Lipid composition of the nervous system in Refsum's disease

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ABSTRACT The compositions of the major lipids and their constituent fatty acids and fatty aldehydes from cerebral gray matter, white matter, and myelin, spinal cord myelin, and sciatic nerve were determined in a 57 yr old woman who died of Refsum's disease. There were deficiencies of ethanolamine glycerophosphatides (EGP) in gray matter and frontal lobe myelin, and a lipid with the chromatographic properties of lyso-EGP accumulated in all tissues. The proportions of the remaining lipids were nearly normal in the central nervous system tissues. In the sciatic nerve the proportions of sphingolipids were small; this observation is consistent with the severe demyelination noted on pathologic examination. Cholesteryl esters were not detected in any tissue.

Phytanate (3,7,11,15-tetramethylhexadecanoate) was present in the glycerophosphatides from each tissue. Higher proportions of phytanate were found in choline glycerophosphatides (CGP) than in EGP or in serine glycerophosphatides (SGP). Hydrolysis with phospholipase established that phytanate was confined to the 1-position of CGP. More phytanate was found in CGP from myelin than from gray or white matter. Fourfold higher proportions of phytanate were found in CGP from sciatic nerve than in CGP from the central nervous system: in sciatic nerve, 24% of the fatty acids of CGP consisted of phytanate. The proportions and compositions of sphingolipid hydroxy fatty acids and odd-numbered fatty acids were normal in each tissue.

These findings argue against a defect in sphingolipid α-hydroxy acid metabolism in Refsum's disease. The results are consistent with the view that the accumulation of phytanate is responsible for the demyelination.

KEY WORDS Refsum's disease · phytanic acid accumulation · demyelination · lipid composition of nervous system · hydroxy acids · plasmalogens

REFSUM'S DISEASE (heredopathia atactica polyneuritiformis) is an inborn error of metabolism characterized by night blindness, severe peripheral neuropathy, cerebellar ataxia, nerve deafness, retinitis pigmentosa, cardiac abnormalities, and bony lesions (1, 2). The clinical picture is often that of a slowly developing, progressive peripheral neuropathy manifested by severe motor weakness and muscular wasting, especially of the lower extremities. An extensive neuropathological study by Cammermeyer (3) demonstrated that the essential pathological change is one of severe interstitial hypertrophic neuropathy. Peripheral nerves exhibit a progressive degeneration of myelin, often proceeding to complete disintegration of myelin sheaths with concomitant proliferation of Schwann cells. The latter are often arranged in a vortical pattern, giving rise to an "onion bulb" appearance. Cammermeyer (3) suggested that Refsum's disease may involve a "delamination" of myelin sheaths due to an inherited anomaly in the biochemical or biophysical properties of myelin.

In 1963, Klenk and Kahlke (4) demonstrated the accumulation of 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid) in serum and tissue lipids from a patient with Refsum's syndrome. High concentrations of phytanic acid have since been found in the plasma lipids of about one dozen patients (5, 6). Recently, it has been shown that tissues from patients with Refsum's disease cannot α-oxidize and decarboxylate phytanic acid (7, 8) (and related branched chain acids [9]); hence, this fatty acid, which is contained in the normal diet, accumulates in the tissues, from which it is normally absent (see reference 10 for discussion of this point and a thorough review of this disorder).

Abbreviations: C–M, chloroform–methanol; DEAE, diethylaminoethyl; EGP, ethanolamine glycerophosphatides; SGP, serine glycerophosphatides; CGP, choline glycerophosphatides. Fatty acids are designated by number of carbon atoms: number of double bonds. The symbol $\omega 6$ indicates that the first double bond is located 6 carbons removed from the terminal methyl group; "h" indicates a hydroxy acid.

The relationship of the phytanic acid accumulation to degeneration of the nervous system is obscure. It has been suggested that if phytanate accumulates in myelin, disruption of the myelin lipid bimolecular leaflet could result (11). It is important, therefore, to know the concentrations of phytanate in myelin in Refsum's disease, its concentrations in nerves with various degrees of demyelination, and the over-all lipid composition of the nervous system in this disease. This report is concerned with the composition of the major lipids and their constituent fatty acids and fatty aldehydes from cerebral gray matter, white matter, and myelin, spinal cord myelin, and sciatic nerve from a patient who died of Refsum's disease.

MATERIALS AND METHODS

Case History

The patient was a 57 yr old woman who suffered from long-standing neurological degeneration. She experienced peripheral neuropathy in her thirties, at which time she also complained of anosmia and ichthyosis. Night blindness was present from age 20, and nerve deafness developed at age 52. She also had diabetes mellitus which was difficult to control, especially when she became disoriented near the end of her illness. On clinical examination at age 57, she was found to have severe cerebellar ataxia, tremor of the head and extremities, retinitis pigmentosa, and severe wasting and weakness of all extremities, with almost no power in the distal muscle groups. Symmetrical epiphysial dysplasia of the bones of the extremities was found on roentgenographic examination.

Terminally, she contracted bronchial pneumonia and died at the University of Washington Hospital, Seattle, Wash. Before her death, a number of clinical studies were performed. These included the demonstration of high levels of phytanic acid in serum and red cells by Dr. Peter Ways; this confirmed the diagnostic impression of Refsum's disease.

At autopsy, the sciatic nerve and the ventral and dorsal roots of the spinal cord all showed marked demyelination, swelling, and loss of axons. There was a striking proliferation of connective tissue in the peripheral nerves involving the endoneurium, perineurium, and epineurium. These changes were most marked in the sciatic nerve. The anterior horn cells in the spinal cord had degenerated and demyelination of the posterial columns was apparent. The degenerative lesions in the spinal cord could be attributed to retrograde changes secondary to the peripheral neuropathy. Sections of the various lobes of the cerebral hemispheres showed normal-looking cortex and white matter, with no evidence of gross or microscopic demyelination. Fatty accumulations were found in the cells of the globus pallidus and in the leptomeninges.

Further details of the clinical history of this patient, the physical findings, analyses of red cells, serum and tissue lipids, as well as more complete details of the systemic and neuropathological examinations will be the subject of a future report by the staff at the University of Washington. We are indebted to them for portions of fresh tissues which were frozen at autopsy and were analyzed in this report.

Isolation of Myelin and Lipid Analysis

Portions of sciatic nerve, spinal cord, and frontal and temporal lobes were analyzed in our laboratory within 7 months after death. Myelin was isolated from frontal lobe white matter, temporal lobe white matter, and spinal cord by differential ultracentrifugation in the method of Autilio, Norton, and Terry (12). No attempt was made to isolate myelin from the sciatic nerve since the specimen was small and severe demyelination was present. Aliquots of each myelin preparation were examined under the light and electron microscopes. Under the light microscope, the myelin preparations were comprised of many long tubules. Contamination by other subcellular organelles was not evident. Electron microscopy was carried out by the processes of fixing the myelin preparations in osmium tetroxide, dehydration, embedding in Vestopal, sectioning, subsequent staining with uranyl acetate, and examination under a Hitachi HS7S electron microscope. Myelin preparations from each tissue consisted almost exclusively of smooth surface membranes. Many of the myelinated fibers were coiled in tightly wound spiral fashion like native myelin, but some membranes were loosely arranged as if the myelin had become uncoiled. This appearance was similar to that of normal myelin. A small amount of granular material was noted in addition to the smooth surface membranes, but no mitochondria, rough surface membranes, or unpaired membranes were seen.

Gray and white matter were manually separated from both the frontal and temporal lobes. The sciatic nerve was dissected free from the connective tissue and adipose tissue which surrounded it. Small portions of adipose tissue were taken from tissue surrounding the nerve. The myelin preparations and the tissue specimens were extracted with 20 volumes of chloroform–methanol (C–M) 2:1 as described previously (13). Each myelin preparation dissolved readily in C–M 2:1, giving a very faintly turbid solution. In the extraction procedure and during all subsequent maneuvers including chromatography, weighing, hydrolysis, and storage, the lipid fractions were kept in a nitrogen atmosphere to prevent the oxidation of unsaturated fatty acids.

Column and Thin-Layer Chromatography

We isolated lipids from each preparation by column chromatography, first on DEAE-cellulose (for fractionation of lipids on the basis of their ionic charge), and then on silicic acid or Florisil (for fractionation of groups of lipids according to their polarity). These procedures are given in detail in previous publications (13, 14). Each lipid was quantified gravimetrically on an analytical balance. In some instances, lipids were quantified on thin-layer chromatograms by densitometric analysis of the charred spots by means of a Photovolt densitometer (15). Pure lipid standards were isolated from human brain specimens. Plots of lipid quantity versus densitometric response were linear between 2 and 20 μg. The densitometric method was also used to quantify the proportions of hydroxy acids in cerebroside and cerebroside sulfate. These glycolipids gave two spots on thin-layer chromatograms, the slower spot being the glycolipid containing hydroxy acids and the faster one, the glycolipid containing unsubstituted fatty acids. Densitometric analysis yielded the hydroxy acid content of these glycolipids. In addition, each glycolipid was hydrolyzed and the liberated fatty acids were chromatographed on thin-layer plates in hexane–ether–acetic acid 85:14:1; unsubstituted and hydroxy acids were determined densitometrically. The thin-layer procedure described was especially useful with cerebroside sulfate, which was difficult to separate into two distinct spots. The estimation of the hydroxy acid content by densitometry of intact standard glycolipids, or of their released fatty acids, agreed within $\pm 10\%$ of values obtained by column-chromatographic isolation of hydroxy acids and weighing (16).

Prior to fatty acid analysis, each lipid was judged to be chromatographically pure, except for contamination of EGP with lyso-EGP. The thin-layer chromatographic system employed Silica Gel H as the adsorbent and the solvent systems C–M–ammonium hydroxide 18:6:1 for polar lipids and hexane–ether–acetic acid 85:14:1 for neutral lipids.

Triglycerides were purified from adipose tissue by preparative thin-layer chromatography. This involved applying the tissue extract in a band, developing the chromatogram in hexane–ether–acetic acid 85:14:1, detecting the triglyceride band with a water spray, scraping the band from the plate, and eluting the triglyceride from the silica gel with C–M 2:1. The fatty acids of the triglycerides were analyzed by gas–liquid chromatography after methanolysis with 5% methanolic HCl.

Fatty Aldehyde and Fatty Acid Analysis

The fatty aldehyde content of each glycerophosphatide was determined by gas–liquid chromatography and measurement of the peak areas of palmitaldehyde dimethylacetal and methyl palmitate in a mixture of aldehyde dimethylacetals and fatty esters released from each glycerophosphatide (14, 17). Glycerophosphatide fatty esters and fatty aldehyde dimethylacetals were analyzed on a 10% diethylene glycol succinate column, while sphingolipid fatty esters were analyzed on a 3% Apiezon L column (17). The fatty acid constituents were identified by determination of carbon numbers (18). Methyl phytanate had a carbon number of 17.2 on diethylene glycol succinate. The fatty acids and fatty aldehydes were quantified by measurement of peak areas; samples were chromatographed in duplicate or triplicate. When standard mixtures were used (National Heart Institute Standard F and a laboratory standard), the quantitative results agree with the known composition with a relative error of $<5\%$ for major components ($>10\%$ of the total mixture) and $<7\%$ for minor components ($<10\%$ of the total mixture).

Venom Hydrolysis

A mixture of choline glycerophosphatides (CGP) and sphingomyelin isolated from both white matter and connective tissue adjacent to the sciatic nerve was subjected to phospholipase A hydrolysis by *Naja naja* (hooded cobra) venom (19). 5 ml of a solution of CGP in diethyl ether (3 mg/ml) was mixed with 0.4 ml of a venom solution (4 mg/ml venom) in 0.1 M borate buffer at pH 7.0, containing calcium chloride at 2.5×10^{-3} M. The reaction was carried out at 27°C for 1 hr with constant shaking. Thin-layer chromatography of the hydrolysate revealed that sphingomyelin was not hydrolyzed, and free fatty acids and lyso-CGP were released. The fatty acids released (from the 2-position of CGP) were isolated by silicic acid column chromatography, the fatty acids being eluted with chloroform, and lyso-CGP plus sphingomyelin with methanol. The lyso-CGP and sphingomyelin mixture was then treated by mild alkaline hydrolysis (17) to yield the 1-linked fatty acids of CGP. After the fatty acids had been methylated with boron trifluoride–methanol (20), they were analyzed by gas–liquid chromatography.

RESULTS

Lipid Analyses

The lipid composition of central nervous system tissues from the patient with Refsum's disease and of those from normal subjects is given in Table 1. The lipid content of gray matter was within normal limits. The concentration of ethanolamine glycerophosphatides (EGP) was diminished in gray matter. The remaining lipids were present in nearly normal proportions except for free fatty acids, which were increased slightly. The lipid content of white matter was lower than normal, as

TABLE 1 LIPID COMPOSITION OF THE CENTRAL NERVOUS SYSTEM IN REFSUM'S DISEASE

	Frontal Lobe Gray Matter		Frontal Lobe White Matter		Frontal Lobe Myelin		Spinal Cord Myelin
	Patient	Normals	Patient	Normals	Patient	Normals	Patient
				% of dry wt			
Total lipid	37.0	37.7 (35.8–39.6)	55.4	63.1 (58.4–66.3)	71.9	79.0 (78.0–80.9)	73.1
Nonlipid residue	63.0	62.3 (60.4–64.2)	44.6	36.9 (33.7–41.6)	28.1	21.0 (19.1–22.0)	26.9
Total glycerophosphatides*	18.1	21.6 (21.1–22.5)	21.6	22.6 (20.4–25.9)	21.0	27.1 (24.6–31.9)	25.2
Total sphingolipids†	4.2	5.0 (3.8–5.6)		20.2 (19.2–21.5)	22.9	26.0 (24.5–28.6)	21.3
Unidentified	6.2	4.1 (2.9–5.8)	5.3	6.4 (5.4–7.3)	3.2	4.6 (1.8–7.6)	6.6
Cholesterol	7.4	7.0 (6.6–7.2)	13.6	13.9 (13.2–15.1)	18.5	19.9 (18.6–21.5)	17.5
EGP	7.6	9.8 (9.2–10.6)	10.3	9.9 (8.6–12.0)	5.2	12.2 (11.2–14.2)	13.0
SGP	3.3	3.1 (2.7–3.6)	5.3	4.3 (3.5–5.1)	8.3	5.0 (4.2–5.5)	5.3
CGP	7.2	8.8 (8.3–9.0)	6.0	8.4 (8.2–8.8)	7.5	9.9 (8.3–12.2)	6.9
Sphingomyelin	2.3	2.0 (1.3–2.8)	6.0	4.3 (2.7–5.2)	5.2	4.5 (4.4–4.6)	5.1
Cerebroside	1.4	1.7 (1.0–2.3)	8.8	11.9 (10.5–12.8)	13.0	16.4 (14.0–19.2)	12.5
Cerebroside sulfate	0.5	0.6 (0.4–0.8)	NA	3.2 (2.7–3.8)	4.7	4.1 (3.4–5.1)	3.7
Free fatty acids	1.1	0.6 (0.5–0.8)	NA	0.7 (0.5–0.9)	4.7	1.3 (0.7–1.4)	2.6

Normal values are from three subjects: 6 yr, 9 yr, and 55 yr old (13). NA, not analyzed.
* Sum of EGP, SGP, and CGP.
† Sum of sphingomyelin, cerebroside, and cerebroside sulfate.

was the concentration of cerebroside. The lipid content of frontal myelin was lower than normal. The lipid content of spinal cord myelin was close to that of frontal myelin. There were slight diminutions in the content of cerebroside in frontal myelin and white matter. The values for EGP in frontal myelin were markedly lower than normal. Thin-layer chromatography showed that the EGP fraction contained a significant proportion (visually estimated at approximately 50% of EGP) of a compound that cochromatographed with lyso-EGP (prepared by phospholipase treatment of gray matter EGP), was reactive with ninhydrin, and which we believe to be lyso-EGP. This compound was also found in the EGP fraction from the other tissues. From visual inspection of the chromatograms, lyso-EGP was estimated to comprise the following proportions of the EGP fraction: frontal lobe gray matter, 1%; whole spinal cord, 40%; spinal cord myelin, 2%; and sciatic nerve, 25%.

The lipid composition of the sciatic nerve from the patient is presented in Table 2. The lipid composition of bovine intradural spinal root nerves is presented for comparison. Most of the lipid present in the sciatic nerve from the patient could be accounted for as triglycerides plus lipids grouped under "unidentified." These unidentified constituents migrated on thin-layer chromatograms with the R_f of hydrocarbons and waxes. Cholesteryl esters were not detected in sciatic nerve, nor in any of the other tissues examined. The proportions of sphingolipids and

TABLE 2 LIPID COMPOSITION OF SCIATIC NERVE IN REFSUM'S DISEASE

	Patient Sciatic Nerve	Bovine Spinal Root Nerves	Patient Sciatic Nerve	Bovine Spinal Root Nerves
	% of dry wt		*% of total lipid**	
Total lipids	21.5	65.8		
Nonlipid residue	78.5	34.2		
Total glycerophosphatides	2.70	28.5	55.5	42.1
Total sphingolipids		18.6		32.5
Unidentified	3.90	4.4	—	—
Cholesterol	0.96	14.5	20.0	25.3
EGP	0.92	10.3	19.1	18.0
SGP	0.83	5.4	17.3	9.4
CGP	0.92	8.4	19.1	14.7
Sphingomyelin	0.79	8.9	20.2	15.6
Cerebroside	0.39	8.3	8.1	14.5
Cerebroside sulfate	NA	1.4	NA	2.4
Triglyceride	12.5	4.4	—	—

Values for bovine spinal root nerve are from O'Brien, Sampson, and Stern (21). NA, not analyzed.
* Excluding triglycerides and unidentified constituents.

TABLE 3 FATTY ALDEHYDE COMPOSITION OF GLYCEROPHOSPHATIDES

	Ethanolamine Glycerophosphatides						Serine Glycerophosphatides		
	Frontal GM	Frontal WM	Frontal Myelin	Cord Myelin	Sciatic Nerve	Normal* Myelin Values	Frontal Myelin	Cord Myelin	Normal* Myelin Values
Total aldehyde content	26.9	43.0	13.2	40.3	28.7	45.7 (40.3–50.3)	7.4	17.5	30.6 (21.8–36.5)
16:br	0.6	3.7	tr.	0.5	4.2	1.1 (tr.–3.4)	tr.	3.7	(tr.–0.1)
16:0	22.1	23.5	31.7	33.4	51.3	35.2 (29.0–38.6)	24.8	34.1	36.1 (28.5–42.5)
17:0	1.8	1.2	1.6	2.2	3.1	1.4 (0.8–2.2)	2.5	1.9	2.2 (1.1–2.4)
18:br	1.2	5.5	1.0	tr.	1.8	1.0 (0.5–1.3)	2.5	2.9	0.6 (tr.–0.9)
18:0	59.0	21.9	17.3	20.4	23.9	18.9 (14.0–21.8)	17.1	28.5	22.2 (17.5–29.2)
18:1	15.3	42.7	48.4	43.5	12.8	41.4 (38.0–44.6)	53.0	28.9	34.3 (20.7–43.0)
20:1	—	—	—	—	2.9	tr.			tr.

Aldehyde content is expressed as a molar percentage of the acyl + alkenyl groups in each lipid. Each aldehyde is expressed as a percentage of the total aldehydes in each lipid. GM, gray matter; WM, white matter; br, branched chain.
* Frontal myelin values from three normal subjects ages 6 yr, 9 yr, and 55 yr (13,17).

glycerophosphatides were very small in sciatic nerve. These low values are in accordance with the severe deficiency of myelin in this nerve noted on pathological examination. In order to make a more meaningful comparison with normal nerves, we compared the polar lipids (and cholesterol) of the patient's sciatic nerve lipids with

TABLE 4 FATTY ACID COMPOSITION

	Ethanolamine Glycerophosphatides						Serine		
	Frontal GM	Frontal WM	Frontal Myelin	Cord Myelin	Sciatic Nerve	Normal* Myelin Values	Frontal GM	Frontal WM	Frontal Myelin
14:0	0.9	3.6	1.4	0.7	1.5	0.7 (0.4–0.8)	0.6	0.3	1.1
16:0	8.7	22.0	16.1	7.8	23.1	9.1 (6.5–13.8)	2.6	2.7	8.3
16:1	0.9	9.6	2.0	1.1	4.0	0.8 (0.4–1.5)	0.5	0.8	1.4
18:0	21.0	11.9	11.6	5.3	10.5	8.9 (7.0–11.9)	37.7	49.0	52.8
18:1	10.6	27.3	33.0	46.0	32.0	57.8 (43.9–72.5)	18.0	36.3	27.4
18:2	1.0	1.0	0.5	tr.	3.0	(tr.–0.3)	0.2		
20:1	0.5	4.8	3.2	16.1	1.4	3.1 (1.7–3.9)	0.7	4.9	2.4
20:3ω6	1.3		1.0	2.4	0.9	0.7 (tr.–1.4)	1.2		
20:4	16.2	4.7	9.2	3.3	12.7	4.7 (1.6–9.6)	1.7	0.9	1.7
22:5ω6	11.0	6.8	12.0	7.9	4.0	9.9 (4.6–15.9)	4.8	1.7	2.3
22:5ω3	1.4			3.3	tr.	0.5 (tr.–1.0)	2.0		
22:6	26.4	3.8	9.0	2.4	5.2	2.0 (0.6–4.0)	30.0	2.6	2.5
Phytanic	0.1	1.0	0.8	tr.	1.2		tr.	0.5	tr.

Each fatty acid is expressed as a percentage of the total fatty acids of each glycerophosphatide. GM, gray matter; WM, white matter.
* Frontal myelin values from three normal subjects ages 6 yr, 9 yr, and 55 yr (17).

those of bovine spinal root nerve on a percentage of total lipid basis, triglycerides and the uncharacterized constituents being excluded from the total. This comparison also revealed a marked diminution of cerebrosides and increased proportions of CGP and sphingomyelin in the patient's sciatic nerve.

Fatty Aldehyde Content and Composition

In the patient, the aldehyde content of EGP and serine glycerophosphatides (SGP) from frontal myelin was lower than normal (Table 3). The low aldehyde content of EGP and the presence of lyso-EGP may suggest the hydrolysis of aldehydes from EGP. This explanation cannot be invoked for SGP, since lyso-SGP was not detected. The fatty aldehyde compositions of EGP and SGP from each tissue were nearly normal. There was no evidence for the presence of a derivative with the chromatographic migration expected for 3,7,11,15-tetramethylhexadecanal.

Fatty Acid Composition of Glycerophosphatides

The fatty acid compositions of the glycerophosphatides and of triglycerides are given in Table 4. The fatty acid compositions of each glycerophosphatide from frontal lobe gray matter were nearly normal. Polyunsaturated fatty acids were present in high proportions in EGP and SGP. There were also no large deviations from normal in the fatty acid compositions of EGP and SGP from white matter, frontal myelin, or spinal cord myelin from the patient. However, small proportions of phytanate were found in EGP and SGP in all tissues, EGP containing more phytanate than SGP. CGP contained much higher proportions of phytanate than either EGP or SGP. CGP from frontal myelin contained more phytanate (7.6%) than CGP from white matter (5.2%) or gray matter (2.7%). CGP from spinal cord myelin contained phytanate in concentrations (5.1%) close to that in white matter. The highest proportions were found in CGP from sciatic nerve, where phytanate constituted 24.0% of its fatty acids. Triglycerides within the nerve sheath and from adipose tissue surrounding the sciatic nerve also contained small but significant proportions of phytanate (1–2%).

Position of Phytanate in CGP

The positional distribution of fatty acids in CGP from frontal white matter and from connective tissue surrounding the sciatic nerve is presented in Table 5. The sample of CGP from the sciatic nerve region was obtained by solvent extraction of the tissue surrounding the nerve after the sciatic nerve had been dissected out. This was necessary because the amounts of CGP obtained from the nerve proper were insufficient for fatty acid positional studies. With CGP, both from white mat-

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Glycerophosphatides			Choline Glycerophosphatides						Triglycerides	
Cord Myelin	Sciatic Nerve	Normal* Myelin Values	Frontal GM	Frontal WM	Frontal Myelin	Cord Myelin	Sciatic Nerve	Normal* Myelin Values	Inside Sheath	Outside Sheath
0.5	tr.	(0.3–0.5)	0.9	1.8	1.4	1.2	0.7	0.5 (0.1–1.0)	2.5	2.4
3.1	10.9	2.5 (1.8–2.7)	58.2	45.5	37.7	49.0	50.4	34.0 (29.9–40.1)	23.7	22.3
0.6	2.9	0.5 (0.4–0.6)	3.3	3.5	3.3	3.8	2.4	1.4 (0.7–2.6)	2.4	3.7
45.7	27.6	42.3 (40.0–44.4)	7.0	8.8	9.5	5.5	3.7	12.7 (6.1–17.3)	5.4	6.2
38.5	53.1	40.1 (36.9–43.3)	24.2	32.1	37.6	31.3	12.8	0.4 (tr.–0.6)	41.3	43.0
	1.7	(tr.–0.3)	1.5	1.2	0.8	0.4	3.6		22.6	20.9
10.2	1.0	3.2 (2.2–3.7)	tr.		0.6	2.9				
		tr.	tr.		0.2	0.3	2.4			
1.4	2.5	3.1 (1.4–4.7) 2.5 (2.3–2.6)	2.2	1.4	1.3	0.5		0.8 (tr.–2.0)		
		tr. 1.7 (1.1–2.3)						0.1 (tr.–0.2)		
tr.	0.3		2.7	5.2	7.6	5.1	24.0		2.2	1.5

TABLE 5 POSITIONAL DISTRIBUTION OF FATTY ACIDS IN CHOLINE GLYCEROPHOSPHATIDES

	Frontal White Matter				Sciatic Nerve*			
	1-linked	2-linked	Total	Expected†	1-linked	2-linked	Total	Expected†
14:0	2.0	1.5	1.8	1.8	2.2	2.7	1.8	2.4
15:0	0.9	0.5	0.5	0.7	tr.	tr.	tr.	tr.
16:0	54.2	35.2	45.5	44.7	27.3	52.8	43.9	40.1
16:1	4.3	4.0	3.5	4.2	4.5	5.7	3.6	5.1
17:0	0.5	0.5	tr.	0.5	tr.	tr.	tr.	tr.
18:0	14.8	tr.	8.8	7.4	9.4	2.9	5.9	6.2
18:1	13.3	54.6	32.1	34.0	8.0	20.5	14.7	14.3
18:2	0.7	1.6	1.2	1.2	1.9	10.0	6.7	6.0
20:4	—	2.1	1.4	1.0	tr.	5.3	3.2	2.7
Phytanic acid	9.3	—	5.2	4.7	46.7	—	20.0	23.3

Fatty acids are expressed as a percentage of the total fatty acids in each position, or of the total fatty acids of CGP.
* Obtained from connective tissue surrounding the sciatic nerve.
† "Expected" results are the average of the 1-linked and 2-linked fatty acids.

TABLE 6 UNSUBSTITUTED FATTY ACID COMPOSITIONS OF SPHINGOLIPIDS

	Sphingomyelin			Cerebroside			Cerebroside Sulfate		Normal* Myelin Values		
	Frontal Myelin	Cord Myelin	Sciatic Nerve	Frontal WM	Frontal Myelin	Cord Myelin	Frontal Myelin	Cord Myelin	Sphingo-myelin	Cerebro-side	Cerebroside Sulfate
Sum of C_{14-18}	36.3	47.7	45.9	18.9	28.8	34.4	37.1	30.0	46.0	20.5	11.2
Sum of C_{19-26}	63.7	52.3	54.1	81.1	71.2	65.6	62.9	70.0	54.0	79.5	88.8
Sum of odd-numbered acids	14.5	4.6	3.5	21.7	18.5	9.6	17.8	7.3	5.9	16.9	14.4

Fatty acids are expressed as a percentage of the total unsubstituted fatty acids of each sphingolipid. WM, white matter.
* Frontal myelin values from three normal subjects ages 6 yr, 9 yr, and 55 yr (17).

ter and the sciatic nerve region, phytanate was found to be exclusively linked to the 1-position. Good agreement was found when an average of the 1- and 2-linked fatty acids was compared with the total fatty acids composition of CGP. This result indicated that the esterase cleaved one-half of the initial ester linkages and that acyl migration was not significant during hydrolysis. The placement of phytanate in the 1-position results in the displacement of saturated fatty acids, principally 16:0. Thus, phytanate-containing CGP is enriched with branched chain and polyunsaturated acids.

Fatty Acid Composition of Sphingolipids

The unsubstituted fatty acid compositions of sphingomyelin, cerebroside, and cerebroside sulfate in the patient were nearly normal in all tissues, and phytanate was not detected. Each fatty acid was quantified, but the data are summarized in Table 6 to save space. There was no deficiency of either long-chain or odd-numbered fatty acids.

The hydroxy fatty acid contents of cerebroside and cerebroside sulfate from each tissue are given in Table 7. The proportions of hydroxy acids were close to normal. A smaller proportion of hydroxy fatty acids was present in cerebroside from sciatic nerve than in that from central nervous system tissues. This may reflect a lower content of hydroxy acids in cerebrosides from peripheral nerves. Cerebrosides from bovine intradural spinal root nerves have also been found to contain relatively small proportions (35.2%) of hydroxy fatty acids (21).

The hydroxy fatty acid compositions of cerebroside from frontal lobe myelin, spinal cord myelin, and sciatic nerve from the patient were not significantly different from normal. Each hydroxy acid was quantified, but the results are summarized in Table 8 to save space. In each cerebroside sample there were high proportions of very long-chain hydroxy fatty acids as well as odd-numbered hydroxy fatty acids.

DISCUSSION

The present study demonstrates that phytanic acid accumulates in lipids from frontal lobe and spinal cord myelin in Refsum's disease. Although the proportions of phytanate in myelin lipids of the central nervous system were small, three- to fourfold higher proportions were found in sciatic nerve (Table 4). Hansen (22) has also demonstrated that phytanate is present in higher proportions in sciatic nerve lipids (13.8%) than in brain lipids (8.5%) in another patient who died with Refsum's disease. The present study indicates that there is no preferential exclusion of phytanate from the myelin

TABLE 7 HYDROXY FATTY ACID CONTENT OF SPHINGOLIPIDS IN REFSUM'S DISEASE

	Frontal Lobe					Normal Values	
	Gray Matter	White Matter	Myelin	Spinal Cord Myelin	Sciatic Nerve	Frontal Myelin*	Spinal Root Myelin†
Cerebroside	78	67	75	61	41	82	35.2
Cerebroside sulfate			14	30		25	32.0

Hydroxy acids are expressed as a percentage of the total fatty acids in each sphingolipid.

* Values from a 55 yr old normal man (17).

† Values from myelin isolated from bovine intradural spinal root nerves (21).

TABLE 8 HYDROXY FATTY ACIDS OF CEREBROSIDE

	Frontal Myelin	Cord Myelin	Sciatic Nerve	Normal* Myelin Values
Sum of $C_{14h-18h}$	1.6	5.9	4.3	4.5
Sum of $C_{14h-26h}$	98.4	94.1	95.7	95.5
Sum of odd-numbered acids	20.5	23.6	21.5	20.6

Fatty acids are expressed as a percentage of the total hydroxy fatty acids of cerebroside.

* Frontal myelin values from three normal subjects ages 6 yr, 9 yr, and 55 yr (17).

membrane since it was present in higher proportions in frontal myelin than in white or gray matter.

The finding that phytanate accumulates preferentially in CGP and is exclusively confined to the 1-position emphasizes once again that each brain lipid has a unique fatty acid composition (17). Little information is available in the literature on the positional distribution of branched-chain fatty acids in glycerophosphatides. Yabuuchi and O'Brien (23) have shown that polyunsaturated fatty acids are preferentially linked to the 2-position in glycerophosphatides from gray matter. Recently, we have found (M. Golditch and J. O'Brien, unpublished) that isosteric acid, fed to rats, accumulates preferentially in CGP in the liver, but this branched-chain fatty acid becomes predominantly linked to the 2-position of CGP.

What is the relationship of the phytanic acid accumulation to the demyelination? It was proposed earlier (11) that if phytanate accumulated in myelin, it would tend to disrupt the packing of the hydrocarbon tails in the center of the bimolecular lipid leaflet due to steric hindrance imposed by its branched methyl groups. It has been shown that, under similar conditions, surface films of phytanic acid are much more highly expanded (by about 80%) than surface films of palmitic acid (see reference 11 for discussion). The interaction energy between hydrocarbon tails packed in parallel array is profoundly affected by the interchain distance. The interchain attractive energies (principally London–van der Waals forces) are "distance-specific"; that is, they are inversely proportional to the fifth power of the distance between interacting chains (24). Thus, even a small expansion of the myelin bilayer in the plane of the lamellae would result in a large diminution in interaction energies between adjacent lipid molecules (24, 25). For this reason, the introduction of 1-phytanoyl 2-palmitoyl CGP (the predominant molecular species) into sciatic nerve myelin will be expected to destabilize myelin and perhaps result in demyelination.

If this hypothesis is correct, phytanate must accumulate in myelin lipids and the degree of accumulation should be roughly proportional to the degree of demyelination. The present study provides some experimental data in support of both predictions, since phytanate was found in myelin and the highest concentrations of it were found in the sciatic nerve, which was the most severely demyelinated. Unfortunately, it was not possible to isolate myelin from sciatic nerve. Nonetheless, it is reasonable to suppose that phytanate accumulated in sciatic nerve myelin as well for the following reasons. Phytanate accumulated in myelin of the central nervous system, which indicates that it is not excluded from the myelin membrane. Secondly, phytanate was found linked to CGP in sciatic nerve, a lipid known to be a major myelin constituent. Whether the magnitude of the accumulation is sufficient to result in myelin destabilization is at present an unanswered question. If the lipid composition of sciatic nerve myelin is the same as that of spinal root myelin (21), and if the phytanate content of sciatic nerve myelin is the same as in the whole nerve, then the phytanate content of sciatic nerve myelin is 6–7% of the total fatty acids. This is somewhat lower than the value of 13.8% found by Hansen in another patient (22). Studies on monolayers containing mixtures of myelin lipids, to which synthetic phytanate-containing lecithins are added in the appropriate concentrations, may shed light on the magnitude of the expansion effect.

It is tempting to suggest that the predilection for the peripheral nervous system in Refsum's disease is due to a greater susceptibility to dietary influences of peripheral than of central myelin. We became aware of this possibility when we found tenfold higher proportions of linoleate in CGP from spinal root myelin than in CGP from central nervous system myelin (21). Nicholls and Rossiter (26) have shown that tritiated cholesterol in-

jected into mature rats becomes incorporated into intact peripheral nerves. These observations suggest a less restrictive blood–brain (or blood–nerve) barrier in the peripheral nervous system. Since phytanate in Refsum's disease is supplied solely from exogenous dietary sources (10), the higher concentrations in peripheral nerves than in central nerves may be explained on this basis. The slow development of peripheral neuropathy in patients with Refsum's disease (the patient in this study was not severely debilitated until her thirties) is also consistent with the idea that a slow build-up of dietarily-supplied phytanate occurs in peripheral nerves. Once phytanate levels reach a critical point, destabilization of the myelin bimolecular lipid leaflet may lead to its subsequent dissolution. Since cholesteryl esters do not accumulate in these demyelinated nerves (and inflammatory cells are not present), the demyelination is not akin to Wallerian degeneration, either chemically or pathologically (3).

It is also tempting to speculate that once high phytanate levels are reached, Schwann cells cannot remyelinate effectively because their plasma membranes have the wrong flow characteristics—that is, the melting point of the Schwann cell plasma membrane is lowered because of the high content of phytanate. The resultant increase in fluidity of the Schwann cell plasma membrane interferes with its manufacture of semirigid, relatively permanent, lamellar myelin spirals. Schwann cells, in an aberrant attempt to myelinate, proliferate and form haphazard tangles of circularly oriented cells with abundant cytoplasmic extensions, giving rise to the characteristic "onion bulb" formations present in nerves in Refsum's disease (3).

Despite the satisfying intellectual features of the above-mentioned conjectures, some alternative possibilities need to be considered in the pathogenesis of the demyelination. It has been suggested that Refsum's disease may involve a systemic error in α-hydroxylation and one-carbon degradation of fatty acids (10). The neuropathy in Refsum's disease could result from defective metabolism of cerebroside (and cerebroside sulfate) fatty acids. The α-hydroxylation and one-carbon degradation system for glyco-sphingolipid fatty acids leads to the production of a fatty acid one carbon shorter than its precursor, with an α-hydroxy fatty acid as an intermediate (27, 28); e.g., $24:0 \rightarrow 24h:0 \rightarrow 23:0 + CO_2$. If the α-hydroxylation and one-carbon degradation of cerebroside fatty acids was defective in our patient, the percentages of hydroxy fatty acids and of odd-number fatty acids should be abnormally low. The data reveal no deficiency of hydroxy fatty acids or of odd-number fatty acids in either cerebroside or cerebroside sulfate in any tissue studied. The evidence indicates that the defect in α-hydroxylation and one-carbon degradation of phytanic acid does not apply to cerebroside fatty acids as well.

The possibility should be considered that damage to the nervous system in Refsum's disease may be due, in part, to the release of hydrolytic enzymes with resultant membrane damage. This suggestion is prompted by the finding that lyso-EGP accumulated in the present patient's tissues. Admittedly, the same result could occur from artifactual hydrolysis due to prolonged storage, and freezing and thawing of the tissues. However, we have not encountered the accumulation of lyso-EGP in cerebral tissues in several diseases of the central nervous system studied thus far, nor in any normal controls. Destabilization of lysosomal membranes with resultant release of hydrolytic enzymes should be considered in Refsum's disease, since when retinol, which also bears a branched isoprenoid side chain, is added to erythrocytes and lysosomes, they are destabilized (29, 30). Further information needs to be gathered on this point.

Finally, if the neuropathy in Refsum's disease does result from the accumulation of phytanic acid in myelin, and if the levels of phytanate can be diminished by eliminating it from the diet, then the neuropathy may be reversible. Eldjarn et al. (31) demonstrated that the exclusion of phytanate from the diet induced a precipitous drop in the serum levels of this fatty acid in patients with Refsum's disease. It may take a considerable time to deplete this fatty acid from relatively slowly metabolizing stores such as those in peripheral nerve membranes, but a beneficial effect on the neuropathy should result, if, as postulated here, the accumulation of phytanate is responsible for the demyelination.

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