Localization of nervonic acid β-oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy

Rajat Sandhir, Mushfiquddin Khan, Amarjit Chahal, and Inderjit Singh[1]

Division of Developmental Neurogenetics, Department of Pediatrics, Medical University of South Carolina, Charleston, SC 29425

Abstract **Studies with purified subcellular organelles from rat liver indicate that nervonic acid ($C_{24:1}$) is β-oxidized preferentially in peroxisomes. Lack of effect by etomoxir, inhibitor of mitochondrial β-oxidation, on β-oxidation of lignoceric acid ($C_{24:0}$), a peroxisomal function, and that of nervonic acid (24:1) compared to the inhibition of palmitic acid (16:0) oxidation, a mitochondrial function, supports the conclusion that nervonic acid is oxidized in peroxisomes. Moreover, the oxidation of nervonic and lignoceric acids was deficient in fibroblasts from patients with defects in peroxisomal β-oxidation [Zellweger syndrome (ZS) and X-linked adrenoleukodystrophy (X-ALD)]. Similar to lignoceric acid, the activation and β-oxidation of nervonic acid was deficient in peroxisomes isolated from X-ALD fibroblasts. Transfection of X-ALD fibroblasts with human cDNA encoding for ALDP (X-ALD gene product) restored the oxidation of both nervonic and lignoceric acids, demonstrating that the same molecular defect may be responsible for the abnormality in the oxidation of nervonic as well as lignoceric acid. Moreover, immunoprecipitation of activities for acyl-CoA ligase for both lignoceric acid and nervonic acid indicate that saturated and monoenoic very long chain (VLC) fatty acids may be activated by the same enzyme. These results clearly demonstrate that similar to saturated VLC fatty acids (e.g., lignoceric acid), VLC monounsaturated fatty acids (e.g., nervonic acid) are oxidized preferentially in peroxisomes and that this activity is impaired in X-ALD. In view of the fact that the oxidation of unsaturated VLC fatty acids is defective in X-ALD patients, the efficacy of dietary monoene therapy, "Lorenzo's oil," in X-ALD needs to be evaluated.—**Sandhir, R., M. Khan, A. Chahal, and I. Singh. **Localization of nervonic acid β-oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy. *J. Lipid Res.* 1998.** 39: **2161–2171.**

Supplementary key words nervonic acid • peroxisomes • Zellweger syndrome • X-linked adrenoleukodystrophy • very long chain fatty acids

The metabolism of very long chain (VLC) fatty acids (>C_{22}) has gained prominence recently because of their excessive accumulation in various peroxisomal disorders (1–3). In fact, the levels of saturated VLC fatty acids have been used as a diagnostic tool in prenatal as well as postnatal diagnosis of peroxisomal diseases (4). VLC fatty acids are the major constituents of sphingolipids (galactocerebrosides and sulfatides), a group of myelin lipids, and can be divided into four groups depending on the state of their unsaturation and α-hydroxylation (5). Studies from our laboratory (6, 7) and others (8) have previously demonstrated that saturated VLC fatty acids are preferentially and possibly exclusively oxidized in peroxisomes, and accumulate in pathognomonic amounts in peroxisomal disorders with defects in their β-oxidation such as Zellweger syndrome (ZS) and X-linked adrenoleukodystrophy (X-ALD). Unsaturated fatty acids, "Lorenzo's oil," have been used as a therapy in X-ALD to decrease the levels of saturated VLC fatty acids, possibly by competition of unsaturated fatty acids for the fatty acid chain elongation system (9); therefore, it is important to understand the metabolic aspects of unsaturated fatty acids.

Nervonic acid (24:1, n–9), a monounsaturated analog of lignoceric acid (24:0), is one of the major fatty acids in brain sphingolipids, normally accounting for approximately 40% of the total fatty acids in sphingolipids (10). In mammalian cells, fatty acids are β-oxidized in both mitochondria and peroxisomes (1, 10–12). Saturated VLC fatty acids are oxidized in peroxisomes, whereas medium and long chain fatty acids (C_6–C_{20}) are oxidized in mitochondria. However, no information is available regarding the subcellular organelle responsible for the oxidation of monounsaturated VLC fatty acids (nervonic acid), a major component of myelin lipids. Unsaturated fatty acids containing *cis* double bonds can be degraded by the β-oxidation cycle to acetyl-CoA. However, their degradation requires auxiliary enzymes, 2, 4 dienoyl-CoA reductase

Abbreviations: VLC, very long chain; ZS, Zellweger syndrome; X-ALD, X-linked adrenoleukodystrophy; RD, Refsum disease; RCDP, rhizomelic chondrodysplasia punctata; ALDP, X-linked adrenoleukodystrophy gene product; FAME, fatty acid methyl ester; TLC, thin-layer chromatography; GC, gas chromatography; MS, mass spectrometry; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

[1]To whom correspondence should be addressed.

(EC 1.3.1.34) and Δ3, Δ2-enoyl-CoA isomerase (EC 5.3.3.8), in addition to the enzymes necessary for the β-oxidation of saturated fatty acids (1, 2, 10, 12).

Excessive amounts of saturated and monoenoic VLC fatty acids accumulate in ZS because of the absence of peroxisomes (13). Saturated VLC fatty acids accumulate in X-ALD as a result of the deficient activity of activation and oxidation of VLC fatty acids (6, 14). Unlike ZS, there is a great deal of ambiguity in reporting the levels of unsaturated VLC fatty acids in X-ALD. Studies from different laboratories have reported decreased (15) or increased amounts (16) of unsaturated VLC fatty acids ($>C_{22:1}$) in X-ALD tissues. The excessive accumulation of saturated VLC fatty acids compared to the ambiguity regarding the status of unsaturated fatty acids implies that unsaturated VLC fatty acids may either be oxidized in an organelle other than peroxisomes or may involve an enzyme system other than the one that activates saturated VLC fatty acids in peroxisomes. Saturated VLC fatty acids are oxidized in peroxisomes and excessive accumulation of saturated fatty acids in X-ALD is due to their impaired β-oxidation because of the abnormality in lignoceroyl-CoA ligase activity (14). Lignoceroyl-CoA ligase is a component of peroxisomal membrane; therefore, the defect in the oxidation of VLC fatty acids is due to the abnormality in the peroxisomal membrane (17). The ALD gene product (ALDP), a 75 kDa protein, is a component of peroxisomal membrane (18), and transfection of human cDNA for ALDP corrects the metabolic defect in X-ALD cells (19, 20). However, the precise function of ALDP in the metabolism of VLC fatty acids is not known. It will be of interest to identify the enzyme system for the catabolism of monoenoic VLC fatty acids (nervonic acid) because recent reports suggest that monoenoic fatty acids may have toxic effects in X-ALD (21, 22). This implies that the therapy of X-ALD patients with "Lorenzo's oil" should be re-evaluated in case monoenoic VLC fatty acids are oxidized by the same enzyme system as the saturated VLC fatty acids.

The present study was undertaken to identify the subcellular site responsible for β-oxidation of unsaturated VLC fatty acids (e.g., nervonic acid) and to understand the similarities/dissimilarities in the metabolism of both saturated (lignoceric) and unsaturated (nervonic acid) VLC fatty acids in X-ALD. The studies described in this manuscript clearly demonstrate that both saturated and unsaturated VLC fatty acids are β-oxidized in peroxisomes in rodents as well as in humans. Saturated (e.g., lignoceric acid) and unsaturated VLC (e.g., nervonic acid) fatty acids are activated by the same VLC acyl-CoA ligase in the peroxisomal membranes and this enzyme activity is deficient in X-ALD. Moreover, this defect in β-oxidation of nervonic acid can be corrected by transfection of the X-ALD gene in cultured skin fibroblasts from X-ALD patients.

MATERIALS AND METHODS

Cell lines

ZS (GM 00228), X-ALD (04934), RD (03896), and RCDP (11347) skin fibroblast cell lines were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, NJ. Other cell lines from patients with peroxisomal disorders (ZS, X-ALD, and RCDP) and control cells from healthy subjects were established in this laboratory.

Materials

Nycodenz was obtained from Accurate Chemical and Scientific Corp., Westbury, NY. Cytochrome c, α-cyclodextrin, FAD, NAD^+, ATP, l-carnitine, l-malate, methyl eruciate, and methyl nervonate, methyl lignocerate, imidazole, sucrose, antipain, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, coenzyme A, fatty acid methyl ester standards, FITC-conjugated anti-rabbit IgG were obtained from Sigma Chemical Co., St. Louis, MO. Etomoxir was obtained from Research Biochemicals, Inc., Natick, MA. [1-^{14}C]palmitic acid (50 mCi/mmol) and K ^{14}C (55 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. ^{125}I-labeled anti-rabbit IgG were from ICN Pharmaceuticals Inc., Irvine, CA. pcDNA3 was obtained from Invitrogen, Carlsband, CA. Lipofectin, G418, Dulbecco's modified Eagle's minimum essential medium, trypsin-EDTA, and Hank's balanced salt solution were obtained from GIBCO BRL, Grand Island, NY. Fetal bovine calf serum was from Hyclone, Logan, UT. 1-Tricosanol was from ICN Biomedical Inc., Aurora, OH. Thin-layer chromatography (TLC) silica gel 60 plates were purchased from Whatman Labsales Inc., Clifton, NJ. All other chemicals and reagents of high purity grade were obtained from Aldrich Chemical Co., Milwaukee, WI. ALDP cDNA clone was provided by Dr. Patrick Aubourg, INSERM, Hospital Saint-Vincent de Paul, Paris, France.

Methods

Antibodies. Antibodies against C-terminal peptide (APSPQG PGGLQGAST) of human ALDP (23) and against N-terminal peptide (MLPVLYTGLAGLL) of rat lignoceroyl-CoA ligase (24) were raised in New Zealand White rabbits after conjugating the peptides with keyhole limpet hemocyanin using glutaraldehyde. The antibody titer was determined by ELISA. The immunoglobulins were precipitated with 45% ammonium sulfate saturation, dialyzed against PBS, followed by dialysis with 100 mm acetate buffer, pH 4.5, to remove albumin. The dialysate was loaded on a protein A-Sepharose column and the immunoglobulins were eluted using 100 mm glycine, pH 4.5, and the pH of the eluent was adjusted to 7.4 with 1 m Tris base.

Synthesis of [1-^{14}C]nervonic acid. [1-^{14}C]nervonic acid was synthesized from erucic acid by 2 carbon chain elongation in two steps. The first step involves the synthesis of unlabeled *cis*-14-tricosenyl methanesulfonate from methyl erucate as described by Richter and Mangold (25). All the compounds prepared were purified and found to have identical values as reported.

In the second step, *cis*-14-tricosenyl methanesulfonate (0.070 mmol) dissolved in 2.5 ml of ethanol and 2 mCi of K^{14}CN (0.037 mmol) dissolved in 400 μl of 50 mm KOH were mixed and refluxed in a Teflon-coated screw-capped test tube for 30 h; 5 ml of ether was added to the reaction mixture, followed by 2 ml water. The contents were mixed and the ether layer was then separated. The aqueous layer was extracted three times with 3-ml portions of ether and pooled with the above ether layer. The pooled ether extract was washed with water and then evaporated to dryness under nitrogen. The radioactive nitrile was obtained in quantitative yield as examined by TLC–radioscanning. The radiolabeled nitrile was dissolved in 2 ml of 95% ethanol followed by addition of 0.2 g KOH and the reaction mixture was refluxed at 100°C for 60 h. The contents were acidified with 10 mm HCl and the radiolabeled product was extracted three times with 5 ml ether. The combined ether pool was washed with water and dried over anhydrous sodium sulfate. The final labeled compound was purified

by TLC [K6 silica gel 60 plates; solvent system, hexane–ether–acetic acid 50:50:1 (by volume)] and the final yield was 80%. A fraction of the radiolabeled product was converted to the methyl ester by treatment with 1.5% (v/v) H_2SO_4 in anhydrous methanol at 80°C for 2 h. The product was compared with the corresponding authentic non-radioactive fatty acid methyl ester by TLC. It was found that the product was identical to the authentic nervonic acid. The purity of the labeled methyl ester was further determined by capillary GC (99.2% purity) and had a high specific activity (54.5 mCi/mmol).

In order to confirm the position and configuration of the olefinic bond in the synthesized radiolabeled nervonic acid, a parallel synthesis of non-labeled nervonic acid under identical conditions was carried out. The methyl ester of the labeled compound had TLC and capillary gas chromatography (GC) properties identical to those of the non-labeled methyl ester. They co-eluted in TLC and GC along with the authentic sample. The spectral analysis corroborated the structure and configuration of the synthesized nervonic acid methyl ester. Infrared (IR, recorded on Mattson Polaris FTIR) spectrum showed characteristic bands at 1735 cm^{-1} (ester function) and 1625 cm^{-1} (olefinic linkage). Proton nuclear magnetic resonance (^1H NMR, $CDCl_3$, ppm, acquired using Varian VXR 400 MHz with quadrature-detection) displayed diagnostic peaks at 5.35 (m, 2H, C*H* = C*H*, J = 4.63 Hz), 3.67(s, 3H, $COOCH_3$), 2.31(t, 2H, CH_2COOCH_3) and 2.03(m, 4H, CH_2–CH = CH–CH_2, J = 5.58 Hz). Mass spectrometry (MS, recorded using a LCQ ion trap mass spectrophotometer in APCI mode) confirmed the structure showing the peaks at *m/z* 381.2 (M + 1, 100%), at *m/z* 382.3(M + 2, 25%) and at *m/z* 349.2 (M–OCH_3, 5%). MS/MS of *m/z* 381 furnished the characteristic M–CH_3OH peak at *m/z* 349 along with sequential cleavage of CH_2 displaying at *m/z* 185, 199, 213, 227, 241, 255, 269, 283, 297, and 311. The spectral and chromatographic data are in good agreement with the structure of the nervonic acid as *cis*-15-tetracosenoic acid.

Synthesis of [1-^{14}C]lignoceric acid. [1-^{14}C]lignoceric acid was made from tricosanyl bromide as described earlier (26) with some modifications. 1-Tricosanol was converted to 1-trocosanyl bromide in the presence of PBr_3 as reported (27). The bromide (0.024 mmol) and K^{14}CN (0.018 mmol, 1 mCi) were dissolved in 1.5 ml absolute ethanol, followed by the addition of 0.2 ml water, and the contents were heated in a Teflon-coated screw-capped glass test tube at 85°C for 48 h. Two ml of water was added to the reaction mixture and was extracted 5 times with 3 ml of ether. The pooled ether extract was washed with water and then evaporated to dryness under nitrogen. The radioactive nitrile was obtained in a quantitative yield as evidenced by TLC–radioscanning. The radiolabeled nitrile was hydrolyzed with 0.2 g KOH in 2 ml 95% ethanol at 100°C for 72 h. The content was acidified with 10 mm HCl and the radiolabeled product was extracted 3 times with 5 ml of ether each time. The pooled ether was washed with water and the ether layer was dried over anhydrous sodium sulfate. The labeled lignoceric acid was purified by TLC [K6 silica gel 60 plates, solvent system, hexane–ether–acetic acid 50:50:1 (by volume)]. The yield was 95% from labeled nitrile. The non-radiolabeled acid was prepared under identical conditions and was converted to the corresponding methyl ester as described for nervonic acid. The identity of the lignoceric acid methyl ester was established by infrared spectroscopy, high performance TLC, and capillary GC. The results were found to be identical to those obtained for the authentic lignoceric acid methyl ester. MS confirmed the structure showing diagnostic peaks at *m/z* 383.4 (M + 1, 12%), *m/z* 382.3 (M, 26%), *m/z* 381.3 (M–1, 100%), and 350.2 (M–CH_3OH, 15%) along with the peaks related to the fatty acid methyl ester.

Synthesis of radiolabeled fatty acyl CoA esters. The CoA esters of 1-^{14}C-radiolabeled fatty acids were prepared and purified as described by Taylor et al. (28).

Isolation of peroxisomes from rat liver. Liver peroxisomes were isolated from Sprague-Dawley rats (200–250 g) by the procedure described previously (29). Briefly, livers were homogenized in homogenizing buffer (0.25 m sucrose, 1 mm EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml antipain, 0.7 μg/ml pepstatin, 0.2 mm PMSF, 0.1% ethanol in 3 mm imidazole, pH 7.4) and fractionated by differential centrifugation to prepare the light mitochondrial fraction, i.e., the lambda fraction. Peroxisomes from 4–5 ml of lambda fraction (80–100 mg protein) were prepared by isopycnic equilibrium centrifugation on 30 ml of continuous gradient of 0 to 50% Nycodenz in homogenizing buffer with 4 ml of 55% Nycodenz in homogenizing buffer as a cushion in a 39-ml tube (29). The gradient fractions were collected from the bottom of the tube and subcellular fractions were identified by their respective marker enzymes: catalase for peroxisomes (30), cytochrome c oxidase for mitochondria (31), and NADPH cytochrome c reductase for microsomes (32).

Fibroblast cell culture. Cell lines were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 15% fetal calf serum (FCS) in a 5% CO_2 air atmosphere at 37°C. The cells were harvested by trypsinization 3–4 days after reaching confluence, centrifuged, and the cell pellet was washed with Hank's balanced salt solution. The cells were suspended in DMEM medium supplemented with 15% FCS and incubated for 1 h at 37°C. The cells were subsequently washed and suspended in Hank's balanced salt solution prior to use.

Isolation of peroxisomes from cultured human fibroblasts. Cultured fibroblast cells were trypsinized, washed with homogenizing buffer (0.25 m sucrose, 1 mm EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml antipain, 0.7 μg/ml pepstatin/ml, 0.2 mm PMSF, 0.1% ethanol in 3 mm imidazole, pH 7.4) and were homogenized using five strokes in a Teflon–glass homogenizer until approximately 90% of the cells were broken. The homogenate was centrifuged at 500 *g* for 5 min to remove the nuclei and the subcellular organelles were isolated from 4–5 ml of post-nuclear fraction (30–35 mg protein) on 30 ml of 0–30% Nycodenz gradient in homogenizing buffer, and 4 ml of 35% Nycodenz in homogenizing buffer as a cushion was used (7, 17). The distribution of subcellular organelles in the gradient was determined by their respective marker enzymes as described above for liver.

Preparation of peroxisomal matrix and membranes. Isolated peroxisomes were sedimented by centrifugation to remove Nycodenz and the peroxisomal pellet was washed with homogenization buffer and again suspended in a hypotonic buffer (50 mm phosphate buffer, pH 7.4) and sonicated to lyse the peroxisomes. Catalase was assayed in matrix and membrane to monitor the lysis of peroxisomes (33). Catalase activity was present only in the matrix and not in the membranes.

Glial cell culture. Mixed glial cell culture was established from 1–2-day-old rat pups as described (34). Cells were maintained in DMEM containing 5% glucose, 10% fetal bovine serum (FBS), and antibiotic/antimycotic mixture. After 10 days of culture, microglia were removed by shaking for 2 h in an orbital shaker at 200 rpm. The medium was changed and the flask was again shaken for 24 h at 240 rpm. The detached cells, predominantly oligodendrocytes, were removed from microglia by seeding on the petri dishes for 30–60 min. The non-attached cells were removed and seeded on poly-l-lysine-coated plates. The flasks containing remaining cells, astrocytes, were trypsinized (0.1% trypsin in 10 mm Tris-EDTA saline, pH 7.4) and further cultured.

Plasmid construction and transfection of human fibroblasts. A full-length ALD cDNA clone was subcloned in a mammalian expression vector, pcDNA3, to generate the plasmid pcDNA3ALD. The orientation of the pcDNA3ALD was verified by sequencing. Con-

trol and ALD fibroblasts were transfected with pcDNA3ALD plasmid using Lipofectin according to the manufacturer's instructions. The cells in a 75-cm^2-culture flask at approximately 60% confluency were transfected with 10 μg of plasmid DNA using 25 μl of Lipofectin reagent. The cells were processed for immunofluorescence microscopy or biochemical assays 48 h after transfection. Subsequently, cells were allowed to grow in G418 antibiotic (200 μg/ml) for 10 days for stable expression.

Immunofluorescence microscopy. Transfected and normal cells were cultured overnight on glass slides in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum under 5% CO_2 and immunofluorescence analysis was carried out as decribed previously (23). Cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde in PBS for 40 min at room temperature. For permeablization, the cells were incubated with 0.1% Triton X-100 in PBS for 15 min, followed by incubation with 0.1% Triton X-100 in PBS containing 2% (v/v) bovine calf serum for 15 min. Cells were then incubated for 2 h with the C-terminal (15 amino acids) anti-ALDP antibodies, followed by an incubation for 1 h with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG. The cells were washed three times after primary and secondary antibody treatment. Finally, cells were mounted in Fluoromount-G and viewed using confocal microscope (Bio-Rad MRC 1000).

Immunoprecipitation of VLC acyl-CoA ligase. Peroxisomes were solubilized in 10 mm potassium phosphate buffer, pH 7.5, containing 0.5 mm EDTA, 0.1% (v/v) hexamethylphosphoric triamide, 2 mm ATP, 0.2% Triton X-100, and 1% n-octyl-α-d-glucopyranoside. The suspension was centrifuged at 100,000 *g* for 60 min and the supernatant was collected. The solubilized peroxisomal protein fraction containing lignoceroyl-CoA ligase activity (500 μg) in a volume of 250 μl was diluted in 2× immunoprecipitation buffer (1x = 0.1% Triton X-100, 150 mm NaCl, 1 mm EGTA, 0.2 mm PMSF, 10 mm Tris-HCl, pH 7.4) and precleared with 50 μl of 10% (w/v) protein A-Sepharose 4B. The precleared peroxisomal preparation was incubated with varying amounts of preimmune sera (IgG) or antibodies (IgG) against lignoceroyl-CoA ligase for 4 h at 4°C followed by precipitation of antigen–antibody complex by addition of 50 μl of 10% (w/v) protein A-Sepharose 4B. The supernatant was assayed for nervonyl-CoA, lignoceroyl-CoA, and palmitoyl-CoA ligase activities.

Assay for activation and oxidation of 1-^{14}C-labeled fatty acids. The activation of fatty acid by ligase to fatty acyl-CoA was measured as described previously (7). The reaction mixture (0.25 ml) contained 12 μm [1-^{14}C]fatty acid (150,000 dpm), 10 mm ATP, 80 μm CoASH, 30 mm KCl, 5 mm $MgCl_2$, and 0.05 mm dithiothreitol in 30 mm MOPS–HCl, pH 7.8. The reaction was terminated by the addition of 1.25 ml of Dole's reagent (isopropyl alcohol–heptane–1N H_2SO_4, 40:10:1, by volume). The denatured protein was removed by centrifugation, followed by addition of 0.45 ml water and 0.8 ml heptane. The lower aqueous layer was washed three times with heptane and the radioactivity in the aqueous phase was measured as an index of acyl-CoA ligase activity. The fatty acid substrates were solubilized with α-cyclodextrin (35). The fatty acids were first dried under nitrogen, suspended in homogenization buffer containing α-cyclodextrin (20 mg/ml), and sonicated for 30 min.

The oxidation of 1-^{14}C-labeled fatty acids to acetate was measured as described previously (6). Briefly, 0.25 ml of the reaction mixture contained 12 μm [1-^{14}C]fatty acid (150,000 dpm), 30 mm KCl, 5 mm $MgCl_2$, 8.5 mm ATP, 0.25 mm NAD^+, 0.17 mm FAD, 2.5 mm l-carnitine, and 0.08 mm CoASH in 20 mm MOPS-HCl buffer, pH 7.8. The reaction was started by the addition of peroxisomal protein and was stopped by adding 0.625 ml of 1 m KOH in methanol. Denatured protein was removed by centrifugation and the supernatant was incubated at 60°C for 1 h, neutralized with 0.125 ml of 6 N HCl , and partitioned by Folch partition. Radioactivity in the aqueous phase was measured as an index of the [1-^{14}C]fatty acid oxidized to acetate.

Measurement of VLC fatty acids in fibroblasts. Fatty acid methyl esters (FAME) were prepared using the method described by Lepage and Roy (36). Isolation and purification of FAME were carried out as detailed by Dacremont, Cocquyt, and Vincent (37). Purified FAME, suspended in chloroform, was analyzed by gas chromatography (GC)-15A attached to the Chromatopac C-R3A Integrator (Schimadzu) on fused silica capillary column (25 M 007 series methyl silicone with with an internal diameter of 0.25 mm from Quadrex Corporation, New Haven, CT). The fatty acid profile was identified by comparison with retention times of standard fatty acid methyl esters. The area under the peaks of identified fatty acid was set as 100% and the individual fatty acids were measured as percent area.

RESULTS

Figure 1 depicts the distribution of marker enzymes for different subcellular organelles from rat liver in Nycodenz gradient: catalase for peroxisomes, cytochrome c oxidase for mitochondria, and NADPH cytochrome c reductase for microsomes, respectively. As judged by the distribution of marker enzymes in the gradient, the mitochondria, microsomes, and peroxisomes were well resolved from each other. The purity of different subcellular fractions was calculated as described by Fujiki et al. (38). Peroxisomes (fractions #4 and 5) prepared by this method were 97% pure with only minor contamination by mitochondria (0.5%) and microsomes (2.8%). In agreement with our previous observations, the activity for oxidation of lignoceric acid was observed in peroxisomes and that for palmitic acid was observed in both peroxisomes and mitochondria (29). The activity for oxidation of nervonic acid paralleled the distribution of catalase and lignoceric acid oxidation activity (peroxisomal markers), indicating that in rat liver nervonic acid is β-oxidized in peroxisomes (Fig. 1). The specific activities for the oxidation of nervonic, lignoceric, and palmitic acids are shown in **Table 1**. The specific activities for oxidation of nervonic and lignoceric acids were 5- and 8-fold higher in peroxisomes as compared with mitochondria, indicating preferential oxidation of both lignoceric and nervonic acids in peroxisomes. These results indicate that peroxisomes are the subcellular organelles responsible for oxidation of nervonic acid.

Activation of fatty acids catalyzed by acyl-CoA ligase is an initial step in fatty acid metabolism. The acyl-CoA ligase activity for the activation of fatty acids is a component of peroxisomal membranes. So far at least two enzymes, one for the activation of VLC fatty acids (lignoceroyl-CoA ligase) and the other for activation of long chain fatty acids (palmitoyl-CoA ligase) have been identified in the peroxisomal membranes (7, 17, 39). The presence of phytanoyl-CoA ligase as a separate enzyme in peroxisomes has not been clearly established so far (40, 41). **Table 2** shows the specific activities of acyl-CoA ligase involved in the activation of nervonic, lignoceric, and palmitic acids. It is clear from the data that both nervonyl-CoA and lignoceroyl-CoA ligases have a bimodal distribution in peroxi-

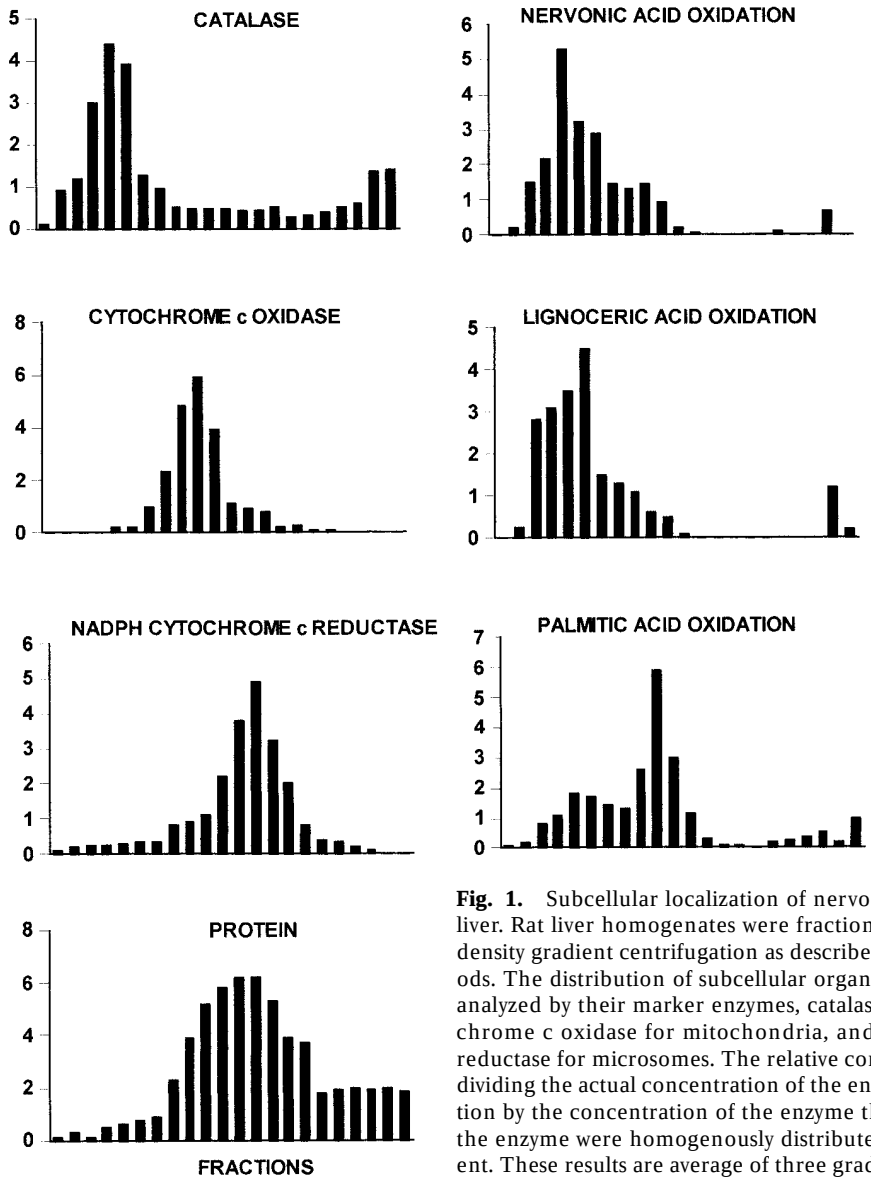

Fig. 1. Subcellular localization of nervonic acid oxidation in rat liver. Rat liver homogenates were fractionated by differential and density gradient centrifugation as described in Materials and Methods. The distribution of subcellular organelles in the gradient was analyzed by their marker enzymes, catalase for peroxisomes, cytochrome c oxidase for mitochondria, and NADPH cytochrome c reductase for microsomes. The relative concentration is derived by dividing the actual concentration of the enzyme in a particular fraction by the concentration of the enzyme that would be observed if the enzyme were homogenously distributed throughout the gradient. These results are average of three gradients.

somes and microsomes and that the specific activity is higher in microsomes. In agreement with previous reports, palmitoyl-CoA ligase had a trimodal distribution in peroxisomes, mitochondria, and microsomes with highest activity being in microsomes (6, 7, 29).

Studies from this laboratory have previously demonstrated that phytanic acid is α-oxidized in different organelles in humans and rodents (42). Therefore, it was thought imperative to study the site of oxidation of nervonic acid in human tissue. **Figure 2** shows the effect of etomoxir, a specific inhibitor of mitochondrial β-oxidation, on nervonic, lignoceric, and palmitic acid oxidation in normal human cultured skin fibroblasts. Consistent with the fact that mitochondria are the site for the oxidation of palmitic acid, etomoxir inhibited palmitic acid oxidation by 97%; on the other hand, etomoxir had no effect on the oxidation of lignoceric acid and nervonic acid, suggesting that these fatty acids are β-oxidized in an organelle different from mitochondria. In fibroblasts from patients with a defect in peroxisomal β-oxidation, the oxidation of nervonic acid was deficient in ZS and was reduced in X-ALD, whereas it was normal in RD and RCDP (**Table 3**). These data further support the fact that the oxidation of nervonic acid is a peroxisomal function.

X-ALD is a peroxisomal disorder characterized by the defective β-oxidation of saturated VLC fatty acids and the results from the present study demonstrate that β-oxidation of monounsaturated VLC fatty acids (nervonic acid) is also impaired in X-ALD. To understand the molecular basis for the deficient activity, the oxidation of nervonic acid was examined in different subcellular organelles purified from control and X-ALD cultured skin fibroblasts. As shown in **Table 4**, the specific activity for the oxidation of nervonic acid in peroxisomes was 7-times higher than mitochondria. The β-oxidation activity for nervonic acid paralleled the β-oxidation activity of lignoceric acid, a peroxisomal function, but not that of palmitic acid, a mitochondrial function. Moreover, the oxidation of nervonic

TABLE 1. Specific actvity for oxidation of fatty acids in subcellular fractions from rat liver

	Fatty Acid Oxidation			
	Homogenate	Peroxisomes	Mitochondria	Microsomes
	nmol/h/mg protein			
Nervonic acid	1.60 ± 0.17	6.05 ± 0.78	1.17 ± 0.23	0.07 ± 0.03
Lignoceric acid	0.35 ± 0.06	1.49 ± 0.32	0.18 ± 0.09	0.01 ± 0.01
Palmitic acid	3.28 ± 0.99	3.52 ± 0.75	11.66 ± 2.62	0.14 ± 0.05

Subcellular organelles were isolated by Nycodenz gradient from rat liver and the rate of oxidation of fatty acids was studied as described in Materials and Methods. Values are mean ± SD of three separate sets of experiments.

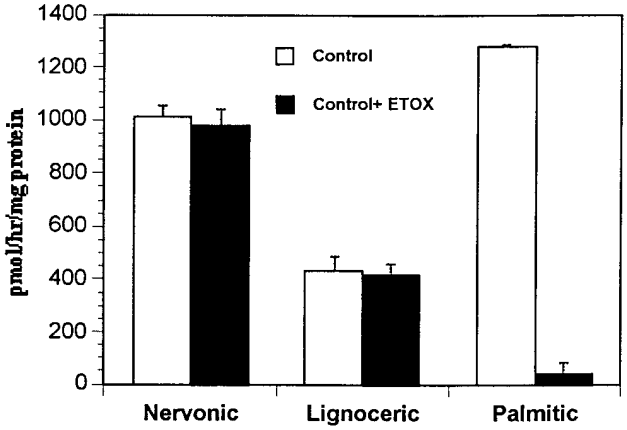

Fig. 2. Effect of etomoxir on the oxidation of nervonic, lignoceric, and palmitic acids in human fibroblasts. Fibroblasts were prepared and incubated in Hank's medium with 0.01 mm etomoxir for 30 min and the oxidation was measured as described in Materials and Methods. Values are mean ± SD of three separate experiments.

acid was deficient in peroxisomes purified from cultured skin fibroblasts of X-ALD patients. The rate of oxidation of lignoceric acid was 22% of the control, while that of nervonic acid was 32% of the control in peroxisomes purified from X-ALD fibroblasts. These results indicate that similar to lignoceric acid (saturated VLC fatty acids), the oxidation of nervonic acid (unsaturated VLC fatty acids) is deficient in peroxisomes from X-ALD. These observations led us to investigate the levels of saturated and unsaturated VLC fatty acids in cultured skin fibroblasts from patients with ZS, X-ALD, rhizomelic chondrodysplasia punctata (RCDP), and Refsum disease (RD). In agreement with previous findings from various laboratories, the levels of saturated VLC fatty acid ($C_{26:0}/C_{22:0}$) were higher in peroxisomal disorders with an abnormality in β-oxidation of VLC fatty acid (9-fold in ZS and 5-fold in X-ALD), and normal in peroxisomal disorders with normal VLC fatty acid β-oxidation activity (RCDP and RD) (**Table 5**). Similarly, levels of monounsaturated VLC fatty acids ($C_{26:1}/C_{22:0}$; $C_{26:1}/C_{22:1}$) were higher in cultured skin fibroblasts from disorders with abnormal peroxisomal β-oxidation (ZS and X-ALD) and normal in cultured skin fibroblasts from disorders with normal peroxisomal β-oxidation (RCDP and RD). These results clearly demonstrate that X-ALD cells accumulate excessive amounts of unsaturated VLC fatty acids due to an abnormality in their β-oxidation in peroxisomes. In X-ALD, there is excessive accumulation of C_{26} fatty acids. The amounts of C_{26} and C_{26}/C_{22} ratio are used as a diagnostic tool. Although ALD cells have little or no increase in C_{24} fatty acids, the enzymatic studies with C_{24} do show a defect in its oxidation in peroxisomal disorders of VLC fatty acid oxidation. Therefore, [1-^{14}C]C_{24} fatty acid (e.g., lignoceric acid) has been the substrate of choice for enzymatic studies because of the unavailability of radiolabeled fatty acids greater than C_{24}.

In peroxisomes, fatty acids are activated to their CoA-derivatives by acyl-CoA ligases present in the limiting membrane and then oxidized by the β-oxidation enzyme system present in the matrix of peroxisomes. Saturated VLC fatty acids (e.g., lignoceric and cerotic acid) accumulate in excessive amounts in X-ALD due to the deficient activity of lignoceroyl-CoA ligase in peroxisomes (7, 14). To understand the cellular basis for the deficient β-oxidation and excessive accumulation of nervonic acid in X-ALD, we examined the activities of nervonyl-CoA ligase and the β-oxidation of nervonic acid in disrupted peroxisomes from X-ALD and control fibroblasts. As shown in **Table 6**, oxidation of nervonyl-CoA was normal in disrupted peroxisomes from X-ALD fibroblasts, whereas oxidation of nervonic acid was deficient. These observations suggest that in X-ALD, the deficient activity of nervonic acid oxidation is due to an abnormality in the activation of nervonic acid to nervonyl-CoA, a peroxisomal membrane function. Next, we examined the rate of activation of nervonic acid and other fatty acids (lignoceric and

TABLE 2. Specific activity for fatty acyl-CoA ligase in subcellular fractions from rat liver

	Fatty Acyl-CoA Ligase			
	Homogenate	Peroxisomes	Mitochondria	Microsomes
	nmol/min/mg protein			
Nervonic acid	3.07 ± 0.05	9.91 ± 0.78	1.50 ± 0.33	39.48 ± 3.89
Lignoceric acid	1.12 ± 0.11	5.14 ± 0.81	0.66 ± 0.04	26.35 ± 1.76
Palmitic acid	17.71 ± 1.55	30.57 ± 2.94	73.75 ± 5.77	84.96 ± 9.27

Subcellular organelles were isolated by Nycodenz gradient from rat liver and the activation of fatty acids catalyzed by acyl-CoA ligase was studied as described in Materials and Methods. Values are mean ± SD of three separate sets of experiments.

TABLE 3. Fatty acid oxidation ratios in fibroblasts from control and patients with peroxisomal disorders

	Fatty Acid Oxidation	
	24:1/16:0	24:0/16:0
Control	0.85 ± 0.11	0.36 ± 0.05
Zellweger syndrome	0.06 ± 0.03	0.03 ± 0.02
X-linked adrenoleukodystrophy	0.32 ± 0.06	0.09 ± 0.03
Refsum's disease	0.87 ± 0.21	0.30 ± 0.04
Rhizomelic chondrodysplasia punctata	0.74 ± 0.11	0.30 ± 0.01

Fatty acid oxidation was studied in freshly prepared fibroblasts suspended in Hank's balanced salt solution as described in Materials and methods. Values are mean ± SD of four separate experiments.

TABLE 4. Rate of oxidation of fatty acid in subcellular fractions from control and X-ALD fibroblasts

	Fatty Acid Oxidation		
	Nervonic Acid	Lignoceric Acid	Palmitic Acid
	nmol/h/mg protein		
Control			
Homogenate	0.23 ± 0.07	0.08 ± 0.02	1.97 ± 0.16
Peroxisomes	0.69 ± 0.14	0.19 ± 0.05	10.53 ± 1.63
Mitochondria	0.09 ± 0.02	0.02 ± 0.01	12.67 ± 1.39
Microsomes	0.03 ± 0.02	0.02 ± 0.01	0.92 ± 0.33
X-ALD			
Homogenate	0.12 ± 0.02	0.04 ± 0.01	1.76 ± 0.25
Peroxisomes	0.22 ± 0.04	0.04 ± 0.02	9.46 ± 1.63
Mitochondria	0.06 ± 0.02	0.02 ± 0.01	11.32 ± 2.44
Microsomes	0.01 ± 0.00	0.01 ± 0.00	0.77 ± 0.55

Subcellular fractions were prepared from control and X-ALD fibroblasts by Nycodenz gradient and the rate of oxidation of different fatty acids was measured as described in Materials and Methods. Values are mean ± SD of three separate sets of experiments.

TABLE 6. Oxidation of VLC fatty acids and their CoA esters in disrupted peroxisomes from control and X-ALD fibroblasts

	Fatty Acid Oxidation	
	Control	X-ALD
	nmol/h/mg protein	
Nervonic acid	0.268 ± 0.03	0.148 ± 0.02
Nervonyl-CoA	5.989 ± 1.27	5.554 ± 0.94
Lignoceric acid	0.065 ± 0.02	0.026 ± 0.01
Lignoceroyl-CoA	0.998 ± 0.22	0.823 ± 0.13

Peroxisomes were purified from control and X-ALD skin fibroblasts by Nycodenz gradient. Oxidation of fatty acids and their CoA esters was carried out as described in Materials and Methods. Values are mean ± SD of three sets of observations.

palmitic acids) in disrupted peroxisomes. As shown in **Table 7**, the activation of nervonic acid to nervonyl-CoA was deficient in X-ALD peroxisomes, whereas nervonyl-CoA ligase activity in microsomes from X-ALD was similar to the control. The observed normal activation of palmitic acid as compared to deficient activation of lignoceric acid in peroxisomes from X-ALD is in agreement with our previous findings (7, 14) and those of others (43).

To understand the differences and/or similarity in the molecular defect in the activation and oxidation of nervonic and lignoceric acid, we examined the oxidation of nervonic and lignoceric acids in X-ALD fibroblasts after transfection with cDNA for human ALDP. Transfected human ALDP cDNA expressed and targeted ALD protein to peroxisomes in ALD cells, as evident from the punctate immunofluorescence detected with antibodies against the C-terminal of ALDP (**Fig. 3**). As shown in **Fig. 4**, transfection of the ALD gene in X-ALD fibroblasts normalized the oxidation of both nervonic and lignoceric acids, indicating that abnormality in the oxidation of nervonic acid as well as lignoceric acid is likely to be caused by the same molecular defect. Next, to understand whether the same VLC acyl-CoA ligase was involved in the activation of lignoceric acid and nervonic acid, preimmune sera (IgG) or antibodies (IgG) against lignoceroyl-CoA ligase were used to immunoprecipitate the acyl-CoA ligase activity. **Figure 5** shows that the addition of antibodies against the N-terminal peptide of lignoceroyl-CoA ligase precipitated both the nervonyl-CoA and lignoceroyl-CoA ligase activities as observed by the loss of the enzyme activities from the supernatant. However, no effect on palmitoyl-CoA ligase activity was observed. Furthermore, IgG from preimmune sera had no effect on any of the ligase activities studied. An interesting question arises from these observations. If the molecular defect in the oxidation of nervonic and lignoceric acids is the same, why is there a greater accumulation of $C_{26:0}/C_{22:0}$ (5.2 times as compared to control) as compared to $C_{26:1}/C_{22:1}$ (2.6 times as compared to control) in X-ALD (Table 5)? One of the explanations may be that nervonic acid in cells or peroxisomes from X-ALD as well as control fibroblasts is oxidized at a higher rate than lignoceric acid (Tables 3 and 4). As VLC fatty acids are mostly present in brain tissue, we also examined the oxidation of lignoceric and nervonic acids in cells isolated from rat brain (**Fig. 6**). Similar to liver (Table 1) and fibroblasts (Table 3), a higher rate of oxidation of nervonic acid than lignoceric acid was also observed in oligodendrocytes and astrocytes isolated from rat brain.

DISCUSSION

The studies described in this manuscript clearly demonstrate that in rodents as well as in humans nervonic acid is oxidized in peroxisomes. This conclusion is based on the following observations. *1*) The subcellular distribution of nervonic acid β-oxidation followed the same distribution pattern as for that catalase and lignoceric acid β-oxidation (peroxisomal markers) in the Nycodenz gradient. *2*) The specific activity for oxidation of nervonic acid in peroxi-

TABLE 5. Composition of saturated and monounsaturated VLC fatty acids in fibroblasts from control and patients with peroxisomal disorders

	24:0/22:0	26:0/22:0	24:1/22:0	26:1/22:0	26:1/22:1
Control	1.32 ± 0.20	0.04 ± 0.01	2.25 ± 0.46	0.05 ± 0.01	0.25 ± 0.05
ZS	2.48 ± 0.19	0.36 ± 0.07	4.95 ± 0.99	0.92 ± 0.30	2.34 ± 0.08
X-ALD	1.98 ± 0.54	0.21 ± 0.02	2.44 ± 0.62	0.15 ± 0.04	0.65 ± 0.07
RCDP	1.74 ± 0.06	0.04 ± 0.01	2.19 ± 0.28	0.04 ± 0.01	0.26 ± 0.06
RD	1.45 ± 0.35	0.04 ± 0.02	2.15 ± 0.38	0.05 ± 0.02	0.28 ± 0.10

Fatty acid analyses of freshly prepared fibroblasts were carried out as described in Materials and Methods. Values are mean ± SD of four sets of experiments.

TABLE 7. Rate of activation of fatty acid in subcellular fractions from control and X-ALD fibroblasts

	Fatty Acid Activation		
	Nervonic Acid	Lignoceric Acid	Palmitic Acid
	nmol/min/mg protein		
Control			
Homogenate	1.06 ± 0.13	0.26 ± 0.04	8.26 ± 2.34
Peroxisomes	3.69 ± 0.72	0.92 ± 0.18	16.67 ± 3.89
Mitochondria	0.46 ± 0.06	0.03 ± 0.01	20.65 ± 2.19
Microsomes	8.41 ± 1.17	1.94 ± 0.29	23.77 ± 6.43
X-ALD			
Homogenate	0.96 ± 0.21	0.22 ± 0.06	8.26 ± 2.34
Peroxisomes	1.29 ± 0.39	0.19 ± 0.07	15.28 ± 2.31
Mitochondria	0.35 ± 0.05	0.04 ± 0.03	21.32 ± 3.98
Microsomes	7.83 ± 1.93	2.07 ± 0.47	24.18 ± 4.86

Subcellular fractions were isolated from control and X-ALD fibroblasts by Nycodenz gradient and the activation of different fatty acids was measured as described in Materials and Methods. Values are mean ± SD of three separate sets of experiments.

somes purified from rat liver or human cultured skin fibroblasts was higher than the activity observed in mitochondria and microsomes, respectively. *3*) Etomoxir, a specific inhibitor of mitochondrial fatty acid oxidation that specifically inhibits the activity of carnitine palmitoyl transferase I, had no effect on the oxidation of lignoceric acid (a peroxisomal function) or on that of nervonic acid, whereas it inhibited the oxidation of palmitic acid by 97% (a predominant mitochondrial function). The lack of an effect of etomoxir on the oxidation of nervonic acid in human cultured skin fibroblasts indicates that nervonic acid oxidation in humans is also a peroxisomal function. Previous studies from our laboratory (6, 7) and others (8) have demonstrated that saturated VLC fatty acids (lignoceric and cerotic acids) are oxidized in peroxisomes. Identification of peroxisomes as the site of nervonic acid oxidation indicates that unsaturated VLC fatty acids are also oxidized in peroxisomes. To demonstrate that nervonic acid is, in fact, oxidized in peroxisomes in rodents and humans is important because the peroxisomal functions are known to differ between species. Phytanic acid, a branched chain fatty acid, was shown to be oxidized in mitochondria in rodents and in peroxisomes in humans (42). Similarly, pipecolic acid was found to be catabolized in mitochondria in rodents and in peroxisomes in monkeys (45).

Excessive accumulation of saturated VLC fatty acids ($>C_{22:0}$) is the hallmark of X-ALD. In fact, levels of saturated VLC fatty acids ($C_{26:0}$ or $C_{26:0}/C_{22:0}$) are used for prenatal as well as postnatal diagnosis of X-ALD (4).

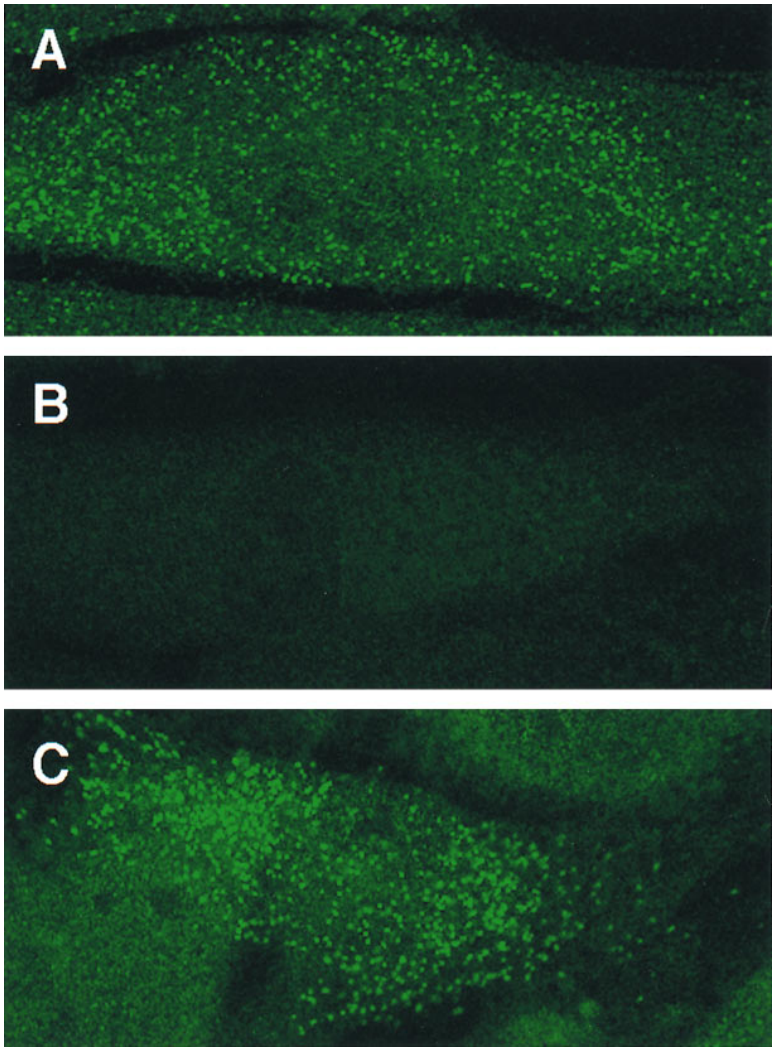

Fig. 3. Immunofluorescence analysis of ALDP in human skin fibroblasts. X-ALD fibroblasts were transfected with ALDP cDNA using Lipofectin. Control, X-ALD, and transfected fibroblasts were treated with 0.1% Triton X-100 and incubated with anti-ALDP antibodies. Indirect immunofluorescence was performed as described in Materials and Methods. A: Control; B: X-ALD; C: X-ALD transfected with ALDP.

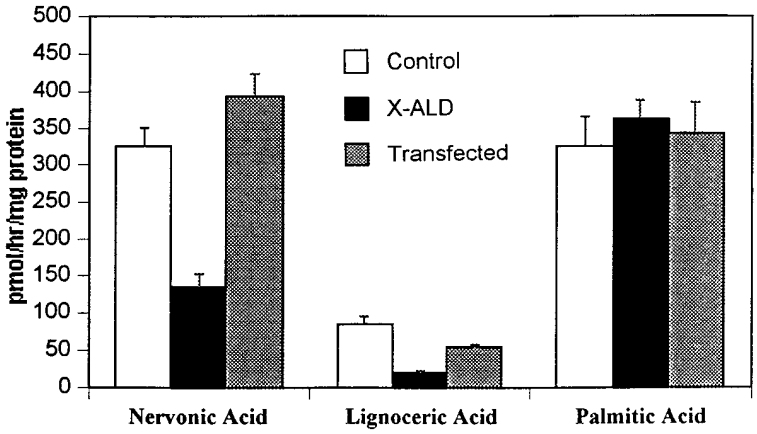

Fig. 4. Nervonic, lignoceric, and palmitic acid oxidation activity of X-ALD fibroblasts transfected with human ALDP cDNA. X-ALD fibroblasts were transfected with ALDP cDNA using Lipofectin and the oxidation of different fatty acids was studied after 10 days as described in Materials and Methods. Values are mean ± SD of three sets of observations.

Higher levels of $C_{24:1}/C_{22:0}$, $C_{26:1}/C_{22:0}$, and $C_{26:1}/C_{22:1}$ in fibroblasts from patients with defective peroxisomal β-oxidation (e.g., ZS and X-ALD) and normal oxidation and levels of VLC fatty acids in peroxisomal disorders with normal peroxisomal β-oxidation activity (RD and RCDP) support the conclusion that similar to the abnormality in saturated VLC fatty acid (e.g., lignoceric acid), the β-oxidation of monoenoic VLC fatty acids (e.g., nervonic acid) is also deficient in X-ALD. Excessive accumulation of monoenoic VLC fatty acids in the phosphatidylcholine fraction of X-ALD brain tissue was also reported previously (16). The defect in the metabolism of fatty acids greater than C_{22} is abnormal in X-ALD. Relatively small or no increase in C_{24} fatty acids as compared to 3- to 6-fold increase in C_{26} in X-ALD may be due to the fact that C_{24} fatty acids are substrates, to some degree, for an enzyme system for oxidation of C_{20}–C_{22} chain length fatty acids, the fatty acids with normal β-oxidation in X-ALD. However, deficient β-oxidation of C_{24} is observed in all disorders of VLC fatty acid β-oxidation. Therefore, C_{24} fatty acids as a representative of VLC fatty acids have been the substrate of choice for enzymatic studies because of the unavailability of radiolabeled fatty acids greater than C_{24}. Moreover, C_{26} is relatively less soluble in aqueous media and gives low activity.

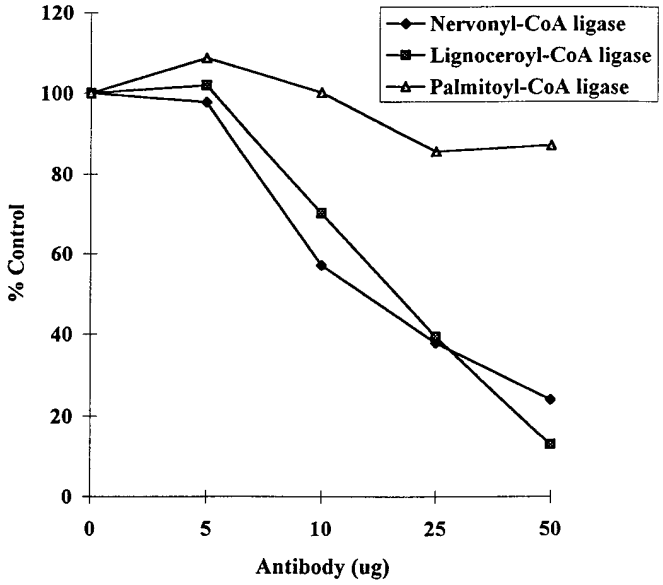

Fig. 5. Immunoprecipitation of peroxisomal VLC acyl-CoA ligase activity. Peroxisomes solubilized in 0.2% Triton X-100, 1% n-octyl-α-d-glucopyranoside, 0.5% mm EDTA, 2 mm ATP, and 10 mm potassium phosphate buffer, pH 7.5, were treated with increasing concentration of anti-lignoceroyl-CoA ligase antibodies. The supernatants were assayed for acyl-CoA ligase activity for palmitic, nervonic, and lignoceric acids as described in Materials and Methods. Values are mean of two sets of experiments.

The enzyme for activation of VLC fatty acids is a component of the peroxisomal limiting membrane, while the enzyme system for the β-oxidation of fatty acids is present in the matrix of peroxisomes (1, 2, 46). Our results showing the normal oxidation of nervonyl-CoA by peroxisomal matrix proteins as compared to the deficient oxidation of nervonic acid and deficient activity of nervonyl-CoA ligase in purified peroxisomes from X-ALD fibroblasts indicates a defect in the peroxisomal membrane component associated with the activation of nervonic acid to nervonyl-CoA. A similar defect in the activation of lignoceric acid was reported previously (6–8, 43). Biochemical studies showing defects in the activation of VLC fatty acids in peroxisomes from X-ALD suggested that the gene for VLC acyl-CoA ligase (lignoceryl CoA ligase) is a candidate for the X-ALD gene (14). Identification of the X-ALD gene by positional cloning led to a different conclusion (47). The X-ALD gene product is a 75 kDa peroxisomal membrane protein with significant homology with the 70 kDa peroxisomal membrane protein (PMP70), a member of the ATP binding cassette (ABC) transporter superfamily (18, 47). Restoration of the oxidation of both lignoceric and nervonic acids in X-ALD cultured skin fibroblasts after transfection of human ALDP cDNA demonstrates that excessive accumulation of VLC fatty acids and deficient oxidation of nervonic and lignoceric acids in X-ALD is most likely due to the same defect in the peroxisomal membrane. Correction of saturated VLC fatty acid metabolism in ALD fibroblasts after 10 days of transfection with cDNA for ALDP was demonstrated previously (19, 20). Although the transfection of cDNA for ALDP corrects the metabolic defect in X-ALD cells, the precise function of ALDP in the peroxisomal membrane is not known at present. Its loss or mutation results in partial loss of activi-

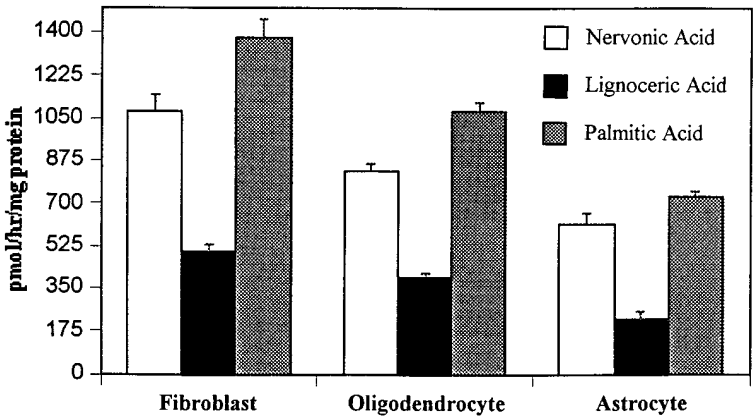

Fig. 6. Oxidation of nervonic, lignoceric, and palmitic acids in fibroblasts and glial cells. Cells were cultured and suspended in Hank's balanced solution and the rate of oxidation of fatty acids was measured in as described in Methods. Values are mean ± SD of three separate sets of observations.

ties of VLC acyl-CoA ligase (e.g., lignoceroyl-CoA ligase) peroxisomal β-oxidation of VLC fatty acids. ALDP, as a component of peroxisomal membranes, may influence the activity of VLC acyl-CoA ligase (lignoceroyl- and nervonyl-CoA ligases) by the following mechanisms. ALDP may be responsible for the entry of substrates (VLC fatty acids, CoASH, and ATP) into peroxisomes or incorporation of VLC acyl-CoA ligase in the peroxisomal membranes. Alternatively, ALDP may serve a structural function in the peroxisomal membrane necessary for the activity of VLC acyl-CoA ligase, probably as a complex or as an anchor for the enzyme.

The relatively higher accumulation of saturated VLC fatty acids than monoenoic VLC fatty acids in X-ALD raises an interesting question. If the abnormal oxidation of nervonic and lignoceric acid is due to the same molecular defect then why should there be a greater accumulation of saturated VLC fatty acids than the monounsaturated fatty acids (Table 5)? The higher rates of oxidation of nervonic acid than lignoceric acid may account, at least in part, for these observed differences. The rate of oxidation of nervonic acid was 3 times higher than that of lignoceric acid in the control fibroblasts (Tables 3 and 4). Moreover, the residual activity for oxidation of nervonic acid (32% of control) was higher than that for lignoceric acid (22% of control) in X-ALD fibroblasts. In fact, the residual specific activity for nervonic acid oxidation (0.219 ± 0.043 nmol/h/mg protein) in peroxisomes from X-ALD fibroblasts was almost equivalent to the activity of lignoceric acid oxidation (0.193 ± 0.054 nmol/h/mg protein) in peroxisomes from control cells (Table 4). As nervonic and lignoceric acids are present predominantly in brain, we examined the rate of their oxidation in primary oligodendrocytes and astrocytes isolated from rat brain (Fig. 6). Similarly, a higher rate of nervonic acid oxidation as compared to lignoceric acid oxidation was observed in primary rat astrocytes and oligodendrocytes. The higher rate for the oxidation of nervonic acid as compared to that of lignoceric acid may be due to the structural differences affecting the affinity of the substrate for the enzyme. Long chain unsaturated fatty acids are known to be oxidized more efficiently by peroxisomes than their saturated counterparts (48).

X-ALD gained prominence in the public domain with the release of the popular film "Lorenzo's Oil," depicting the life of an X-ALD patient and the development of a specialized oil therapy for X-ALD patients. Treatment of X-ALD patients with trioleate resulted in 50% reduction of saturated fatty acid. Subsequently, treatment of X-ALD patients with a mixture of trioleate (GTO) and trieruciate (GTE), popularly known as "Lorenzo's oil," led to normalization of the plasma levels of VLC fatty acids (9, 49, 50). The fatty acid therapy was developed on the assumption that saturated VLC fatty acids are toxic and that unsaturated VLC fatty acids are nontoxic. The monoenoic fatty acids (oleic and erucic acids), when given in excess inhibit the chain elongation of palmitic and stearic acids to saturated VLC fatty acids by competing for the same enzyme system with the result that there is higher accumulation of unsaturated VLC fatty acids in X-ALD patients on diet therapy (50). Unfortunately, the clinical efficacy has been unsatisfactory as no proof of favorable effects has been observed. However, recent reports showing the toxicity of erucic acid in neonatal ALD and Zellweger cells (21) and induction of superoxide, a highly reactive oxygen free radical, in neutrophils by poly or monoenoic VLC fatty acids (22) suggest that unsaturated fatty acids may have a detrimental effect for X-ALD patients, especially when studies reported in this manuscript clearly show that monoenoic VLC fatty acids (e.g., nervonic acid) are oxidized in peroxisomes and this activity is impaired in X-ALD.

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