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PELIZAEUS–MERZBACHER DISEASE: BRAIN LIPID AND FATTY ACID COMPOSITION

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Abstract—Biochemical analysis of the leukodystrophic brain from a case of Pelizaeus–Merzbacher disease, classical type, was performed. A decrease in the amount of solid material present was found. The lyophilized brain weight was reduced to 76% of normal with a slightly greater decrease in the amount of extractable lipid. Total myelin was diminished to 7% of normal. Among specific lipids plasmalogens were present in slightly lowered amounts. Cerebrosides and sulphatides were drastically reduced to 8% of normal, whereas sphingomyelin was less severely affected. Fatty acids from phospholipids were close to normal, only enols being slightly diminished. Analysis of pure cerebrosides and sulphatides revealed that the α-hydroxylated compounds as well as very long chain fatty acids (over C_{18}, especially C_{23} to C_{26}) were greatly reduced. For chain lengths over C_{18}, the ratio of leukodystrophic fatty acid to normal fatty acid was close to 10%. The defect in very long chain fatty acids is estimated at 99.2% in total brain.

Thus, we have found a marked decrease in the amount of very long chain fatty acids and a less marked decrease in sphingolipids. The reduced amount of these acids appears to be partially offset by an increase in the amount of medium-chain fatty acids in sphingolipids. We conclude that one aspect of Pelizaeus–Merzbacher disease may be a defect in the synthesis of myelin very long chain fatty acids (as these acids are far much reduced than any other myelin molecule).

THE TERM Pelizaeus–Merzbacher disease originally described a peculiar leukodystrophic process affecting one family in which 14 cases, 12 male and 2 female, one a sibling of the original case, were observed over 4 generations (PELIZAEUS, 1885; MERZBACHER, 1910; SPIELMEYER, 1923). Onset occurred during the first few months of life with nystagmus and tremor of the head. The disease progressed slowly, eventually including bradylalia, scanning speech, ataxia and intention tremor of the arms combined with spasticity of the legs, occasional athetotic movements and tic-like twitching, pallor of the optic discs and trophic disturbances of bones with secondary scoliosis. In most cases there was only mild dementia. Death occurred in the second decade (LIEBERS, 1928). The peculiar neuropathological findings included a diffuse demyelination with residual myelin islands, often around blood vessels. This gave a tigroid appearance to a myelin stain preparation of the central white matter. Preservation of most nerve cells and processes, especially the axons, and the presence of minimal amounts of neutral lipids (SPIELMEYER, 1923 as quoted by SEITELBERGER, 1970) also occurred.

A number of similar cases have since been reported. Many of these differed from the above description because of very early (congenital) onset with findings of total demyelination, or delayed onset and partial myelination. NORMAN *et al.* (1961) proposed that a distinction be made between the completely demyelinating connatal type, the classical type described above and an adult form of Pelizaeus–Merzbacher disease. After a thorough review of the literature, SEITELBERGER (1970) has recognized 11 reported cases representing the classical type. Until now, of 15 reports mentioned for Pelizaeus–Merzbacher disease (61 patients) 9 dealt with familial cases and 6 with sporadic (MERZBACHER, 1923; SPIELMEYER, 1923; LIEBERS, 1928; BOSTROËM, 1927; BIELSCHOWSKY & HENNEBERG, 1928; BODECHTEL, 1929; JOSEPHY, 1935; BONHOFF, 1954; EINARSON & NEEL, 1938; NORMAN & TINGEY, 1963; GARCIN *et al.*, 1965; ALLIGRANZA *et al.*, 1968; WATANABE *et al.*, 1969; SCHNABEL, 1970; WATANABE *et al.*, 1973; LIANO *et al.*, 1974; BORNHOFEN *et al.*, submitted to *Acta Neuropath.*).

Previous biochemical investigations performed on 2 cases of the classical type in which the brains had been formalin-fixed (NORMAN & TINGEY, 1963; ALLEGRANZA *et al.*, 1968) showed a reduction of total lipids, including cholesterol, cerebrosides and sulphatides. However, analysis of a cerebral biopsy performed at 14 weeks of age on another case believed to be of the classical type showed a normal sphingolipid content (WATANABE *et al.*, 1973); no sulphatides and a 50% decrease in cerebrosides were found in a presumed Pelizaeus–Merzbacher disease (WATANABE *et al.*, 1969).

MATERIAL AND METHODS

Case report. The patient, a female, was one of two siblings who had demonstrated a slowly progressive neuro-

logical handicap beginning with nystagmus and ataxia at 2½ years and progressing to include bradylalia, scanning speech, optic atrophy, and spasticity in the lower extremities with relative preservation of intellect. Death at 11 years of age was attributed to bronchopneumonia. Neuropathological findings, on both the patient and her brother, supported the diagnosis of Pelizaeus–Merzbacher disease, classical type. There was no similar family history and the marriage was not consanguinous. A detailed report of the clinical and the neuropathological findings is given separately (BORNHOFEN *et al.*, submitted to *Acta Neuropath.*).

Biochemical techniques. The biochemical studies were carried out on fresh autopsy material (autopsy performed 1 h after death) that had been kept frozen at −60°C for 18 months. The frontal lobe was analyzed using total brain, as it was not possible to separate the white from the gray matter in this case of leukodystrophy. The control brain was from a normal 11 year old girl. This specimen had been kept frozen at −60°C for 10 months. The fragments were thawed for 24 h at −30°C and for 6 h at 4°C. A large number of the pieces were used to isolate myelin according to NORTON & PODUSLO (1973) with slight modification for the diseased brain: three layers from the first gradient were pooled to increase the yield of the preparation. The myelin was lyophilized after suspension in water and weighed.

Another part of the brain was used for lipid analysis. The technique used for lipid extraction as well as the methods used for quantitative lipid analysis have been described previously (BAUMANN *et al.*, 1968; JACQUE *et al.*, 1969). Briefly, brain was homogenized in chloroform–methanol (2:1, v/v) according to FOLCH (1951); total lipid extract was chromatographed using the solvent system of SUZUKI *et al.* (1966) chloroform–methanol–water (70:30:4, by vol.). The chromatograms were sprayed with bichromate (ROUSER *et al.*, 1964), and they were also viewed under ultra-violet light (245 nm) or stained with molybdic reagent for phospholipids (DITTMER & LESTER, 1964), α-naphtol for glycolipids (JACIN & MISHIN, 1965), 2-4 dinitrophenyl hydrazine for plasmalogens (REITSEMA, 1964) antimony trichloride for sterols (STAHL, 1964). An extract, filtered of most of the proteins, was used for quantitative lipid analysis. Phosphorus was measured according to BARTLETT (1951); glycolipids were quantified by orcinol reagent (RADIN *et al.*, 1955); cholesterol was measured according to SEARCY & BERQUIST (1960) and plasmalogens according to GOTTFRIED & RAPPORT (1962). The proportion of the galacto and gluco moieties in cerebrosides was determined by the method of CHAMBERS & CLAMP (1971) after estimation of thin layer chromatogram impregnated by borate (KEAN, 1966).

A large part of the lipid extract was used to isolate cerebrosides, sulphatides and sphingomyelin. Phospholipids were eliminated by selective methanolysis (HAJRA & RADIN, 1963) slightly modified: lipids were dissolved in chloroform–methanol (2:1, v/v) (200 mg), evaporated to dryness and dissolved in 2.5 ml chloroform and 1 ml of methanolic sodium hydroxide, 0.5 N. Methanolysis was allowed to proceed for 30 min at 37°C with mechanical stirring; the mixture was acidified to pH 1 with concentrated HCl and left for 2 h at room temperature to obtain complete methanolysis of the plasmalogen fraction of phosphatides. The methanolysate was washed three times by 0.8 ml of Folch upper phase. The lower phase was checked for the absence of phospholipids by TLC and visualisation by molybdic reagent. There was no loss of sphingolipids during this methanolysis. Each individual sphingolipid was then purified by column chromatography. By this procedure 4 g of unisil (100–200 mesh) in chloroform were used. The volume was washed twice by chloroform before applying the lipids dissolved in this solvent. Chloroform 75 ml eluted cholesterol and fatty acids; 125 ml chloroform–methanol (95:5, v/v) eluted cerebrosides; 138 ml of chloroform–methanol (80:20) eluted sulphatides; 75 ml of methanol eluted sphingomyelin. The purity of each fraction was checked by thin layer chromatographies sprayed by α-naphtol and molybdic reagent. No cross-contamination was detected. Each lipid fraction was methylated according to MORRISON & SMITH (1964). Eventually a preparative TLC separated non-hydroxylated fatty acid methyl esters from the α-hydroxy fatty acids (solvent: 20% ether in petroleum ether, b.p. 45–60°C).

Fatty acid analysis was performed by gas liquid chromatography on 3% SE 30 column (on chromosorb WAW 100/120) with temperature programming at 2°C/min between 155 and 288°C with a Packard apparatus No. 800. Fatty acids were identified by retention time, equivalent chain length and co-chromatography with commercial standards. The ratio hydroxy/non-hydroxy fatty acids was determined by quantification on the same run of $C_{24:0}$ and h-$C_{22:0}$ or by adding known quantities of $C_{20:0}$ and h-$C_{20:0}$ in the mixture before TLC (taking into account the very low quantity of the natural occurrence of these acids). The two techniques gave the same results. The lower yield of hydroxy acids (71% of non-hydroxy) in the flame detector was taken into account. Peak area was determined by triangulation. Each experiment was performed three times.

RESULTS

Thin layer chromatography of brain lipids

Qualitative abnormalities of cerebrosides, sulphatides and sphingomyelin are detected in leukodystrophy material (Fig. 1). In the leukodystrophy material, the slower moving spot of cerebrosides (phrenosine) is greatly reduced and the faster spot (kerasin) has completely disappeared. Sulphatides are nearly undetectable and sphingomyelin is reduced in the pathological brain. No abnormality in diacylphosphatide or cholesterol is detected. However, ethanolamine plasmalogen is slightly reduced. The results are in good agreement with the lack of myelin demonstrated by histologic methods because cerebrosides and sulphatides are essentially constituents of white matter (O'BRIEN & SAMPSON, 1965). Sphingomyelin (O'BRIEN, 1964) and ethanolamine plasmalogens (WELLS & DITTMER, 1967) are found in the gray matter as well as in the white matter.

Quantitative analysis

The results of quantitative analysis are presented in Table 1. A diminished amount of solids is found in the pathological material (as the lyophilized weight of leukodystrophy brain is 76% of the normal value). The amount of lipids is 63% of the normal, expressed by gram of lyophilized tissue; it is higher if based on wet material. Galactolipids are drastically reduced in the leukodystrophy brain (8% of the normal value

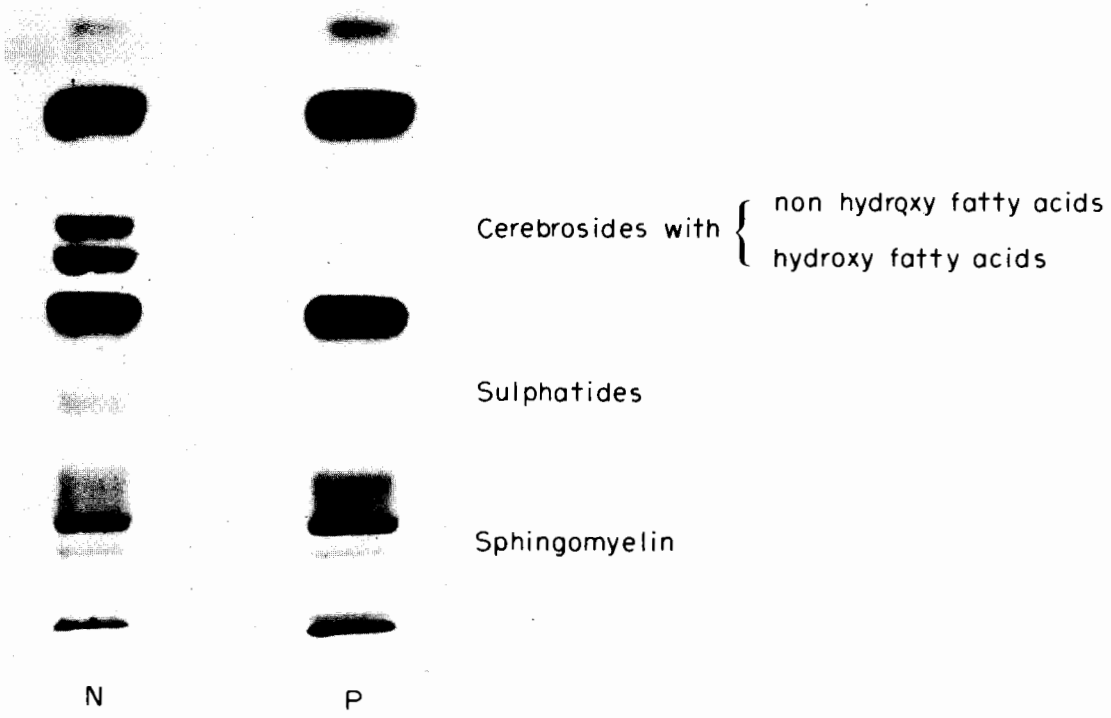

FIG. 1. Thin layer chromatography of 'normal' and leukodystrophy brain lipids. Precoated TLC plates silicagel 60 F 254 from Merck. Solvent: chloroform–methanol–water (70:30:4, by vol), 0,600 mg lipid extract was spotted. Sprayed with bichromate reagent.

TABLE 1. QUANTITATIVE ANALYSIS OF TOTAL LIPID EXTRACT AND MYELIN RECOVERY

	Normal	Leukodystrophy
Lyophilized brain (mg)/wet brain (g)	218 ± 4	165 ± 18
Lipid extract (mg)/lyophilized brain (g)	605 ± 5	380 ± 19
Isolated myelin (mg)/wet brain (g)	33 ± 2	2.5 ± 1
Lipids (mg)/wet brain (g)		
Galactolipids	24	2
Galactocerebrosides (%)	98.3	82.6
Glucocerebrosides (%)	1.7	17.4
Cholesterol	37	17
Phospholipids	61	36
Lipids (mg)/lipid extract (100 mg)		
Galactolipids	18 ± 4	3 ± 1
Cholesterol	28 ± 3	28 ± 4
Phospholipids	46 ± 2	58 ± 3

Each value is the mean of 3 measurements ± S.D.

in fresh brain), consistent with the reduction of myelin.

There is a relatively high proportion of glucocerebrosides in the leukodystrophy material (but the absolute amount is about the same as in the control).

Fatty acid methyl esters analysis of brain lipids

Phospholipids fatty acid profile is essentially normal in the leukodystrophy brain (including poly-unsaturated chains). However, dimethyl-acetals with 16 and 18 carbon atoms are slightly reduced corresponding to the reduction of plasmalogens (dimethyl acetals derive from enols of plasmalogen during methanolysis).

Initial sphingolipid investigations by thin layer chromatography suggested that less hydroxy fatty acids were present in the pathological material. We were able to measure these difference by gas chromatography. In cerebrosides (Table 2), the fatty acid profile is completely disturbed in the leukodystrophy material, with considerable reduction in fatty acids with very long chains (over C_{18}); for instance C_{24} fatty acids are drastically reduced (about 10% of the normal value). Hydroxy fatty acids are largely affected since they consist mainly of very long chains. Using 100 as an arbitrary basis for saturated fatty acids in both normal and pathological material, Table 3 shows that other fatty acid families are even more reduced in the pathological material, unsaturated fatty acids (both hydroxylated and non-hydroxylated) being most affected.

In sulphatides (Table 3 and 4) essentially the same anomalies are seen, very long chain fatty acids being reduced by approx 90%. Here again the large reduction of C_{24} fatty acids is particularly evident. Hydroxy fatty acids are reduced in nearly the same proportion in cerebrosides and sulphatides, although these acids are quantitatively more important in normal cerebrosides (50% of total fatty acids) than in sulphatides (30% of total fatty acids).

The main defect for fatty acids is the extreme reduction of the very long chain fatty acids, the ratio of very long chain ($\geq C_{19}$) over short and medium chains ($< C_{19}$) being about 1% of the normal value, this being between 0.5 and 1.5% according to the lipid considered (cerebroside or sulphatide) and the class (saturated or mono unsaturated) referred to.

In sphingomyelin, hydroxy fatty acids were hardly detected in normal material, and qualitative analysis shows that these acids are not detected in leukodystrophy brain. Because of the very low quantities available, the profile was not determined. Reduction in very long chain fatty acids (saturated and mono unsaturated) is observed, but in lower proportion than in cerebrosides and sulphatides. The ratio of very long chains ($\geq C_{19}$) to short and medium chains ($< C_{19}$) is about 25% of the normal value (Table 5).

Figure 2 shows the value of the ratio of pathological to normal tissue for fatty acids of cerebrosides and sulphatides according to their chain length, hydroxy and non-hydroxy fatty acids, and saturated and mono-unsaturated. It must be pointed out that this ratio is constant for all chain lengths over C_{22}.

DISCUSSION

Analyses was performed on whole brain, not on isolated white and gray matter, because we were unable to separate the white matter. This should not seriously affect our findings since cerebrosides and sulphatides have nearly the same fatty acid profile in both constituents (RADIN & AKOHORI, 1961; SVENNERHOLM & VANIER, 1973). However, in sphingomyelin the concentration of very long chain fatty acids is somewhat higher in white matter than in the cerebral cortex (SVENNERHOLM & VANIER, 1973). This is possibly due to the fact that sphingomyelin is not found only in myelin but also in other membranes.

Our findings for lipid composition and for the fatty acid profile in the normal brain compare favorably with previously published results for both man and animals. These findings, however, stress the relative concentration of fatty acid chains longer than C_{24}, which are not generally given by other authors. By contrast, short chain fatty acids (C_{12} and C_{14}) are barely detected in the normal brain sphingolipids, a

Table 2. Cerebrosides fatty acids

	Chain length		16	18	19	20	21	22	23	24	25	26	Total	Ratio $>C_{19}/<C_{19}$
Non-hydroxylated fatty acids	Saturated	N%	1.4	4.6	0.05	0.44	0.13	1.4	1.9	7.8	2.4	0.86	20.98	2.50
		P%	29.3	51.2	*t*	0.50	*t*	0.45	0.18	1.0	0.17	0.12	82.72	0.03
	Monounsaturated	N%		0.76		0.24		0.16	0.40	17.7	4.6	4.3	28.16	38.4
		P%		6.6	—	0.38	—	0.15	0.046	1.0	0.27	0.29	8.74	0.32
α-Hydroxylated fatty acids	Saturated	N%	*t*	0.39	—	0.43	—	3.9	6.4	20.5	3.1	0.82	35.54	
		P%	*t*	0.5	—	0.12	—	1.21	1.14	4.2	0.50	0.13	7.80	
	Monounsaturated	N%	—	—	—	—	—	*t*	0.29	9.7	1.6	1.7	13.29	
		P%	—	—	—	—	—	*t*	*t*	0.45	0.09	0.073	0.62	

The chain length refers to the number of carbon atoms of the considered fatty acid. N: normal, P: pathological. Ratio $\geq C_{19}/<C_{19}$ means ratio of chain equal and longer than 19 carbon atoms over chains smaller. −: not detected. *t*: <0.05%. Each value is the mean value from 3 experiments (18 chromatograms at least).

Table 3. Estimation of each family of fatty acids related to saturated fatty acids

		Saturated	Monounsaturated	α-OH-saturated	α-OH-monounsaturated
Cerebrosides	Normal	100	140	175	65
	Pathological	100	10	9	0.7
	Ratio $\frac{\text{Pathological}}{\text{Normal}}$	3.9	0.3	0.2	0.05
Sulphatides	Normal	100	148	71	28
	Pathological	100	14	6	1.5
	Ratio $\frac{\text{Pathological}}{\text{Normal}}$	2.8	0.28	0.25	0.15

The content of saturated fatty acids was arbitrarily set to be 100 for galactolipids derived from normal and pathological tissue and all other families of fatty acids are referred to this.

TABLE 4. SULPHATIDES FATTY ACIDS

Chain length			16	18	19	20	21	22	23	24	25	26	Total	Ratio $\geq C_{19}/<C_{19}$
Non-hydroxylated fatty acids	Saturated	N%	5.2	6.0	0.16	0.31	0.17	1.2	1.8	9.8	3.1	1.0	26.74	1.60
		P%	28.4	50.7	0.33	0.39	0.12	0.39	0.16	0.84	0.18	0.14	81.65	0.03
	Monounsaturated	N%	—	4.9	—	2.0	—	0.23	0.50	24.1	5.2	5.8	42.73	7.6
		P%	—	6.5	—	2.4	—	0.11	0.075	1.8	0.43	0.53	11.85	0.8
α-hydroxylated fatty acids	Saturated	N%	*t*	0.14	—	0.57	—	1.4	3.5	11.7	2.1	0.94	20.35	
		P%	*t*	0.17	—	1.7	—	0.56	0.49	1.7	0.23	0.18	5.03	
	Monounsaturated	N%	—	—	—	—	—	*t*	0.11	5.8	0.86	1.2	7.97	
		P%		—	—	—	—	—	0.022	0.94	0.17	0.18	1.22	

See legend to Table 2.

TABLE 5. SPHINGOMYELINS FATTY ACIDS

Chain length		16	18	19	20	21	22	23	24	25	26	Total	Ratio $\geq C_{19}/<C_{19}$
Saturated	N°/₀	5.7	42.9	0.29	1.7	—	1.8	1.7	6.8	1.9	0.53	63.32	0.30
	P°/₀	6.0	79.0	0.62	3.6	—	1.4	0.43	1.1	0.1	—	92.25	0.08
Monounsaturated	N°/₀	—	3.1	—	1.2	—	0.36	0.33	23.3	4.1	3.7	36.09	10.3
	P°/₀	—	1.9	—	1.0	—	0.16	0.10	3.9	0.26	—	7.32	2.8

See legend to Table 2.

finding at variance with previous reports (O'BRIEN & ROUSER, 1964). We feel with SVENNERHOLM & VANIER (1973) that the appearance of these acids is due to contamination from organic solvents.

These results show that in the leukodystrophy brain the reduction of galactolipids parallels the diminution of myelin (Table 1); but there is a much more drastic reduction in very long chain fatty acids. It is to be expected that saturated and mono-unsaturated fatty acids are jointly reduced since these fatty acids share a common synthetic pathway (BOURRE *et al.*, 1976*a*). A lack of substrate probably accounts for the reduction of hydroxylated compounds which have been shown to be synthesized from their non-hydroxylated homologues (HOSHI & KISHIMOTO, 1973). Myelin fatty acids are synthesized in endoplasmic reticulum (BOURRE *et al.*, 1973*b*, 1976*b*, 1977) and not in mitochondria (BOURRE *et al.*, 1977). In endoplasmic reticulum there are at least three different multi-enzyme complexes, one de novo system producing C_{16} (POLLET *et al.*, 1973; BOURRE *et al.*, 1973*a*) and two elongating systems synthesizing respectively C_{18} and very long chain fatty acids (BOURRE *et al.*, 1970). The deficiency of very long chain fatty acids suggests a deficiency of the latter enzyme complex. A similar sphingolipid profile is seen in the murine leukodystrophy of the Quaking mouse, in which the latter elongating enzyme is indeed affected (BOURRE *et al.*, 1973*a*, 1975). One may suspect that one aspect of human Pelizaeus–Merzbacher disease may be a defect in the synthesis of myelin very long chain fatty acids due to a deficiency of one of the elongating enzyme complexes.

It is worth noting that in this case the disease appears to have been inherited as an autosomal recessive, i.e. both children were affected, one female and one male, but neither parent, and there was no similar family history. Although similar autosomal recessive cases have been reported, and the occurrence of dominant heredity cannot be excluded, most families clearly demonstrate a sex-linked mode of inheritance. It is known that myelination in the mouse is controlled by at least two chromosomes. Two mutants

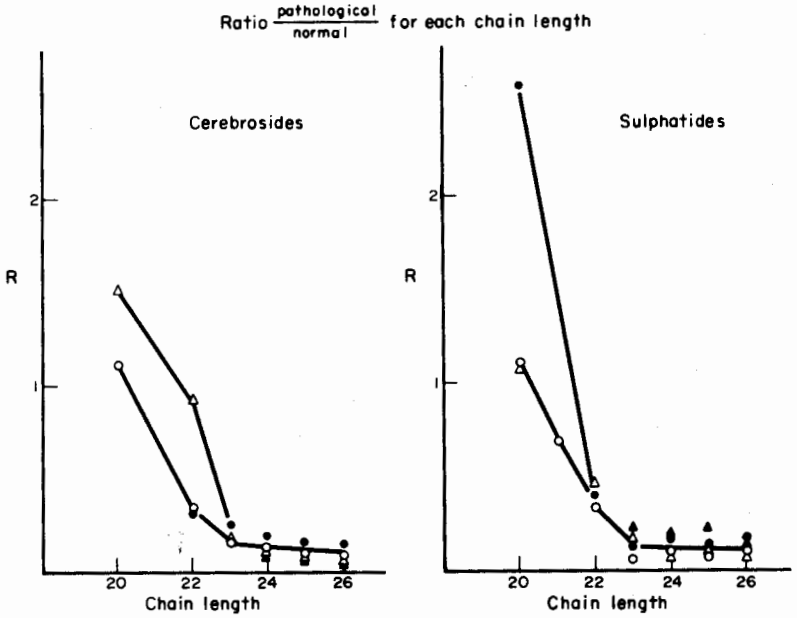

FIG. 2. Ratio R = (Pathological/Normal) for each brain length of fatty acids in cerebrosides and sulphatides. Normal fatty acids, saturated (○) and mono-unsaturated (△) α-hydroxylated fatty acids, saturated (●) and mono-unsaturated (▲).

with hereditary defective myelination have been identified (SIDMAN *et al.*, 1964), Quaking referred to above, and Jimpy, with mutation on autosome and gonosome respectively.

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