxisomes	Mitochondria	Endoplasmic	Peroxisomes	Mitochondria	Endoplasmic
Catalase						
Control	19.6 ± 4.4	0.6 ± 0.3	0.2 ± 0.1		1.5 ± 0.6	0.3 ± 0.1
CHRS	0.3 ± 0.1	0.5 ± 0.3	0.2 ± 0.1		1.3 ± 0.5	0.4 ± 0.2
X-ALD	18.3 ± 3.9	0.5 ± 0.2	0.2 ± 0.1		1.3 ± 0.4	0.4 ± 0.2
Cytochrome c oxidase						
Control	0.3 ± 0.1	4.8 ± 0.9	0.6 ± 0.2	5.6 ± 2.4		12.4 ± 4.2
CHRS	0.2 ± 0.1	4.9 ± 1.0	0.7 ± 0.2	4.8 ± 2.2		14.2 ± 4.2
X-ALD	0.3 ± 0.1	5.1 ± 0.8	0.8 ± 0.3	6.2 ± 2.0		16.4 ± 7.2
NADPH cytochrome c reductase						
Control	0.11 ± 0.06	0.9 ± 0.4	5.9 ± 0.9	2.2 ± 1.2	18.6 ± 8.2	
CHRS	0.12 ± 0.04	1.0 ± 0.5	5.6 ± 1.0	2.4 ± 0.8	18.8 ± 10.6	
X-ALD	0.09 ± 0.05	0.8 ± 0.3	5.4 ± 0.9	1.8 ± 1.0	16.2 ± 6.5	
N-Acetyl-β-glucosaminidase						
Control	0.4 ± 0.2	1.6 ± 0.5	2.3 ± 0.5	0.9 ± 0.4	3.3 ± 1.0	4.5 ± 1.1
CHRS	0.5 ± 0.2	1.7 ± 0.6	2.4 ± 0.4	1.0 ± 0.4	3.4 ± 1.2	4.7 ± 0.8
X-ALD	0.5 ± 0.2	2.2 ± 0.6	2.8 ± 0.4	1.1 ± 0.4	4.4 ± 1.1	5.6 ± 0.9

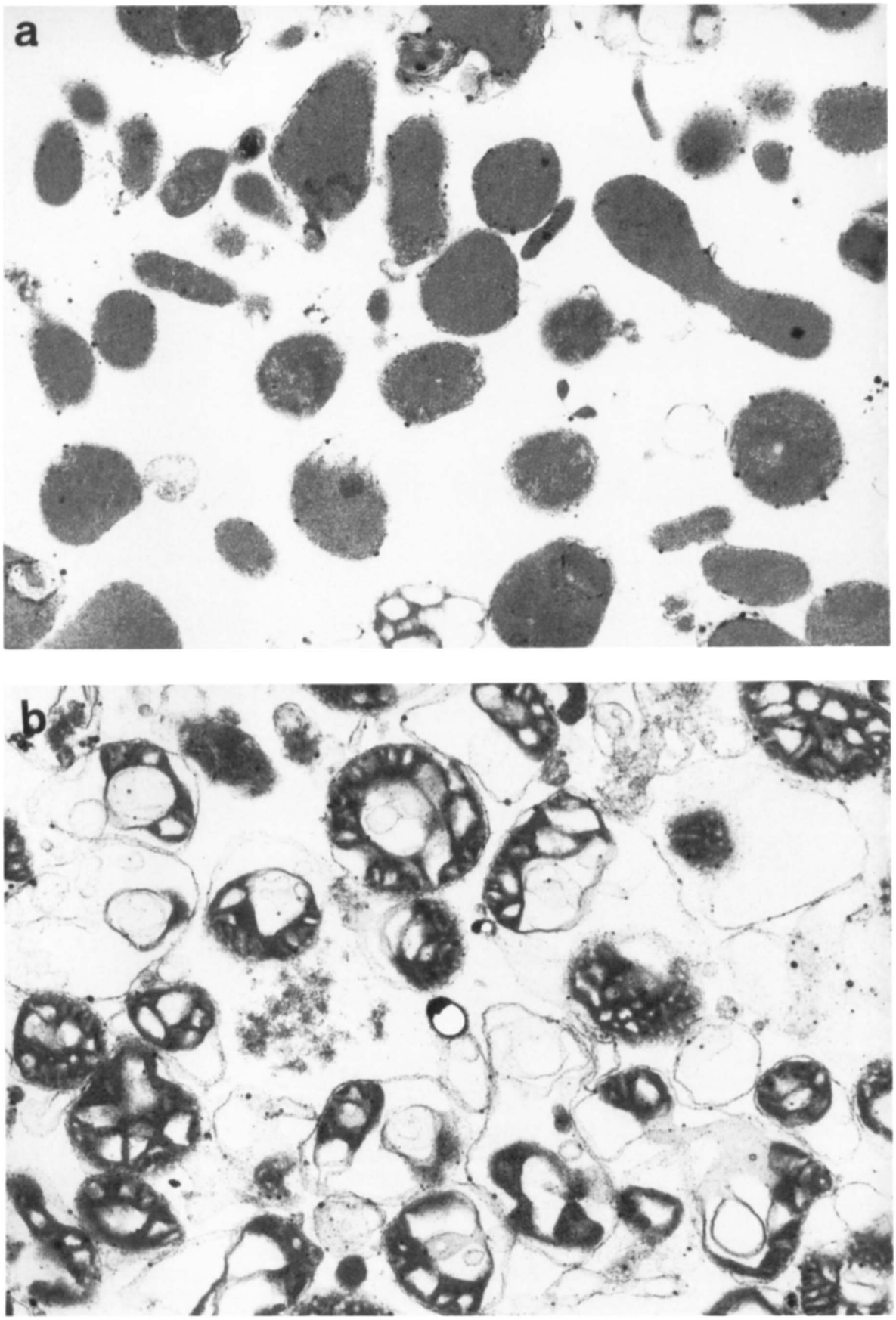
Relative specific activities and percent of contamination are expressed as the means ± SD in different cell lines (nine of controls, three of CHRS, and seven of X-ALD.) Approximate calculation using the protein values that represent mitochondria, microsomes, lysosomes, and peroxisomes in liver, according to Leighton et al., 1968 (54). Significant differences between control and CHRS were observed only for the activity of catalase in the peroxisomal fraction. There were no significant differences between control and X-ALD.

were found in the mitochondria from X-ALD and CHRS. These results demonstrate that while lignoceric acid is mainly oxidized in peroxisomes, lignoceroyl-CoA can be oxidized in both mitochondria and peroxisomes. The higher mitochondrial rate of oxidation of lignoceroyl-CoA as compared to lignoceric acid and the low mitochondrial activity of lignoceroyl-CoA ligase suggests that the low rate of oxidation of lignoceric acid in the mitochondria may be due to a relatively small amount of activity for the synthesis of lignoceroyl-CoA in that organelle. To test this hypothesis we examined the effect of exogenously added lignoceroyl-CoA ligase on the mitochondrial ability to oxidize lignoceric acid. The addition of lignoceroyl-CoA ligase (ER) increased the oxidation of lignoceric acid in mitochondria from control, CHRS, and X-ALD fibroblasts (**Fig. 3**). However, it had very little effect, if any, on the mitochondrial oxidation of palmitic acid. This further suggests that mitochondria can efficiently oxidize palmitic acid, but that it can oxidize lignoceric acid efficiently only if it is provided in an activated form (lignoceroyl-CoA). Thus, the oxidation of lignoceric acid may be regulated by the distribution of lignoceroyl-CoA ligase in different subcellular organelles.

Subcellular distribution of lignoceroyl-CoA ligase

Acyl-CoA ligases have wide substrate specificity (41) and palmitoyl-CoA ligase (76 kDa) is present in mitochondria, peroxisomes, and ER (42). Our previous studies have demonstrated that in ER (35, 43) and peroxisomes (20) palmitoyl-CoA ligase and lignoceroyl-CoA ligase are different enzymes. Furthermore, in contrast to palmitoyl-CoA ligase activity, lignoceroyl-CoA ligase activity is lower in mitochondria than in ER and peroxisomes (Table 4 and ref. 20). Since the oxidation of lignoceric acid in mitochondria might require its activation in that organelle, it was important to identify the acyl-CoA ligases in mitochondria. An antibody raised against ER palmitoyl-CoA ligase (76 kDa), in agreement with previous studies (35), inhibited palmitoyl-CoA ligase activity in mitochondria, ER (**Fig. 4**) and peroxisomes (20, 44) in control and X-ALD as well as in mitochondria and ER in CHRS (Fig. 4). This antisera completely inhibited lignoceroyl-CoA ligase activity in mitochondria from X-ALD, CHRS, and control fibroblasts but had very little effect on this activity in ER (Fig. 4). The inhibition of lignoceroyl-CoA and palmitoyl-CoA ligase activities in mitochondria with antibody to palmitoyl-CoA ligase suggests that lignoceric and palmitic acids in mitochondria are activated by the same enzyme, palmitoyl-CoA ligase.

A similar study demonstrating the effect of this antibody on the oxidation of palmitic and lignoceric acids in mitochondria from these three cell types is shown in **Fig. 5**. Antibody to palmitoyl-CoA ligase inhibited the oxidation of palmitic and lignoceric acids in mitochondria from



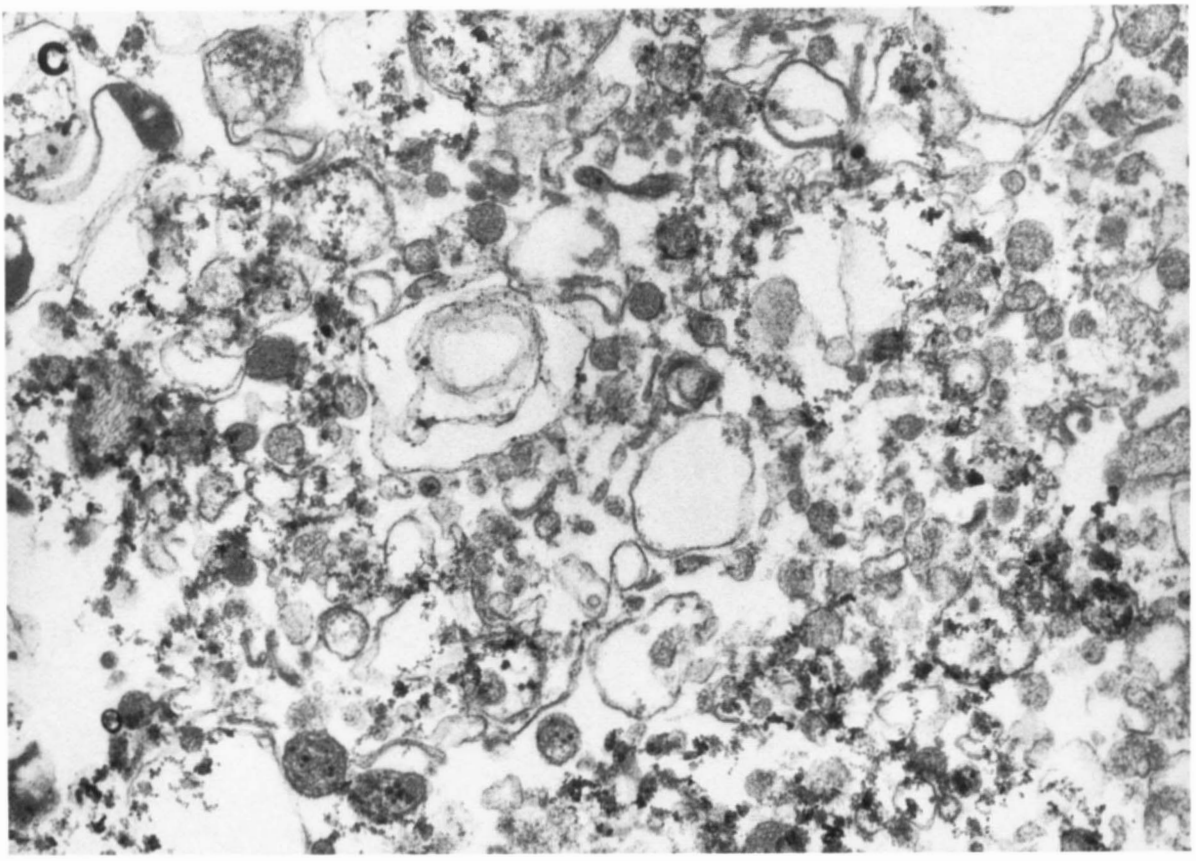


Fig. 2. Electron micrographs of peroxisomes, mitochondria, and endoplasmic reticulum enriched fractions from Nycodenz gradient of control fibroblasts. a: Section through peroxisomal-enriched fraction. The field is filled with peroxisomes of normal appearance with small percent contamination of lysosomes. Magnification 32,616 × . b: Section through mitochondrial-enriched fraction. Most mitochondria are in a condensed state, while others are swollen and damaged. There is a background of amorphous, membranous material, some of which is probably of lysosomal origin. Magnification 32,616 × . c: Section through ER-enriched fraction. The composition of this fraction is very heterogenous. Some swollen mitochondria, many small vesicles, and a very heterogenous background of amorphous material are also present. Magnification 32,616 × .

TABLE 3. Subcellular distribution of the enzyme markers and oxidation and activation of palmitic and lignoceric acids in cultured skin fibroblasts.

	Control			CHRS			X-ALD		
	p	M	m	p	M	m	p	M	m
Catalase	54.4	19.4	26.2	0.9	1.9	97.2	59.3	17.6	23.1
Cytochrome c oxidase	4.6	54.4	41.0	6.0	53.0	41.0	3.6	59.0	37.3
NADPH cytochrome c reductase	2.2	18.5	79.3	1.1	24.2	74.7	2.2	20.2	77.5
N-Acetyl-glucosaminidase	2.9	31.0	66.1	2.1	32.3	65.6	2.4	38.1	71.4
Protein	5.6	11.2	83.2	2.7	9.1	88.2	5.5	12.8	81.7
Palmitoyl-CoA ligase	12.9	22.6	64.5	1.9	21.4	76.7	10.6	30.9	58.5
Palmitic acid oxidation	45.4	41.0	13.6	4.5	80.7	14.8	42.0	42.9	15.2
Lignoceric acid oxidation	79.5	15.3	15.2	0.0	100.0	0.0	60.4	28.1	11.5
Lignoceric-CoA ligase	17.0	8.6	74.4	1.0	16.5	82.5	5.3	12.4	82.4
Lignoceroyl-CoA oxidation	24.6	63.8	11.5	4.6	82.6	12.8	25.0	60.2	14.8

The percent of enzyme activities for activation and oxidation of fatty acids was calculated by dividing the gradient into three fractions so that one fraction had a higher activity for catalase, the marker enzyme for peroxisomes (p), cytochrome c oxidase, the marker enzyme for mitochondria (M), and cytochrome c reductase, the marker enzyme for endoplasmic reticulum (m). These fractions represented 36.4, 18.2, and 45.4% of the gradient volume (from the bottom) for peroxisomes, mitochondria, and microsomes, respectively. The fatty acid activation and oxidation enzyme activities that co-distributed with the areas of marker enzymes for different organelles were calculated by integration of the area for the respective enzymes. The endoplasmic reticulum fraction contained 100% activity of phosphoglucomutase.

TABLE 4. Rates of activation and oxidation of lignoceric acid in different subcellular organelles from cultured skin fibroblasts

	Peroxisomes	Mitochondria	Endoplasmic Reticulum
	nmol/h per mg protein		
Lignoceric acid oxidation			
Control	0.17 ± 0.07	0.004 ± 0.001	0.00
CHRS	0.00	0.004 ± 0.001	0.00
X-ALD	0.02 ± 0.01	0.003 ± 0.001	0.00
Lignoceroyl-CoA ligase			
Control	0.86 ± 0.12	0.30 ± 0.02	0.78 ± 0.07
CHRS	0.00	0.32 ± 0.02	0.79 ± 0.12
X-ALD	0.15 ± 0.04	0.31 ± 0.04	0.80 ± 0.13
Lignoceroyl-CoA oxidation			
Control	4.57 ± 0.22	5.97 ± 0.22	0.00
CHRS	0.00	5.81 ± 0.12	0.00
X-ALD	4.70 ± 0.37	5.85 ± 0.34	0.00

The oxidation and activation of lignoceric acid was measured as described in the text. The results are expressed as the mean ± SD in different cell lines (nine of controls, three of CHRS, and seven of X-ALD). Activities were absent in samples from CHRS gradients with densities corresponding to normal peroxisomes. Significant differences between control and X-ALD were observed only for the oxidation of lignoceric acid and the activity of lignoceroyl-CoA ligase in peroxisomes ($P < 0.001$).

control, X-ALD, and CHRS (Fig. 5) as well as peroxisomes from control and X-ALD (44). The inhibition of lignoceric acid oxidation in mitochondria from control, X-ALD, and CHRS suggests that the limited oxidation of lignoceric acid observed in mitochondria is dependent on activation by palmitoyl-CoA ligase. Lignoceric acid oxidation in peroxisomes from X-ALD was also inhibited by this antiserum, suggesting that the limited activation of lignoceric acid to lignoceroyl-CoA and its subsequent oxidation in X-ALD peroxisomes is also catalyzed by palmitoyl-CoA ligase (44). The inability of this antiserum to inhibit lignoceroyl-CoA ligase activity in isolated ER (Fig. 4) and peroxisomes (44) from control fibroblasts suggests that ER and peroxisomal lignoceroyl-CoA ligase is a different enzyme from palmitoyl-CoA ligase. These studies demonstrate that palmitoyl-CoA ligase is present in mitochondria, peroxisomes, and ER while lignoceroyl-CoA ligase is present only in peroxisomes and ER and that these two acyl-CoA ligases are different enzymes.

Fatty acid oxidation in monolayers of fibroblast cultures from control, X-ALD, and CHRS

To understand the physiological role the acyl-CoA ligases play in the subcellular metabolism of fatty acids, we examined the in situ oxidation of fatty acids in monolayer fibroblast cultures. In agreement with the data from fibroblast homogenates, the oxidation of palmitic acid in fibroblast monolayers from X-ALD and CHRS was not statistically different from controls (**Table 5**). The deficient oxidation of lignoceric acid in CHRS fibroblast monolayers is consistent with the observed pathogno-

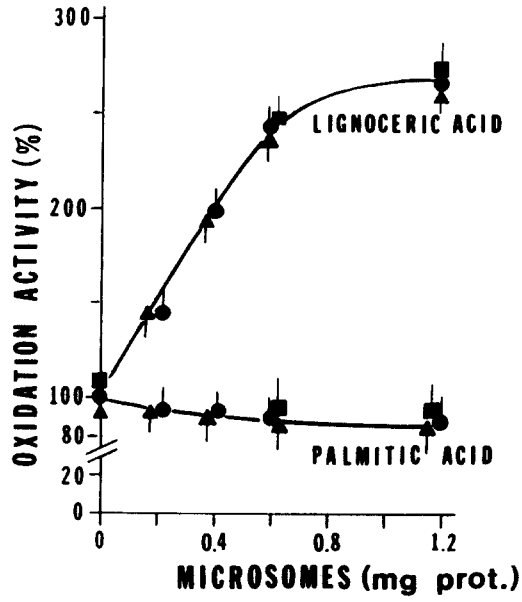


Fig. 3. Effect of exogenously added microsomal acyl-CoA ligases on the mitochondrial oxidation of palmitic and lignoceric acids. The oxidation of palmitic acid and lignoceric acid in isolated mitochondria from different cells lines (six of control (●), three of X-ALD (■), and three of CHRS (▲)) was measured in the presence of different amounts of ER as described in the text.

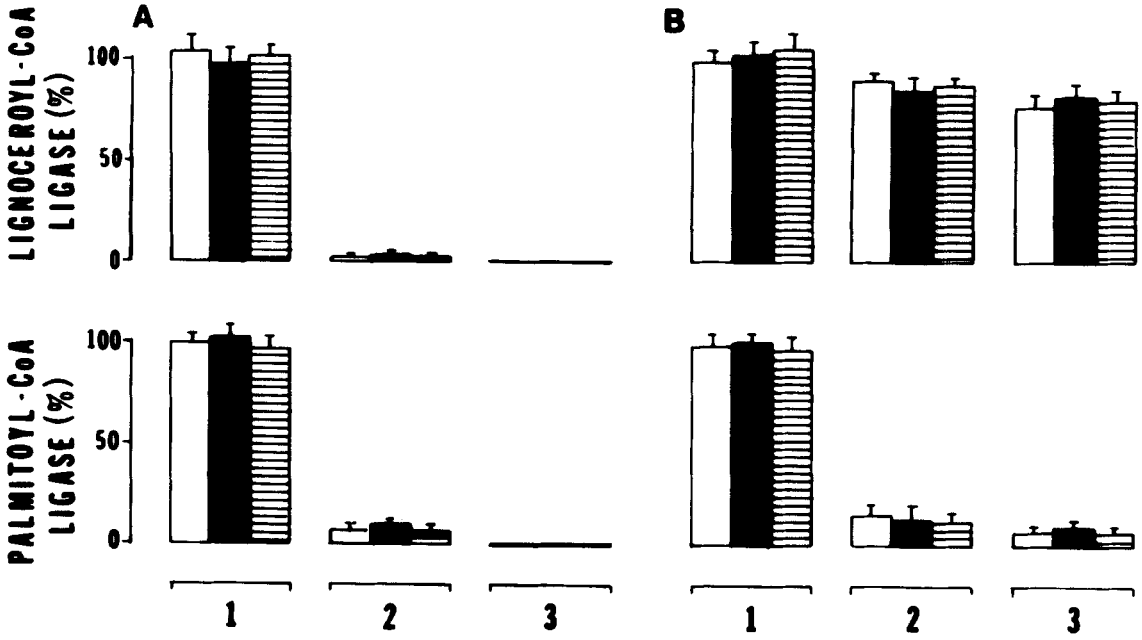


Fig. 4. Effect of antisera to palmitoyl-CoA ligase on lignoceroyl-CoA ligase and palmitoyl-CoA ligase activities in mitondria and endoplasmic reticulum. Mitochondria (A) and endoplasmic reticulum (B) from four different cell lines each of control (clear bar graph), CHRS (dark bar graph), and X-ALD (shaded bar graph) were incubated with 2 mg of preimmune (**1**), 1 mg of antisera (**2**), and 2 mg of antisera (**3**) as described in the Materials and Methods section. Lignoceroyl-CoA ligase and palmitoyl-CoA ligase activities were measured as described in the text. The specific activity of palmitoyl-CoA ligase in mitochondria and ER were, respectively, 15.8 ± 1.6 and 40.9 ± 3.2 in control; 15.5 ± 0.9 and 40.9 ± 2.1 in CHRS; and 18 ± 3.1 and 41.9 ± 3.2 nmol/h per mg protein in X-ALD. The specific activities for lignoceroyl-CoA ligase activities in mitochondria and ER were, respectively, 0.30 ± 0.02 and 0.78 ± 0.07 in control; 0.32 ± 0.02 and 0.79 ± 0.12 in CHRS; and 0.31 ± 0.04 and 0.80 ± 0.13 nmol/h per mg protein in X-ALD. These values were considered 100% and the results are expressed as the mean ± SD.

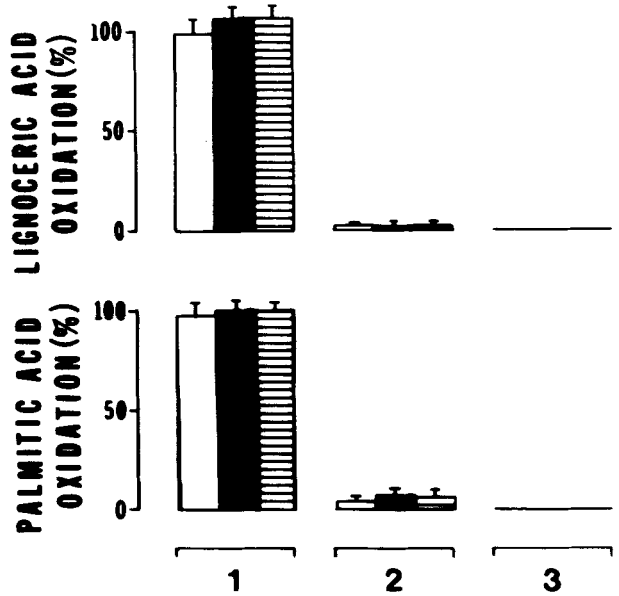


Fig. 5. Effect of antisera to palmitoyl-CoA ligase on the mitochondrial oxidation of palmitic and lignoceric acids. Mitochondria isolated from four different cell lines of control (clear bar graph), three of CHRS (dark bar graph), and four of X-ALD (shaded bar graph) were incubated with 2 mg of preimmune sera (**1**), 1 mg of antisera (**2**), and 2 mg of antisera (**3**), and the fatty acid oxidation was measured as described in the text. The specific activities for mitochondrial oxidation of palmitic and lignoceric acids were, respectively, 11.2 ± 1.2 and 0.004 ± 0.001 in control; 12.0 ± 1.0 and 0.004 ± 0.001 in CHRS; and 12.1 ± 3.2 and 0.003 ± 0.001 nmol/h per mg protein in X-ALD. These values were considered 100% and the results are expressed as the mean ± SD.

monic accumulation of this fatty acid in this disease. Similar to our results with fibroblast homogenates from CHRS, lignoceroyl-CoA was oxidized efficiently, as compared to lignoceric acid, in fibroblast monolayers. This indicates that while the majority of the cellular lignoceroyl-CoA ligase activity is located in ER (Tables 3 and 4 and Fig. 1B) the lignoceroyl-CoA synthesized by ER is not available for oxidation in mitochondria. The accessibility of exogenously added lignoceroyl-CoA to mitochondria for its subsequent oxidation, compared to the inaccessibility of lignoceroyl-CoA synthesized in ER in CHRS fibroblast monolayers, suggests that the catabolism of lignoceric acid in vivo is controlled by organelle specific acyl-CoA ligases. The oxidation of lignoceroyl-CoA in X-ALD as compared to lignoceric acid in fibroblast monolayers agrees with our previous conclusion that peroxisomal lignoceroyl-CoA ligase is deficient in this disease (20, 44).

DISCUSSION

By using an enriched peroxisomal fraction from rat liver, we previously demonstrated that VLC fatty acids are mostly and possibly exclusively oxidized in peroxisomes (6). VLC fatty acids accumulate when there is either a single enzyme defect in the peroxisomal β-oxida-

TABLE 5. Rate of fatty acid oxidation in monolayers of cultured skin fibroblasts

	Oxidation Activity		
	Control	CHRS	X-ALD
	pmol/h per mg protein		
Palmitic acid	2381 ± 496	1849 ± 469 (77.6)[a]	2087 ± 517 (87.4)
Lignoceroyl-CoA	71.4 ± 13.8	27.4 ± 14.0 (38.4)	65.5 ± 16.1 (91.7)
Lignoceric acid	7.6 ± 1.2	0.14 ± 0.11 (1.8)	2.3 ± 0.8 (30.3)

The oxidation of [1-^{14}C]-labeled fatty acid for monolayers of cultured skin fibroblasts was measured as described in the text. The results are expressed as the mean ± SD in three different control, CHRS, and X-ALD cultures. Significant differences ($P < 0.001$) between control, CHRS, and X-ALD were observed for lignoceric acid oxidation activity and between the control and CHRS for lignoceroyl-CoA oxidation activity.

[a]Figures in parentheses represent activity expressed as percent of control.

tion pathway (e.g., X-ALD) or when there is a general deficiency in the biogenesis of normal peroxisomes (e.g., CHRS). The activation of lignoceric acid by lignoceroyl-CoA ligase is the first and obligatory step in the β-oxidation of this VLC fatty acid. In X-ALD, lignoceroyl-CoA ligase activity is deficient (16–20, 44). The lack of peroxisomal β-oxidation enzymes (45) and the deficient oxidation of lignoceric acid (8) in CHRS is consistent with the observation that normal peroxisomes are absent in this disease (22). However, when lignoceroyl-CoA is used as a substrate for oxidation, we found a significant amount of oxidation (55% of the control) compared to the deficient oxidation of lignoceric acid (>15% of the control) in CHRS. These observations suggest that lignoceroyl-CoA may be oxidized by organelles other than peroxisomes.

There are differences in the enzymatic composition of human and rat liver peroxisomes (46). There also seem to be differences in the subcellular localization of enzymes between human and rat liver (47–50). Although phytanic acid oxidation takes place in rat liver mitochondria (49), the deficient oxidation of phytanic acid in a number of peroxisomal disorders (50) suggests it may be a peroxisomal function. Therefore, to understand the subcellular site for the oxidation of lignoceric acid and its derivatives in human tissue, we examined the catabolism of VLC fatty acids in different subcellular organelles derived from cultured skin fibroblasts using our newly developed procedure (20). The marker enzyme activities for mitochondria (cytochrome c oxidase), endoplasmic reticulum (cytochrome c reductase), lysosomes (N-acetyl-β-glucosaminidase), and cytosol (phosphoglucomutase) were similar in gradients from control, X-ALD, and CHRS except that catalase (marker enzyme for peroxisomes) was present in the cytosolic peak of the CHRS gradient rather than the peroxisomal peak because peroxisomes are absent in these cells. It is known that peroxisomal β-oxidation enzymes are absent in liver from CHRS patients (45). This distribution of catalase in the cytosolic peak in CHRS is consistent with the previous observations (39, 51).

Palmitoyl-CoA ligase (76 kDa) is known to be present in peroxisomes, mitochondria, and ER (42). We have also observed a trimodal distribution of this enzyme in these three organelles in our gradient (Fig. 1). Lignoceroyl-CoA ligase activity was mostly present in the ER and peroxisomal fractions (Fig. 1 and Table 4). We found that the mitochondrial fraction showed a reduced ability to synthesize lignoceroyl-CoA, having only one-third the specific activity present in ER and peroxisomes (Table 4). It is possible, however, that this limited mitochondrial activity may be functionally significant because the number of mitochondria per cell in rat liver is approximately 10 times higher than peroxisomes. The inhibition of activation and oxidation of lignoceric acid in mitochondria with antibody to palmitoyl-CoA ligase (76 kDa), however, indicates that the lignoceroyl-CoA ligase activity observed in mitochondria was derived from the activity of palmitoyl-CoA ligase (Figs. 4 and 5). In addition, the absence of lignoceric acid oxidation as compared to lignoceroyl-CoA in CHRS cell homogenates (Table 1) and monolayers (Table 5) further indicates that mitochondrial lignoceroyl-CoA oxidation activity may have no physiological role in the cellular oxidation of lignoceric acid. Moreover, an increase in the in vitro oxidation of lignoceric acid in mitochondria by the addition of ER supports this conclusion (Fig. 3). The inability to inhibit lignoceroyl-CoA ligase activity in ER (Fig. 4) and peroxisomes (20, 44) from normal cultured skin fibroblasts by antibody to palmitoyl-CoA ligase suggests that lignoceroyl-CoA ligase and palmitoyl-CoA ligase are separate enzymes. These studies demonstrate that palmitoyl-CoA ligase is present in mitochondria, peroxisomes, and ER, while lignoceroyl-CoA ligase is present only in ER and peroxisomes. The normal lignoceroyl-CoA ligase activity in ER as compared to the impaired activity in X-ALD peroxisomes suggests that the lignoceroyl-CoA ligases in these organelles may also be different enzymes (Table 4 and references 20, 44).

Sixty-five percent of the total palmitoyl-CoA ligase and 74% of the total lignoceroyl-CoA ligase (Table 3) activities found in cultured human skin fibroblasts are localized in the ER fraction. Since the orientation of the active site of these enzymes is on the cytoplasmic surface of ER membranes (34, 52, 53), the acyl-CoA synthesized by ER then should be accessible to mitochondria and/or per-

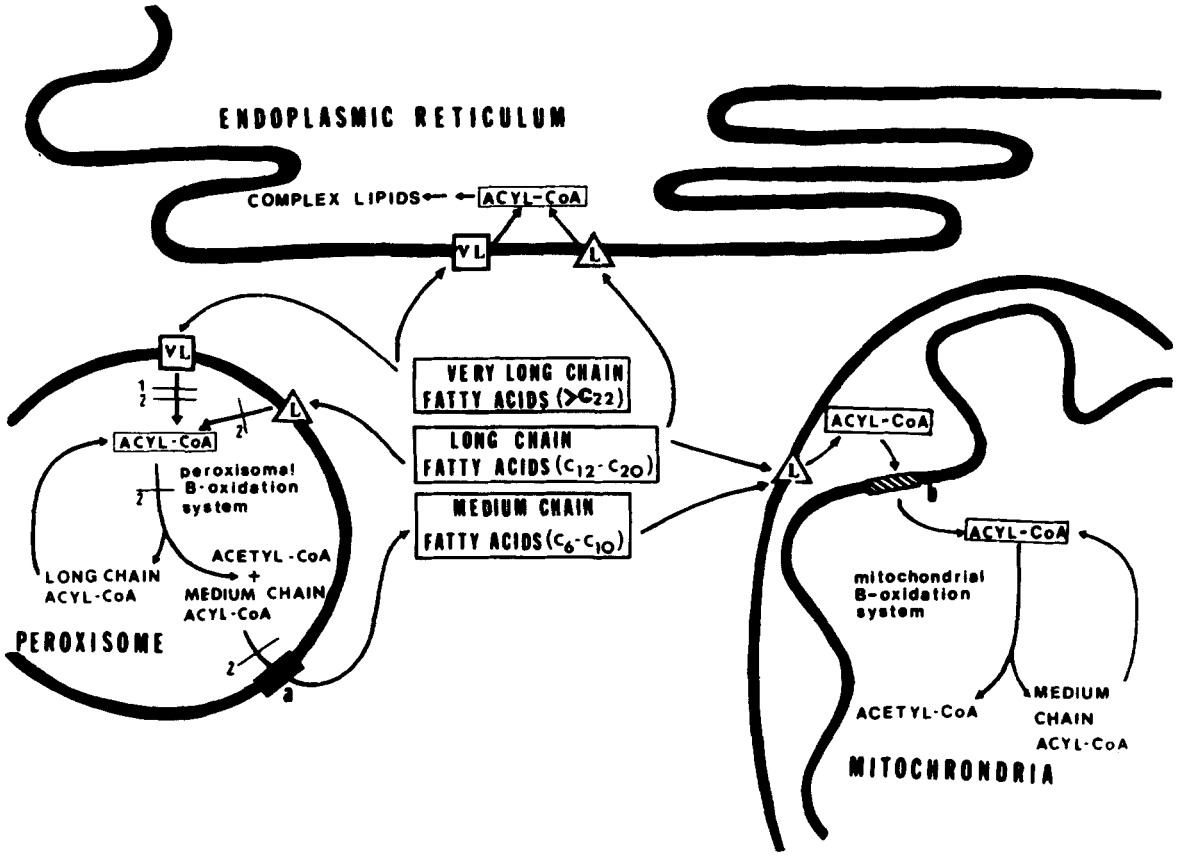


Fig. 6. Model representing the subcellular distribution of palmitoyl-CoA (76 kDa) and lignoceroyl-CoA in fibroblasts. VLC is very long chain acyl-CoA (lignoceroyl-CoA) ligase, and L is long chain acyl-CoA (palmitoyl-CoA) ligase. Enzymatic abnormalities in X-ALD and CHRS are represented by 1 and 2, respectively. Peroxisomal carnitine acyl-transferase ($\leq C_8$) and mitochondrial carnitine acyl-transferase system are represented by (a) and (b), respectively.

oxisomes for its subsequent oxidation. In this case, the preferential oxidation of lignoceroyl-CoA in mitochondria (Fig. 1 and Table 4) should not result in an accumulation of VLC fatty acids in X-ALD and CHRS cells since peroxisomes contain only 17% of the total cellular lignoceroyl-CoA ligase activity (Table 3). However, the deficient in situ oxidation of lignoceric acid in fibroblast monolayers of X-ALD cells and the almost total absence of in situ oxidation of lignoceric acid in CHRS cells monolayers compared to exogenously added lignoceroyl-CoA (Table 5) suggests that the lignoceroyl-CoA synthesized in ER may not be available to mitochondria and peroxisomes. Therefore, the observed oxidation of exogenously added lignoceroyl-CoA in cellular homogenates and isolated mitochondria as well as the in vivo oxidation in monolayers of cultured skin fibroblasts are nonphysiological processes. The oxidation of VLC fatty acids in mitochondria and/or by peroxisomes, then, must be controlled by the presence of specific acyl-CoA ligases in these organelles.

Based on these studies, we are proposing a model for the subcellular localization of various acyl-CoA ligases in different organelles and a description of how these acyl-CoA ligases control the metabolism of fatty acids in the cell (**Fig. 6**). Palmitoyl-CoA ligase is present in mitochondria, ER, and peroxisomes, and lignoceroyl-CoA ligase is present in peroxisomes and ER. The acyl-CoA ligases in ER provide substrates (acyl-CoA) for the synthesis of complex lipids in the ER, while the acyl-CoA ligases in mitochondria and peroxisomes provide acyl-CoA for the β-oxidation of fatty acids. Palmitic acid is oxidized both in mitochondria and peroxisomes because palmitoyl-CoA ligase is present in both of these organelles. Lignoceric acid is oxidized only in peroxisomes, since lignoceroyl-CoA ligase is present in peroxisomes but not in mitochondria. This model provides an explanation for the pathognomic accumulation of VLC fatty acids in a peroxisomal disorder characterized by a deficiency for peroxisomal lignoceroyl-CoA ligase activity (e.g., X-ALD) as well as in disorders lacking peroxisomes (e.g., CHRS).

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