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The occurrence of polyenoic very long chain fatty acids with greater than 32 carbon atoms in molecular species of phosphatidylcholine in normal and peroxisome-deficient (Zellweger's syndrome) brain

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The $n-6$ tetra- and pentaenoic fatty acids with carbon chain lengths > 32 found in normal brain are located predominantly in a separable species of phosphatidylcholine. A similar phospholipid is found in increased amounts in the brain of peroxisome-deficient (Zellweger's syndrome) patients, but the fatty acid composition differs in that penta- and hexaenoic derivatives predominate. Our data strongly suggest that the polyenoic very long chain fatty acids are confined to the *sn*-1 position of the glycerol moiety, while the *sn*-2 position is enriched in saturated, monounsaturated and polyunsaturated fatty acids with < 24 carbon atoms. It is postulated that these unusual molecular species of phosphatidylcholine may play some, as yet undefined, role in brain physiology.

INTRODUCTION

Polyenoic fatty acids with carbon chain lengths > 22 (very long chain fatty acids, VLCFA) have been reported to be present in rat testes (Bridges & Coniglio, 1970), human brain (Poulos *et al.*, 1986*b*), mammalian spermatozoa (Poulos *et al.*, 1986*a*), and the retina of a number of different mammalian species (Aveldaño, 1987; Aveldaño & Sprecher, 1987) and to be synthesized by mouse spermatocytes and spermatids (Grogan & Huth, 1983), and human endothelial cells in culture (Rosenthal & Hill, 1984).

In bovine retina these lipids are predominantly $n-3$ series acids and occur in unusual dipolyunsaturated molecular species of phosphatidylcholine (PC) (Aveldaño & Sprecher, 1987); in contrast, the corresponding fatty acids in ram spermatozoa are found almost exclusively in a novel molecular species of sphingomyelin (Poulos *et al.*, 1987).

We have recently observed that the polyenoic VLCFA in the brain of patients with Zellweger's syndrome, a rare inherited disorder characterized by a marked deficiency of peroxisomes, are $n-6$ series fatty acids and are confined to two lipid classes, cholesterol esters and an unidentified phospholipid (Sharp *et al.*, 1987). In contrast to the cholesterol esters, the latter contained relatively high concentrations of polyenoic VLCFA with 34, 36 and 38 carbon atoms. Similar VLCFA were also detected in normal neonatal brain, indicating that these fatty acids are normal brain components.

We report here that the brain lipid which contains such a high proportion of 34–38 carbon polyenoic fatty acids is an unusual molecular species of PC.

MATERIALS AND METHODS

Post-mortem samples were obtained from patients with biochemically and clinically confirmed diagnoses of Zellweger's syndrome (Poulos *et al.*, 1985), and stored at −70 °C for periods ranging from a few months to 8 years, prior to analysis. The ages of these patients ranged from 4 days to 6 weeks. Control brain samples were obtained from patients (aged 10 days to 5 months) who had died with sudden infant death syndrome.

Lipids were extracted from small amounts (< 2 g) of brain and individual components isolated as described in an earlier report (Sharp *et al.*, 1987). For extractions from larger amounts of brain, the following procedure was adopted: 45 g of brain was extracted by the Bligh & Dyer (1959) technique except for the substitution of 0.1 M-KCl for water at the partitioning stage. The total lipid (approx. 1.6 g was extracted from both control and Zellweger brain) was applied to a 3 cm diameter column of silicic acid (40 g, 325 mesh; Sigma) and eluted successively with 1000 ml of chloroform, 600 ml of chloroform/methanol (4:1, v/v), 800 ml of chloroform/methanol (1:1, v/v) and 600 ml of methanol. The chloroform/methanol (1:1, v/v) eluate was evaporated to dryness in a rotary evaporator at 40 °C, and the residue was applied to a 2 cm diameter column of DE-52 (20 g, Whatman) and the non-acidic lipids were eluted with 450 ml of chloroform/methanol (1:1, v/v). The lipid eluted from the DE-52 column (400–450 mg) was applied to eight 20 cm × 20 cm silica gel 60 t.l.c. plates (Merck) and chromatograms were developed in chloroform/methanol/water (14:6:1, by vol.). After spraying with 0.2% dichlorofluorescein in 95% ethanol to visualize

Abbreviations used: VLCFA, fatty acids with carbon chain lengths > 22; PC, phosphatidylcholine; VLCFA-PC, phosphatidylcholine enriched in VLCFA; FAB, fast atom bombardment.

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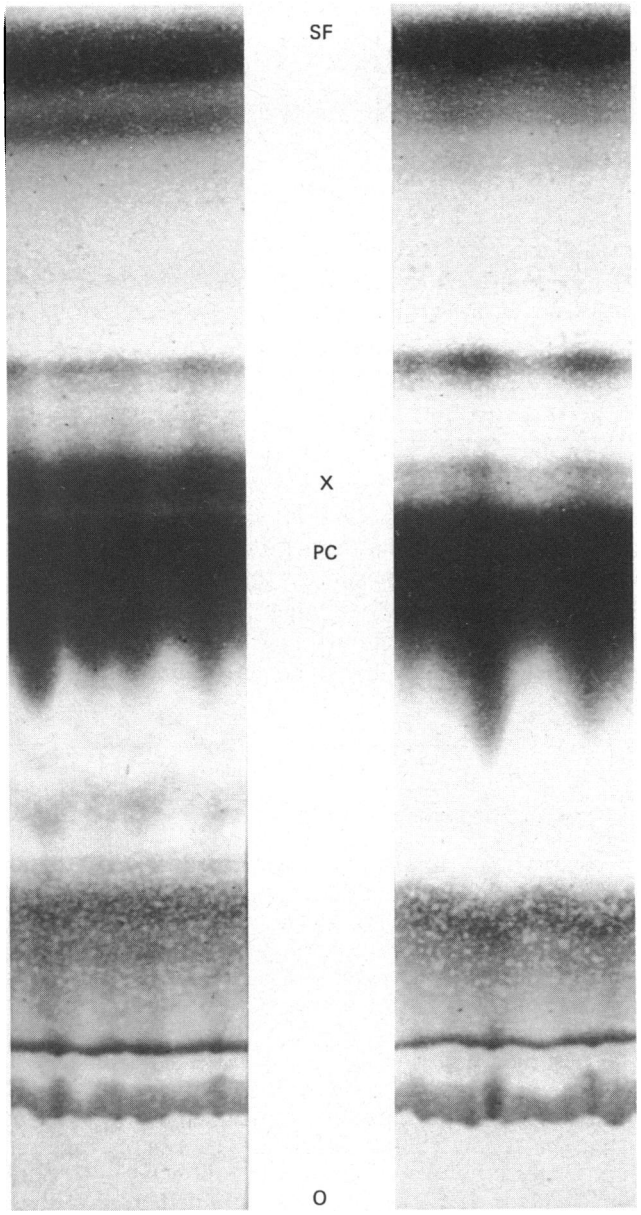

Fig. 1. T.l.c. of phospholipids from normal and Zellweger brain

Lipids were extracted from normal and Zellweger brain and were partially purified by silicic acid column chromatography as described in the text. The chromatograms shown above were obtained using aliquots of the chloroform/methanol (1:1, v/v) eluates. The various zones were detected with a phosphomolybdic acid spray reagent. Zellweger brain, left lane; normal brain, right lane. O, origin; SF, solvent front; X, phospholipid X; PC, phosphatidylcholine.

the various lipids, the zone migrating directly above the major PC zone was scraped from the plates, pooled, and eluted with 2 × 30 ml of chloroform/methanol/water (5:5:1, by vol.). The eluate was partitioned (Folch *et al.*, 1957) to remove the dye and evaporated to dryness in a rotary evaporator at 40 °C. The dried residue was dissolved in a small volume of chloroform/methanol (2:1, v/v). Further purification was achieved by t.l.c. on 20 cm × 10 cm high performance thin-layer silica gel 60 plates (HPTLC Kieselgel 60, Merck) in chloroform/methanol/water (14:6:1, by vol.). Zones were located and eluted in a similar fashion to that described for the t.l.c. on ordinary silica gel 60 plates.

Lipids were extracted from retina and purified by preparative t.l.c. as described for the brain. Aliquots of the various eluates were transesterified (Poulos *et al.*, 1986*b*) and the resulting fatty acid methyl esters were subjected to the combined g.c.-m.s. technique described earlier (Fellenberg *et al.*, 1987). This technique permits the assignment of the *n*-series for individual polyenoic fatty acids. Phosphorus analyses were carried out as described by Owens & Hughes (1970). Phospholipase A_2 hydrolysis was carried out using a commercial preparation of the bee venom enzyme (Sigma, lyophilized enzyme). Lipid samples (0.07 μmol lipid phosphorus) were shaken vigorously at room temperature (20–22 °C) for 30 min with 500 μl of diethyl ether and 150 μl of 25 mM-Tris/HCl buffer, pH 8.3, containing 5 mM-$CaCl_2$ and 0.65 units of enzyme. At the end of the incubation period, 2.5 ml of chloroform/methanol (2:1, v/v) was added and the mixture was evaporated to dryness under N_2 at 40 °C. The residue was dissolved in a small volume of chloroform/methanol (2:1, v/v), applied as a 2 cm zone to a silica gel 60 (Merck) thin-layer plate and a chromatogram was developed in chloroform/methanol/water (14:6:1, by vol.). The reaction products (1-acyl-*sn*-glycero-3-phosphocholine and unesterified fatty acid) were located with dichlorofluorescein and the lipids were eluted with chloroform/methanol/water (5:5:1, by vol.). After elution the lipids were transesterified and the resulting methyl esters were subjected to g.l.c. (Poulos *et al.*, 1986*b*). The specificity of the enzyme for the *sn*-2 position was confirmed by using 1-stearoyl 2-arachidonoyl *sn*-glycero-3-phosphocholine (Sigma) as a model substrate. Under the conditions described, the products were 1-stearoyl *sn*-glycero-3-phosphocholine and arachidonic acid, indicating that there had been little cleavage of the *sn*-1 position by the enzyme.

Tentative identification of lipids was based on a comparison of t.l.c. mobility with authentic standards using chloroform/methanol/water (14:6:1, by vol.) as the developing solvent and either normal or high performance t.l.c. plates. Lipids were detected either with iodine or with a phosphomolybdic acid spray reagent (Skipski & Barclay, 1969). For more definitive identification the techniques of n.m.r. spectrometry and fast atom bombardment (FAB) mass spectrometry were employed. Proton n.m.r. spectra were recorded on a Fourier Transform Bruker CXP-300 spectrometer operating at 300 MHz. Spectra were determined in deuteriochloroform/deuterated methanol, using tetramethylsilane as an internal standard.

Collision activation mass analysed ion kinetic energy spectroscopy (CAMIKES) of ions produced by FAB was carried out as described by Easton *et al.* (1988). For these studies mass spectra were measured on a Vacuum Generator ZAB 2 HF mass spectrometer operating in the FAB mode.

RESULTS

Polyenoic VLCFA with 32 or more carbon atoms were previously detected in significant amounts in normal and Zellweger brain in a phospholipid (designated as 'X') which migrated between PC and phosphatidylethanol-

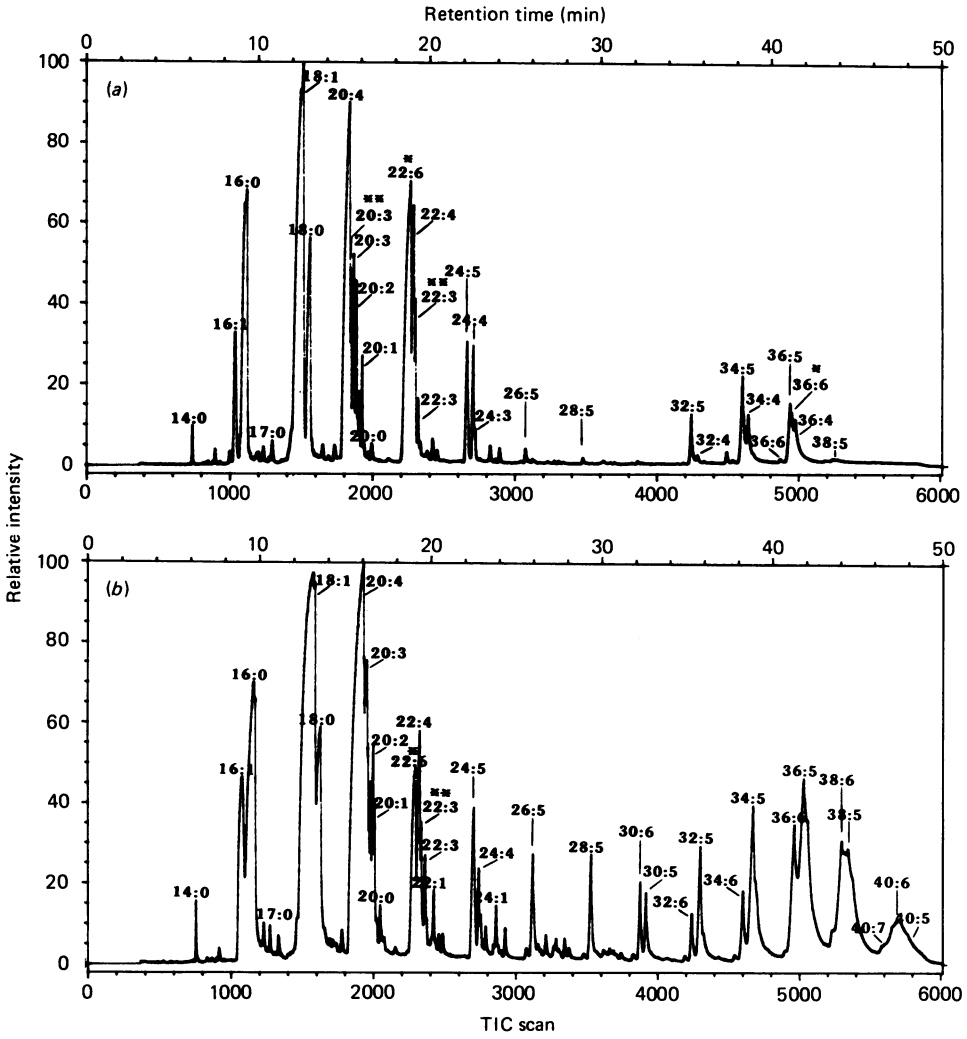

Fig. 2. G.c.-m.s. of normal and Zellweger brain phospholipid-bound fatty acids

Fatty acids (methyl esters) were released from normal (*a*) and Zellweger (*b*) brain polyenoic VLCFA-PC and were subjected to g.c.-m.s. analysis as described in the text. The figures shown above represent the total ion chromatogram. *, $n-3$ polyenoic fatty acids; **, $n-9$ polyenoic fatty acids; all other fatty acids with three or more double bonds are $n-6$ series acids.

amine zones on t.l.c. (Sharp *et al.*, 1987) (Fig. 1). (R_F lipid X, 0.61; PC, 0.52; R_F relative to PC 1.17.) This lipid was only a trace component of normal brain but was increased in Zellweger brain. Based on phosphate analysis, 0.9 and 2.8 μmol of the lipid were isolated from 45 g of control and Zellweger brain respectively. It generally migrated on normal silica gel t.l.c. plates as a single band, but high performance t.l.c. demonstrated that X isolated from normal and Zellweger brain may have comprised a number of closely migrating compounds. The complete band was used for analysis.

Although the normal and Zellweger brain lipid contained polyenoic VLCFA with 32, 34, 36 and 38 carbon fatty acids, there were subtle differences. In particular, Zellweger brain contained predominantly penta- and hexaenoic derivatives while tetra- and pentaenoic fatty acids were the major 32–38 carbon fatty acids in normal brain (Fig. 2). Zellweger brain also contained small amounts of $n-6$ polyenoic fatty acids with 40 carbon atoms. M.s. analysis confirmed that both normal and Zellweger brain fatty acids were predominantly $n-6$ series derivatives (Fellenberg *et al.*, 1987).

FAB mass spectra were obtained from the Zellweger and control brain lipid, and MIKES experiments were performed on the strong ion at m/z 184. These experiments demonstrated that the quaternary nitrogenous base choline was a constituent (Easton *et al.*, 1988) and suggested that the lipid was probably a species of PC enriched in polyenoic VLCFA (polyenoic VLCFA-PC) similar to that described in bovine retina (Aveldaño, 1987). T.l.c. of a bovine retina extract confirmed the presence of a lipid which migrated with a similar mobility to Zellweger and control polyenoic VLCFA-PC. The bovine retina lipid also showed a strong ion at m/z 184.

The p.m.r. spectrum of bovine retina PC (Fig. 3*a*) contained a number of easily identifiable peaks. These included a sharp singlet at δ 3.2 from the nine choline methyl hydrogens, a complex multiplet centred at δ 5.4 due to olefinic hydrogens, and a complex multiplet centred at δ 2.8 attributed to doubly allylic hydrogens.

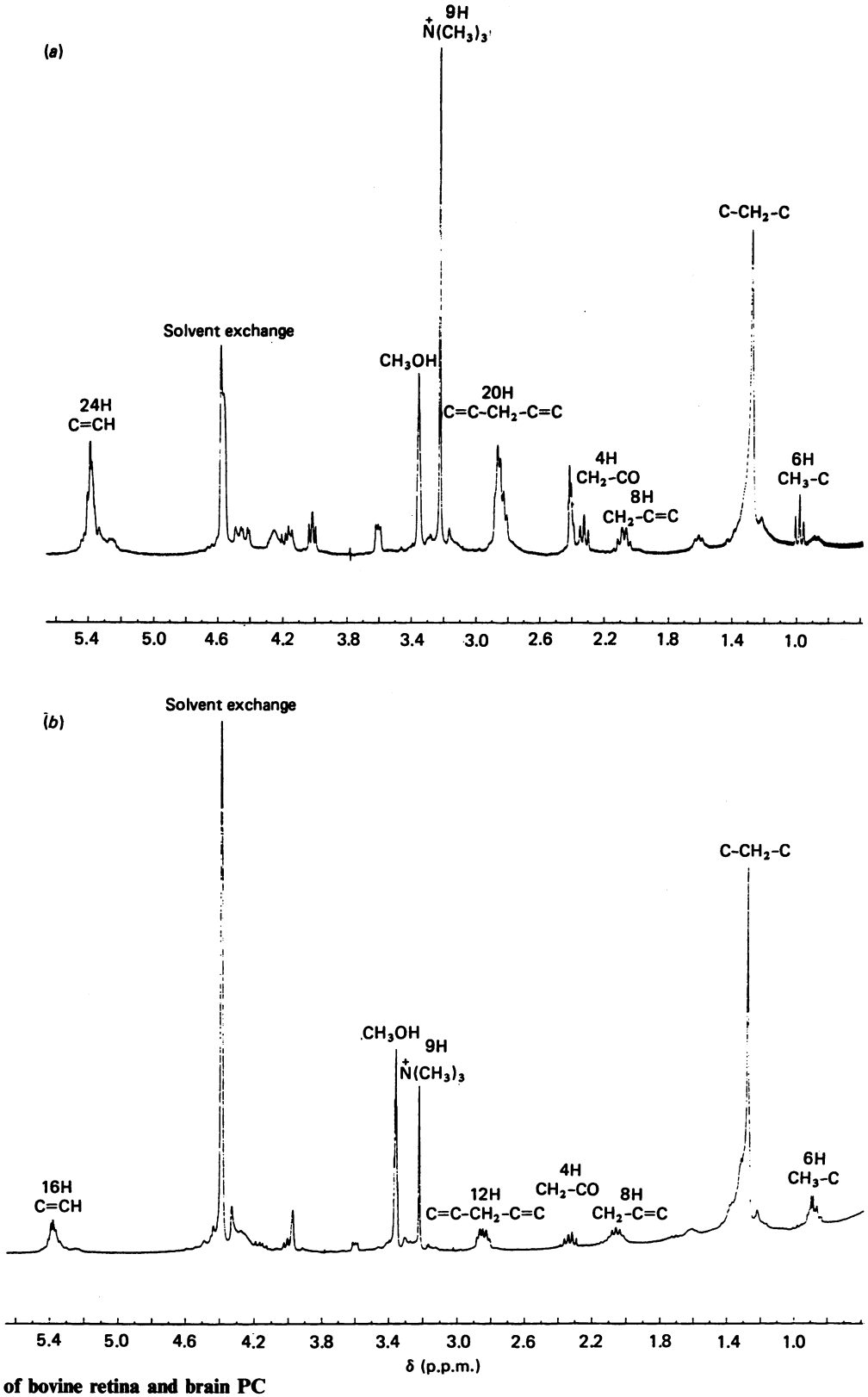

Fig. 3. P.m.r. of bovine retina and brain PC

P.m.r. spectrometry of bovine retina (*a*) and Zellweger brain (*b*) polyenoic VLCFA-PC was carried out as described in the text.

The p.m.r. spectrum of the Zellweger brain lipid (Fig. 3*b*) contained those same peaks with different relative integrated areas. There was insufficient of the normal brain lipid to obtain a p.m.r. spectrum.

The high mass region of the FAB mass spectrum of bovine retina polyenoic VLCFA-PC (Fig. 4*a*) showed three major clusters. Our analysis of the fatty acid composition of this lipid confirmed that 22:6 ($n-3$), 32:6 ($n-3$) and 34:6 ($n-3$) were major components. The major ion of each cluster neatly fits with a PC

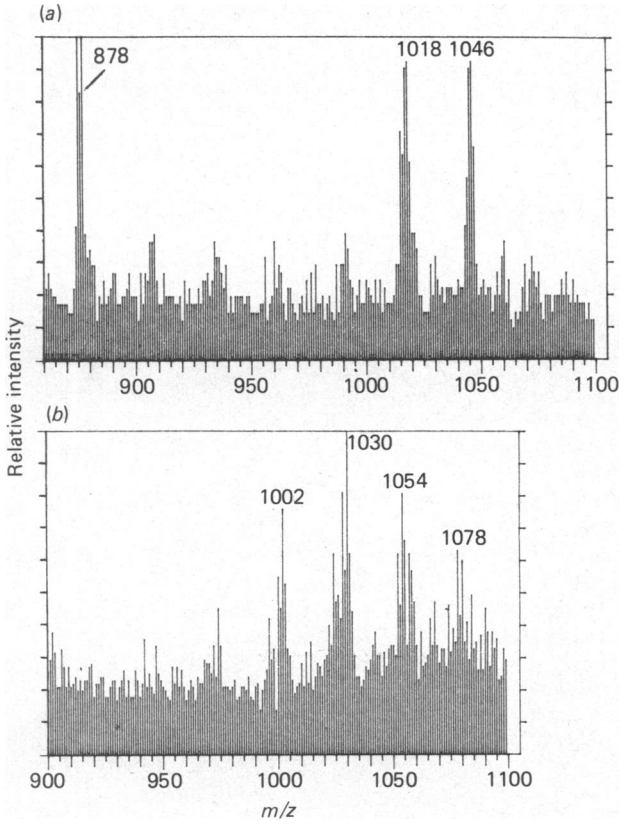

Fig. 4. FAB mass spectrometry of bovine retina and brain PC

FAB mass spectrometry of bovine retina (*a*) and Zellweger brain (*b*) polyenoic VLCFA-PC was carried out as described in the text.

containing two 22:6 (*m/z* 878), 22:6 and 32:6 (*m/z* 1018) and 22:6 and 34:6 (*m/z* 1046) fatty acids.

The corresponding high mass region of the FAB mass spectrum of Zellweger brain PC (Fig. 4*b*) contained four clusters of ions. Again it was simple to match the ions to a series of PCs, each of which contained a long chain (18–22) fatty acid and a very long chain (34–36) polyenoic fatty acid. The major ion of each cluster could be rationalized to belong to a PC containing either 18:1 and 34:5, or 18:0 and 34:6 (*m/z* 1002), 18:1 and 36:5, or 18:0 and 36:6 (*m/z* 1030), 20:3 and 36:5, or 20:2 and 36:6 (*m/z* 1054), and 22:5 and 36:5, or 22:4 and 36:6 (*m/z* 1078) fatty acids respectively. Similar ions could not be detected in the mass spectra of normal brain polyenoic VLCFA-PC, probably because the amount of lipid was insufficient to generate ions in the high mass region of the spectra.

Two products were formed by phospholipase A_2 hydrolysis of the normal and Zellweger polyenoic VLCFA-PC, unesterified fatty acid, and a lipid migrating with a slightly greater mobility than the product formed by hydrolysis of brain PC containing fatty acids with carbon chain lengths < 24, i.e. 1-acyl-*sn*-glycero-3-phosphocholine. No unreacted PC was detected on t.l.c. indicating complete hydrolysis. More than 95% of the polyenoic VLCFA with carbon chain lengths > 32 were found in the released 1-acyl-*sn*-glycero-3-phosphocholine indicating that these fatty acids were bound to the *sn*-1 position of the glycerol moiety. The major fatty acids released were 18:1, 20:4, 20:3, 20:2, 20:1, 22:4 and 22:6.

DISCUSSION

These investigations were prompted by our earlier observation that Zellweger brain contains a homologous series of polyenoic VLCFA (Poulos *et al.*, 1986*b*). Later studies demonstrated that most of these fatty acids were distributed in two lipids, cholesterol esters and an unidentified polar lipid (Sharp *et al.*, 1987). Although the latter is only a minor lipid in Zellweger brain, comprising $< 0.2\%$ of the total extracted lipid, it contains virtually all of the long chain fatty acids with carbon chain lengths > 32. Our m.s. and p.m.r. data show clearly that this lipid is a highly hydrophobic species of PC containing up to 58 carbons with as many as ten olefinic centres. Our earlier inability to detect a choline moiety using the Dragendorff reagent as a spray reagent may have been due to the lack of sensitivity of the reagent at the low lipid levels used (Sharp *et al.*, 1987). A similar molecular species is also present in bovine retina, but the polyenoic VLCFA are mostly $n-3$ rather than $n-6$ series acids (Aveldaño, 1987). The retina differs as well in that the major molecular species are dipolyunsaturated and contain docosahexaenoic acid in at least one of the positions (Fig. 4*a*).

The greatly increased t.l.c. mobility of the unusual PC species is perhaps not surprising, although the resolution of individual molecular species of phospholipids has been thought to require more specialized techniques such as argentation t.l.c. and reverse-phase h.p.l.c. (Christie, 1982). Our recent detection of a unique molecular species of ram sperm sphingomyelin containing 28–34 carbon $n-3$ polyenoic fatty acids, which migrated considerably faster on t.l.c. than conventional sphingomyelin species (Poulos *et al.*, 1987), indicates the phenomenon is not confined to glycerophospholipids but affects sphingolipids as well. It is possible that the increased mobility of phospholipids containing polyenoic VLCFA is an indication that, with increasing carbon chain length, partition assumes a proportionally greater importance in influencing chromatographic mobility due to the increased solubility of the lipid in the mobile solvent phase.

It is clear that polyenoic 34, 36 and 38 carbon fatty acids are present in both normal and in Zellweger brain with increased levels in the latter. Of particular interest is the subtle difference which exists between normal and mutant tissue. Thus tetra- and pentaenoic $n-6$ derivatives are the major 32–38 carbon fatty acids in normal brain, although we have detected smaller amounts of $n-3$ hexaenoic acids as well. In contrast, Zellweger polyenoic VLCFA-PC contains mostly pentaenoic and hexaenoic fatty acids, while the corresponding tetraenoics are barely detectable. These differences are intriguing but difficult to explain, particularly in view of the lack of information on the mechanisms for biosynthesis of the polyenoic VLCFA. However, it is likely that the carbon chains of the tetraenoic acids in normal brain are all derived by chain elongation of the shorter chain $n-6$ precursors, 20:4 $n-6$, 22:4 $n-6$, 24:4 $n-6$, all of which occur in significant amounts in normal and Zellweger brain. Similarly, the pentaenoic acids are probably formed by chain elongation of 22:5 $n-6$ and 24:5 $n-6$. The origin of the corresponding hexaenoic acids whose

synthesis requires both elongation as well as desaturation steps remains unclear. Whether the relative absence of tetraenoic VLCFA in Zellweger syndrome is due to an abnormality in the elongation of shorter chain precursors, or whether it simply reflects an increased synthesis of penta- and hexaenoic fatty acids, is clearly worthy of further investigation.

The relatively high degree of specificity of incorporation of 34–38 carbon polyenoic VLCFA into the *sn*-1 position of PC is of considerable interest because this position is thought to be mostly occupied by saturated fatty acids (Christie, 1982), and therefore indicates the existence of enzymological specificity at some stage along the biosynthetic pathway. Further supporting evidence for some degree of enzyme specificity is provided by comparing the fatty acid compositions of Zellweger brain cholesterol esters and PC (Poulos *et al.*, 1986*b*). Whereas the former lipid contains few longer (i.e. > 32 carbon) chains, and large proportions of 26–30 carbon compounds, the more polar PC is enriched in the longer chain polyenoic fatty acids. At present the exact stage at which the polyenoic VLCFA are introduced into the phospholipid molecule is not known. There have been reports that PCs enriched in polyenoic fatty acids in rat brain are formed by methylation of phosphatidylethanolamine (Blusztajn & Wurtman, 1981; Tacconi & Wurtman, 1985). However, as we have been unable to detect the longer chain polyenoic VLCFA in brain phosphatidylethanolamine (Sharp *et al.*, 1987), it would seem unlikely that this particular lipid is a precursor, although we cannot discount the possibility that there is a rapid turnover of molecular species of phosphatidylethanolamine enriched in polyenoic VLCFA.

The function of these unusual molecular species of PC remains unknown. It is probable however, that any biological role is associated with the presence of the polyenoic VLCFA. Whether the latter are released from the phospholipid and possibly converted to some as yet undefined physiologically active compounds, as has been suggested by some workers (Rosenthal & Hill, 1984), or whether the diacylglycerol or the parent phospholipid is the active molecule remains to be established.

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