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Structure and lipid distribution of polyenoic very-long-chain fatty acids in the brain of peroxisome-deficient patients (Zellweger syndrome)

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The polyenoic fatty acids with carbon chain lengths from 26 to 38 (very-long-chain fatty acids, VLCFA) previously detected in abnormal amounts in Zellweger syndrome brain have been shown to be $n-6$ derivatives and therefore probably derived by chain elongation of shorter-chain $n-6$ fatty acids such as linoleic acid and arachidonic acid. Polyenoic VLCFA are also present in Zellweger syndrome liver, but this tissue differs significantly from brain in that the saturated and mono-unsaturated derivatives are the major VLCFA. Zellweger syndrome brain polyenoic VLCFA are present in the neutral lipids predominantly in cholesterol esters, with smaller amounts in the non-esterified fatty acid and triacylglycerol fractions. These fatty acids are barely detectable in any of the major phospholipids, but are present in significant amounts in an unidentified minor phospholipid. The polyenoic VLCFA composition of this lipid differs markedly from that observed for all other lipids, as it contains high proportions of pentaenoic and hexaenoic fatty acids with 34, 36 and 38 carbon atoms. A polar lipid with the chromatographic properties in normal brain contains similar fatty acids. It is postulated that the polyenoic VLCFA may play an important role in normal brain and accumulate in Zellweger syndrome brain because of a deficiency in the peroxisomal β-oxidation pathway, although a possible peroxisomal role in the control of carbon-chain elongation cannot be discounted.

INTRODUCTION

Igarashi *et al.* (1976) reported that tissue from patients with X-linked adrenoleukodystrophy contained elevated amounts of saturated long-chain fatty acids with carbon chain lengths greater than 22 (very-long-chain fatty acids, VLCFA). More recently Brown *et al.* (1982) showed that VLCFA were increased in Zellweger syndrome, a severe neurodegenerative disorder characterized ultrastructurally by a deficiency of tissue peroxisomes, and we have reported an increase in these lipids in the infantile form of Refsum's disease (Poulos & Sharp, 1984) as well as in other clinically distinct phenotypes (Poulos *et al.*, 1985). Current evidence suggests that VLCFA accumulate because of a deficiency in their β-oxidation, a reaction now thought to take place exclusively in peroxisomes (Singh *et al.*, 1984, 1987). In addition to the increase in saturated VLCFA, Zellweger syndrome and infantile-Refsum's-disease patients show significant increases in mono-unsaturated VLCFA in plasma, skin fibroblasts and liver (Moser *et al.*, 1984*b*; Poulos *et al.*, 1986*c*; Solish *et al.*, 1985). Until recently it was thought that the inherited abnormalities in these disease states only affected saturated and mono-unsaturated fatty acids. However, we reported that the brain from Zellweger syndrome patients contains significant amounts of a homologous series of polyenoic VLCFA with carbon chain lengths 26–38, but neither the structure nor the lipid distribution of these fatty acids was examined (Poulos *et al.*, 1986*a*). In the present paper we report that the polyenoic VLCFA in Zellweger syndrome brain are $n-6$ series fatty acids, and those with carbon chain lengths 26–32 are located predominantly in the cholesterol ester fraction, whereas those with carbon chain lengths 34–38 are located almost exclusively in an uncharacterized minor polar lipid (PL).

MATERIALS AND METHODS

Samples were obtained at postmortem from patients with Zellweger syndrome and stored at either −20 °C or −70 °C for periods ranging from a few months to approx. 7 years before analysis. The diagnosis of Zellweger syndrome was based on clinical history and examination, and was subsequently confirmed by showing (*a*) a marked deficiency in skin fibroblast dihydroxyacetone phosphate acyltransferase (Schutgens *et al.*, 1984), (*b*) an increase in plasma and skin fibroblast $C_{26:0}/C_{22:0}$ and $C_{26:1}/C_{22:0}$ fatty acid ratios (Brown *et al.*, 1982; Moser *et al.*, 1984*b*; Poulos *et al.*, 1986*c*; Solish *et al.*, 1985), (*c*) a marked decrease in ether lipid concentration in brain, liver and cultured skin fibroblasts (Heymans *et al.*, 1984), (*d*) increased pipecolic acid concentrations in urine and/or plasma (Danks *et al.*, 1975; Poulos *et al.*, 1984, 1985). The ages of these patients ranged from 4 days to 6 weeks. Age-matched control samples were obtained at autopsy from two patients who had died from sudden infant death syndrome, from a patient with a congenital heart defect and from a patient who had died from an unrelated

Abbreviations used: VLCFA, very-long-chain fatty acids; PL, polar lipid.

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Table 1. VLCFA in normal and Zellweger syndrome brain

The ratios of the various saturated and pentaenoic VLCFA to docosanoic acid in the brain of age-matched control and Zellweger syndrome patients were measured as described in the Materials and methods section. Mean values are given, with ranges in parentheses. Ratios of hexaenoic and heptaenoic VLCFA were also clearly elevated, but are not shown.

	$C_{24:0}/C_{22:0}$	$C_{26:0}/C_{22:0}$	$C_{24:5}/C_{22:0}$	$C_{26:5}/C_{22:0}$	$C_{28:5}/C_{22:0}$	$C_{30:5}/C_{22:0}$	$C_{32:5}/C_{22:0}$
Control ($n = 4$)	0.73 (0.33–1.0)	0.05 (0.02–0.08)	7.0 (3.2–11.7)	0.21 (0.08–0.31)	0.04 (0.02–0.08)	0.05 (0.03–0.07)	0.02 (0.01–0.03)
Zellweger syndrome ($n = 3$)	0.64 (0.60–0.65)	0.17 (0.14–0.19)	8.6 (4.7–11.2)	3.3 (1.4–4.5)	6.2 (3.4–8.0)	1.3 (0.7–1.8)	0.8 (0.4–1.1)

inherited neurological disease (Krabbe's disease). The ages of control patients ranged from 10 days to 5 months.

Lipids were extracted from whole brain and liver, and fatty acid methyl esters were prepared from the extracts and subjected to g.l.c. as described previously (Poulos *et al.*, 1986*a*,*b*). For some experiments the total lipids extracted from 1 g of brain were applied to a column (1 cm diam.) of silicic acid (Sigma) at a loading of 25 mg of lipid/g of silicic acid. The neutral lipids, glycolipids and phospholipids were eluted with 10 column vol. of chloroform, 40 column vol. of acetone and 10 column vol. of methanol respectively (Christie, 1982). A ganglioside fraction was isolated from the brain extract as described by Igarashi *et al.* (1976). The phospholipid fraction was resolved into acidic and non-acidic components by applying it to a column (2 cm × 1 cm diam.) of DEAE-cellulose DE52 (acetate form; Whatman). Non-acidic phospholipids were eluted with 10 column vol. of chloroform/methanol (1:1, v/v). Acidic phospholipids were eluted with 10 column vol. of chloroform/methanol (1:1, v/v) made 0.05 M with respect to ammonium acetate, to which was added 20 ml of 28% (w/v) NH_3/litre (Christie, 1982). Both the neutral lipids and the phospholipids were further resolved by preparative t.l.c. on silica-gel 60 plates (Merck) in hexane/diethyl ether/acetic acid (90:10:1, by vol.) for neutral lipids, or chloroform/methanol/water (35:15:2, by vol.) (solvent A) for phospholipids. For some experiments an alternative phospholipid solvent, B [chloroform/methanol/28% (w/v) NH_3, 12:6:1, by vol.] was used. Lipids were detected by spraying the plates with 0.2% (w/v) dichlorofluorescein in 95% (v/v) ethanol, and the various zones were eluted with chloroform/methanol (2:1, v/v). After removal of the dye by partitioning, the lipids were trans-esterified and the resulting fatty acid esters were subjected to g.l.c. (Poulos *et al.*, 1986*a*). G.l.c. analysis and quantification of fatty acids with up to 24 carbon atoms was carried out as described by Gibson & Kneebone (1981).

VLCFA data were expressed as the ratio of the peak height of a particular component relative to the peak height of docosanoic acid, e.g. $C_{24:0}/C_{22:0}$ ratio represents the ratio of the peak height of lignoceric acid ($C_{24:0}$) relative to docosanoic acid ($C_{22:0}$). Data were also expressed as the peak height of an individual fatty acid as a percentage of the combined peak heights of all fatty acids with carbon chain lengths greater than 14. Absolute amounts of VLCFA were determined by a comparison of the peak heights of a particular fatty acid relative to a $C_{30:0}$ fatty acid internal standard (11–15 μg of $C_{30:0}$ fatty acid added/g wet wt. of brain). It should be emphasized that the values obtained are an approximation, since polyenoic VLCFA standards were not available to permit us to determine their detector response.

G.l.c./mass spectrometry was performed on a JEOL JMS DX-303 mass spectrometer operating in electron-impact mode and equipped with a Hewlett–Packard 5890 gas chromatograph and a JEOL JMA-DA 5000 data system. Separation of the fatty acid methyl esters to determine chain length was accomplished with a Scientific Glass Engineering (SGE) BP-1 bonded-phase silica column (12 m × 0.22 mm internal diam. × 0.25 μm phase thickness), temperature programmed from 160 to 320 °C at 4 °C/min, with He gas (1 ml/min) as the carrier, which was directly inserted into the mass-spectrometer source.

Characterization of the degree of unsaturation by argentation chromatography and subsequent hydrogenation was performed as described previously (Poulos *et al.*, 1986*a*).

Identification of the various lipids was based on a comparison of their t.l.c. mobility with that of authentic standards. In some cases, additional confirmation was obtained by t.l.c. identification of the products released after acid or alkaline hydrolysis.

Acid hydrolysis of lipids was carried out in 3 M-HCl for 3 h at 100 °C. After extraction of lipids with hexane, the aqueous layer was dried under a stream of nitrogen at 50 °C. The residue was dissolved in ethanol/water (1:1, v/v), and samples were chromatographed on silica-gel 60 plates in butanol/acetic acid/water (4:2:1, by vol.). Alkaline hydrolysis was carried out in 10% (w/v) KOH dissolved in ethanol/water (9:1, v/v) for 18 h at 110 °C. After neutralization, the lipid products were extracted into hexane, and samples were chromatographed on silica-gel 60 plates (Merck) in dichloromethane. Dragendorff and ninhydrin spray reagents (for quaternary and primary amines respectively) were prepared as described by Skipski & Barclay (1969). An iodoplatinate spray agent for nitrogenous bases was prepared as described by Clarke (1969). Lipid phosphate (P) was assayed as described by Owens (1966).

Phospholipase C hydrolysis was carried out with non-purified commercial preparations of enzyme (from *Bacillus cereus*; Sigma) as described by Kiyasu & Kennedy (1960). The lipid products of the reaction were applied as a 3 cm band to a 20 × 20 cm silica-gel 60 plate (Merck) and developed up to 10 cm with chloroform/methanol/water (35:15:2, by vol.). After air drying for

a few minutes, the plate was developed in the same direction to the top of the plate with hexane/diethyl ether/acetic acid (70:30:1, by vol.). The various zones were located by spraying with 0.2% dichlorofluorescein in 95% ethanol and then eluted with chloroform/methanol (2:1, v/v). After removal of the dye by partitioning, the eluted lipids were trans-esterified and any resulting fatty acid esters were subjected to g.l.c.

RESULTS AND DISCUSSION

As reported previously Zellweger syndrome brain contains a greatly increased proportion of polyenoic fatty acids with carbon chain lengths from 26 to 38 and with five, six or seven double bonds (Poulos *et al.*, 1986*a*). The ratio of the saturated VLCFA, hexacosanoic acid ($C_{26:0}$), to docosanoic acid ($C_{22:0}$) ($C_{26:0}/C_{22:0}$), a biochemical marker for abnormalities in VLCFA metabolism (Moser *et al.*, 1980, 1984*a*), was moderately elevated, but much greater ratios were observed for the polyenoic VLCFA (Table 1). The increases in polyenoic acid/$C_{22:0}$ ratios were paralleled by equally marked increases in absolute amounts of these fatty acids in Zellweger syndrome brain. For example, one of the Zellweger syndrome brains examined contained 16, 40, 8 and 4 μg of $C_{26:5}$, $C_{28:5}$, $C_{30:5}$ and $C_{32:5}$ fatty acids respectively/g wet wt., compared with 2.6, 0.7, 0.2 and 0.2 μg/g wet wt. from an age-matched control brain. Shorter-chain (< 24 carbon atom) polyenoic fatty acids were present in Zellweger syndrome brain in relatively normal proportions, except for decreases in $C_{22:6,n-3}$ (5.8 versus 1.5% of total fatty acids; means of two control versus two Zellweger syndrome brains) and $C_{22:4,n-6}$ (4.9 versus 2.1% total fatty acids), with a moderate increase in the amounts of mono-unsaturated fatty acids compared with controls also being observed. The position of the various double bonds has been confirmed by mass-spectrometric analysis, which indicates that the fatty acids are members of the $n-6$ series and are therefore probably derived from linoleic acid and arachidonic acid. Evidence for their structure is based on the detection of an ion fragment at m/z 150 in the spectra of all of the fatty acids, a fragment that we have observed in high abundance only in $n-6$ series fatty acids (Fellenberg *et al.*, 1987). Zellweger syndrome liver differs considerably from brain in that it contains a mixture of saturated, mono-unsaturated and polyenoic VLCFA (Fig. 1). The saturated and mono-unsaturated fatty acids consist of both even and odd numbers of carbon atoms.

Fractionation of the lipids isolated from Zellweger syndrome brain by silicic acid column chromatography demonstrates that the polyenoic VLCFA are components of the three major lipid species, i.e. neutral lipids, glycolipids and phospholipids, but they could not be detected in the gangliosides. Although the triacylglycerol and non-esterified fatty acids contained significant proportions, the most dramatic increase was observed in cholesterol esters (Fig. 2*a*) where approx. 25% of the total fatty acids were polyenoic VLCFA with carbon chain lengths of 26 or greater. These fatty acids were detected in significant amounts (up to 10% of total fatty acids) in only one polar lipid (PL) (Fig. 2*b*), a non-acidic phospholipid which migrates with a slightly greater R_F on t.l.c. in neutral solvents (solvent A) than phosphatidylcholine and which moves near phosphatidylethanolamine in alkaline solvents (solvent B). By using a combination of silicic acid and DE-52 column chromatography and preparative t.l.c., we have succeeded in isolating sufficient PL from Zellweger syndrome brain (20 nmol of lipid P/g wet wt. of brain) to undertake a preliminary structural investigation. PL is alkali-labile, contains P, and is hydrolysed by phospholipase C to produce a lipid with a similar fatty acid composition to the parent lipid, and which we have identified on the basis of its chromatographic mobility as a diacylglycerol. Acid hydrolysis of PL releases a water-soluble product which does not react with ninhydrin or the Dragendorff reagent, but which reacts weakly with iodoplatinate reagent, indicating that it may possess a nitrogenous base. Further studies are currently hampered by our inability to obtain sufficient material from patients. A similar compound may also be present in normal neonatal brain, because we have observed polyenoic VLCFA in a phosphorus-containing lipid which possesses the same chromatographic properties as PL. Although its identity is unknown, thus far we have been able to exclude all of the major polar lipids found in brain, including phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolamine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol and cardiolipin, as well as the more usual molecular species of phosphatidylcholine. Comparison of the composition of the cholesterol ester and PL fractions revealed significant differences. Whereas $C_{26:5}$ and $C_{28:5}$ were the major polyenoic VLCFA in cholesterol esters, ultra-long-chain fatty acids with 34 and 36 carbon atoms and with five and six double bonds were quantitatively the most important in Zellweger syndrome brain PL. These last fatty acids were not detected in cholesterol esters.

Our results demonstrate clearly that the VLCFA which accumulate in Zellweger syndrome brain are predominantly polyenoic with even numbers of carbon atoms, whereas in Zellweger syndrome liver all VLCFA species, i.e. saturated, mono-unsaturated and polyenoic fatty acids, are present. Although most of the liver VLCFA were also fatty acids with even numbers of carbon atoms, pentacosanoic acid ($C_{25:0}$) was clearly present (Fig. 1). The differences between brain and liver are marked and are not easy to explain. Both Zellweger syndrome brain and liver contain substantial amounts of the presumed precursors of the saturated and mono-unsaturated VLCFA, i.e. stearic acid and oleic acid respectively (Bourre *et al.*, 1978). Yet elongation of these precursors, to $C_{26:0}$ or $C_{26:1}$ and beyond, only appears to have taken place to any degree in the liver. It is likely that the relatively low concentration of saturated and mono-unsaturated VLCFA in Zellweger syndrome brain is a reflection of the low activity of the C_{18} elongase in the neonatal period. The activity of this enzyme (Bourre *et al.*, 1978) and the concentrations of its products, notably $C_{26:0}$ and $C_{26:1}$, increase markedly with age in brain (Svennerholm & Stallberg-Stenhagen, 1968). Our data appear to suggest that there may be considerable C_{18} elongase activity in liver even in the neonatal period, although the exact contribution of the diet remains unclear. Our data also strongly support the hypothesis that there is another elongase, one which is able to elongate polyenoic fatty acids, and it may be active in the pre-natal and/or neonatal brain. We have detected some of the presumed products of this enzyme, i.e. $C_{24:4}$, $C_{24:5}$ and $C_{26:5}$, in normal neonatal brain, but not in liver

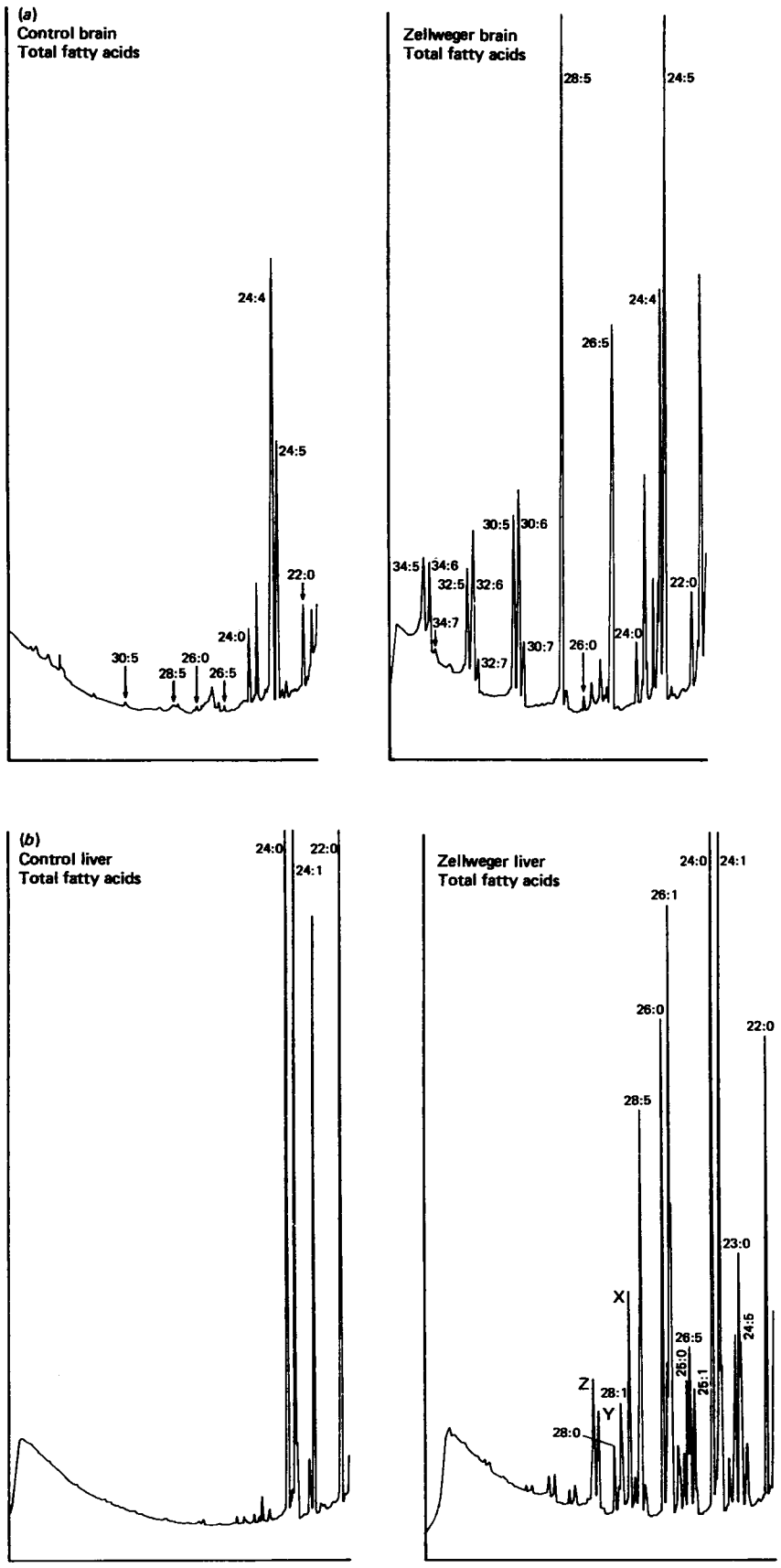

Fig. 1. Brain and liver fatty acids in Zellweger syndrome

Fatty acid methyl esters were prepared from (*a*) brain and (*b*) liver samples taken from a patient with Zellweger syndrome and from an age-matched control, and were subjected to g.l.c. as described in the text. The chromatogram shown above depicts only the VLCFA. X may be an artefact, possibly produced from cholesterol during the methanolysis procedure; Y and Z are probably $C_{30:6}$ and $C_{30:5}$ fatty acids respectively, although their structure has not been confirmed by mass spectrometry.

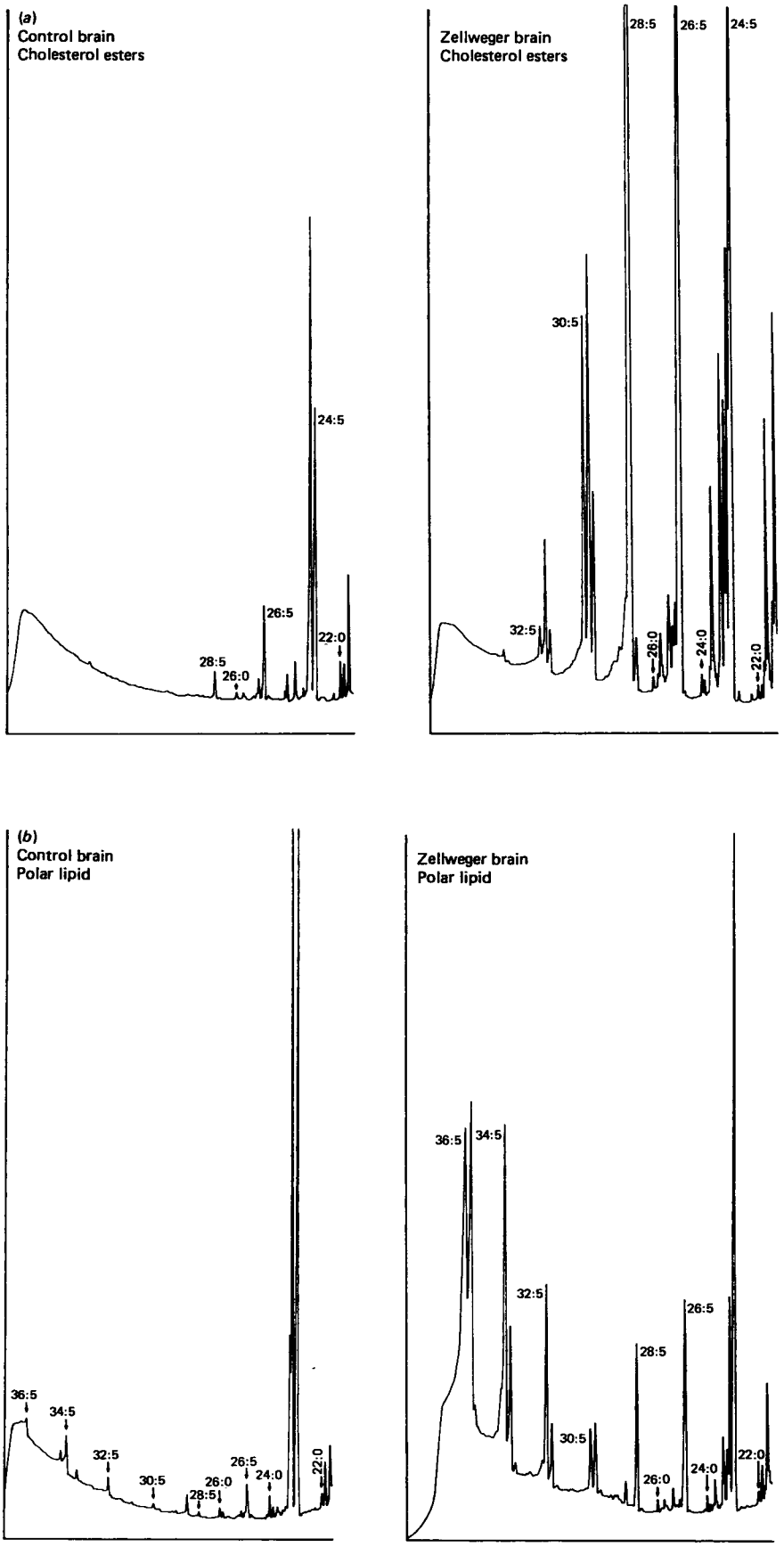

Fig. 2. Fatty acid composition of Zellweger syndrome brain cholesterol esters and polar lipid

The fatty acid composition of (*a*) cholesterol esters and (*b*) and unidentified polar lipid (PL) isolated from Zellweger syndrome brain was determined by g.l.c. as described in the text. The chromatogram shown above depicts only the VLCFA.

(Table 1, Fig. 1), indicating that this pathway may be important in developing brain.

It is likely that the accumulation of polyenoic VLCFA in Zellweger syndrome is related to the peroxisomal defects described in this disease (Goldfischer *et al.* 1973); in particular, these defects result in a much decreased VLCFA β-oxidation activity (Poulos *et al.*, 1986*c*). The β-oxidation of saturated, and probably monounsaturated VLCFA is thought to take place in peroxisomes (Singh *et al.*, 1984, 1987), but it is not known whether the corresponding polyenoic fatty acids are also degraded in these organelles, although peroxisomes are able to β-oxidize shorter-chain polyenoic fatty acids (Hiltunen *et al.*, 1986) (i.e. < 22 carbon atoms).

Our mass-spectrometric studies suggest that the polyenoic VLCFA are $n-6$ series and are thus probably formed from linoleic acid ($C_{18:2,n-6}$) and arachidonic acid ($C_{20:4,n-6}$) by successive elongation and desaturation reactions (Naughten, 1981). Similar fatty acids have been reported to be present in mammalian spermatozoa (Poulos *et al.*, 1986*b*) and rat testis (Bridges & Coniglio, 1970) and to be synthesized by mouse spermatocytes and spermatids (Grogan & Huth, 1983). Human endothelial cells in culture are able to synthesize polyenoic VLCFA from $C_{20:3,n-6}$ or $C_{20:4,n-6}$ (Rosenthal & Hill, 1984). Presumably these fatty acids are also $n-6$ series.

Although the biosynthesis of shorter-chain polyenoic fatty acids has been investigated in great detail (Naughten, 1981), relatively little is known about the biosynthesis of the polyenoic VLCFA. Apart from their importance as components of membrane lipids, the shorter-chain polyenoic fatty acids also act as potential substrates for the synthesis of a great variety of highly active oxygenated metabolites (Samuelsson, 1983). In view of the similarities in their structure, it is possible that the corresponding VLCFA may take part in, or interfere with, these processes. This hypothesis is supported by reports that polyenoic VLCFA are synthesized in the cell and are rapidly excreted (Rosenthal & Hill, 1984). If this is the case, then the accumulation in the brain could be an important factor in the development of the severe neurodegenerative changes observed in Zellweger syndrome patients. As these patients present very early in the neonatal period and rarely survive for longer than 12 months (Kelley, 1984) (one of our patients only survived for 4 days), it is tempting to speculate that these lipids may be particularly important in developing brain.

Our data show that polyenoic VLCFA accumulate in brain cholesterol esters (Fig. 2). Igarashi *et al.* (1976) have reported a similar predilection of saturated VLCFA for the brain cholesterol ester fraction in another disease state characterized by abnormalities in VLCFA oxidation, i.e. X-linked adrenoleukodystrophy. They reported that the major phospholipid species in brain in adrenoleukodystrophy, including presumably phosphatidylcholine and phosphatidylethanolamine, did not contain abnormal amounts of these VLCFA. Although we were unable to find polyenoic VLCFA in Zellweger syndrome brain phosphatidylcholine and phosphatidylethanolamine, or in any other of the quantitatively important phospholipids, we did detect them in a minor non-acidic polar lipid (PL). The identity of this lipid, and the reason for the marked difference in polyenoic VLCFA distribution between it and cholesterol esters, is not known, but is clearly worthy of further investigation. Current evidence suggests that this lipid is not an abnormal metabolite, and studies are needed to determine its structure and possible function.

Note added in proof (received 15 September 1987)

From the n.m.r. spectrum and the fast atom bombardment mass spectrum of the minor polar lipid (PL), we now believe that it is a series of molecular species of phosphatidylcholine each containing an ultra-long-chain polyenoic fatty acid.

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