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Biochemical and Physicochemical Determinations in a Premyelin Fraction Obtained by Zonal Centrifugation in Normal Mouse and in Dysmyelinating Mutants (Quaking, Shiverer, and Myelin-Deficient)

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ABSTRACT

Myelin and premyelin material denser than myelin were obtained from quaking (Qk), shiverer (Shi), and myelin-deficient (mld) mutant and control mice, using zonal centrifugation on zonal rotor. On these fractions, we performed biochemical analysis (lipids and fatty acid), and, in parallel, we determined the physical structure of membranes by the spin-label method. The hyperfine splitting constant ($2T_{\parallel}$) was used to determine the order of membranes and their rigidity, and frequency of rotation (V_c) was used to measure fluidity.

In control mice, the premyelin material contained a lesser amount of sphingolipids than pure myelin, but the relative proportions between hydroxy- and nonhydroxy-cerebroside and sulfatides were similar in the premyelin material and in pure myelin. The

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premyelin material contained half the alkanes found in the pure myelin and much less very-long-chain fatty acids. The ($2T_{\perp}$) was lower in the premyelin material, but the (V_c) was similar in myelin and premyelin material.

In mutants, the amount of material recovered in the premyelin fraction was reduced in qk, and increased in both shi and mld. The relative amount of sphingolipids were normal in mld, but not in shi mutants, especially in cerebrosides formed with α-hydroxylated fatty acids and sulfatides formed with unsubstituted fatty acids. The absolute amounts of sphingolipids were nearly normal in both shi and mld.

In the premyelin fraction from qk mutants, both relative and absolute amounts of sphingolipids were drastically altered. In percentage, cerebrosides and sulfatides formed with nonhydroxyfatty acids were dramatically reduced, and, conversely, cerebrosides and sulfatides formed with hydroxyfatty acids were increased. In terms of absolute amount, only cerebrosides and sulfatides formed with nonhydroxyfatty acids were dramatically reduced.

In the premyelin fraction, polyunsaturated fatty acids were increased in shi and mld, but decreased in qk. In this mutant, lignoceric (24:0) and nervonic (24:1) acids were drastically reduced.

The amount of alkanes in the premylin material from qk and mld was reduced by 50%. The shi fraction was nearly free of alkanes.

The maximal apparent coupling constant (hyperfine splitting constant, $2T_{\perp}$) was not affected in the mld and qk mutant, but was reduced in the shi mutant premyelin fraction. The V_c was dramatically increased in the qk, slightly decreased in the shi, and close to control in the mld.

This work provides additional data on premyelin material prepared in various neurological mutants using continuous gradients in zonal rotor. Moreover, it reopens the discussion on the possible primary defect in the qk at the level of a defective synthesis of nonhydroxyfatty acids–sphingolipids. The role of alkanes in membrane organization is discussed; in the shi mutant, defective myelination could be a result of the defective synthesis of alkanes or defective incorporation of alkanes during myelin assembly (in addition or absence of myelin basic protein).

Index Entries: Myelin, in dysmyelinating mutants; quaking mice, and premyelin and myelin fraction; shiverer mice, and premyelin and myelin fraction; myelin deficient mice, and premyelin and myelin fraction; fatty-acid composition, lipids from dysmyelinating mutants; alkanes, lipids from dysmyelinating mutants; fluidity, of myelin membranes from dysmyelinating mutants; membrane fluidity, of myelin from dysmyelinating mutants.

INTRODUCTION

A large body of evidence has been accumulated to support the idea that the myelin membrane is assembled by successive addition of its

component molecules to the plasma membrane of the oligodendrocyte in the central nervous system and the Schwann cells in the peripheral nervous system. A number of papers has shown that zonal centrifugation on a continuous sucrose gradient is a useful method for separating brain myelin particles and premyelin material according to their density (Waehneldt, 1978; Reiber and Waehneldt, 1978; Bourre et al., 1978, 1980a; Shapira et al., 1981; Ginalski–Winkelmann et al., 1982). This technique can provide interesting data in animal research in determining the density and composition of myelin from neurological mutants (Waehneldt, 1979; Bourre et al, 1980a; Ginalski–Winkelmann et al., 1982) and the degree of myelin maturation in animals (Bourre et al., 1980b); it is also useful in human research (Konat and Offner, 1982).

In a previous paper we have shown that the particulate material of brain homogenate is separated by zonal centrifugation on a continuous 0.4–1.2*M* sucrose gradient into four major peaks corresponding to 1.1 (peak A), 0.68 (peak B), 0.35, and 0.12*M* (Bourre et al., 1980a). Peak B consisted of pure compact myelin, and peak A consisted of vesicles, some having a double membrane. In both shiverer (shi) and myelin deficient (mld) neurological mutants, peak B was absent and peak A was present in the 0.88*M* region and was made of noncompact lamellar membranes and myelin figures with an abnormal major dense line. In the quaking mutant (qk), there were two shoulders in the myelin layer and a peak at 0.85*M*. Thus, this work was undertaken to isolate and analyze the premyelin peak A in both normal and dysmyelinating mutants and to compare their composition and physicochemical properties to the myelin peak B.

Examination of dysmyelinating mutants as a tool to study myelination has been recently reviewed (Baumann, 1980; Hogan and Greenfield, 1984). A method that has proven fruitful in other systems entails analyzing mutations in order to define the normal mechanism they disrupt:

(A) The alterations found in brain of qk seem most consistent with a failure in maturation of the myelin sheath, which can also be termed an arrest in myelinogenesis. This mutant presents (1) an histological appearance of a failure to develop a compact multilamellar myelin structure; (2) an attainment of plateau in myelin-associated lipids during development; (3) an "immature" fatty acid composition, with reduction of long-chain saturated and monoenoic fatty acids of the myelin-associated sphingoglycolipids; (4) an "immature" composition of myelin proteins; and (5) a selective reduction in biosynthetic capacity for myelin-associated galactolipids, sulfolipids, long-chain fatty acids, and sterols without detectable alteration in the structure of the relevant enzymes.

(B) The prominent change in the brain of shi is the paucity of myelin and an associated virtual absence of the major dense

line. The biochemical correlate is the reduction of the content of myelin basic protein to less than 3% of control. The association of myelin basic protein and the major dense line of myelin is established by their virtual absence in shi, but the peripheral nerve myelin also lacks myelin basic protein. This casts doubt on hypotheses that have been advanced postulating that myelin basic protein has an adhesive function in maintaining compact myelin.

(C) The mld is an allele of the shi. Moreover, dissimilarities between these two strains appear to exist: mld has higher concentrations of myelin basic protein than does shi. Thus, mld may represent a more limited penetrance of this mutation.

MATERIAL AND METHODS

Animals

We obtained the quaking mutants from the C57-Bl6 strain, the shiverer from the C3H SWV, and the mld from an unidentified background.

Zonal Centrifugation

The preparation of the fractions have been previously published (Bourre et al., 1980a). Briefly, the forebrains were weighed and washed with saline. Small fragments were obtained with a razor blade, washed again, and pelleted at 1000 rpm (5 min). The pellet (approximately 2 g fresh brain) was homogenized in 50 mL water and spun at 1000 rpm for 5 min to discard unbroken material. The supernatant was centrifuged for 60 min at 100,000g, to eliminate soluble material. The pellet was homogenized in 50 mL of water and placed onto a 0.4–1.2M-sucrose gradient (550 mL) previously prepared with the aid of an automatic pump. No cushion was added, but the suspension was overlaid with 50 mL water. Centrifugation was carried out in an Al 14 rotor (Beckman) for 2 h in an L5-65 Beckman centrifuge. Thereafter, 60 10-mL fractions were collected by pumping 1.2M sucrose into the rotor. The optical density was continuously monitored at 280 nm (Isco UV 4 apparatus) and subsequently checked in the 60 fractions by determination of the absorbance at 260 nm. Sucrose molarity was determined by measurement of refractive index.

In controls, fraction A banded at 0.85–0.95M sucrose and B at 0.62–0.72. In the mutants, A was found at 0.74–0.95M. The A and B fractions were obtained by pooling the collected fractions from the rotor at the adequate sucrose molarity; the samples were dialyzed for 24 h and pelleted at 100,000g for 1 h.

Lipid Analysis

The techniques for lipid extraction have been previously described (Bourre et al., 1977a; Pollet et al., 1978). For sphingolipid measurements, lipids were separated on thin-layer chromatography, using chloroform–methanol–water (70:30:4 v/v/v). After α-naphthol spraying, plates were scanned with an auto-scanner using a 610-nm filter (Helena). Absolute determinations were performed by running a known amount of standards on the same plate. Quantities were determined by measuring the weights of the peaks.

The fatty-acid composition was determined by gas–liquid chromatography of methyl esters on an open tubular column (50 m length, 0.2 mm diameter; Carbowax 20*M*). Quantifications were performed by a computer (IPAC 10; LTT).

The alkane fraction was prepared and analyzed by gas–liquid chromatography on a CP Si/5 column (25 m length, 0.2 mm diameter), and quantification was performed using an internal standard, as previously described (Bourre et al., 1977b; Darriet et al., 1978a), using SP 4100 integrator.

Spin-Labeling Method

Two spin-label lipids were used. The 5-nitroxide stearic acid method (5-NS), exploring a region close to the polar head of the biological membrane; and the 16-NS method, allowing for measurement in the hydrophobic region of the bilayer. These two probes were inserted in parallel with the fatty-acid chains within a few minutes when incubated with membranes; they were used in this study at 5×10^{-5} mol/L. Spectra were recorded on an electron-spin resonance (esr) spectrometer Varian E 3, equipped with a temperature control. Spectra were interpreted as previously described (Viret et al., 1979; Leterrier et al., 1984). Eventually, alliquots of the membrane pellets were suspended and incubated 10 min in the presence of basic protein (generous gift of Dr. C. Jacque) or alkanes.

Statistical Procedure

Comparisons were performed using the Student's *t*-test.

RESULTS

Lipid Analysis

The amount of material recovered in fraction A was reduced in qk and increased in both shi and mld (Table 1). The lipid content in fraction A in mutants did not differ from the normal fraction and represented approximately 50% of the material. In myelin (fraction B), lipids accounted for 75% of the dry weight.

TABLE 1
Cerebroside and Sulfatide Content in the Premyelin Fraction A in Normal and Dysmyelinating Mice: Comparison with the Myelin Fraction B*

	Control		A		
	B	A	shi	mld	qk
Amount of material, mg dry wt/mouse	3.8	2.2	2.7†	3.5†	1.8†
Lipid extract, mg/mg dry wt	0.75	0.45	0.49	0.50	0.48
% Sphingolipids					
cerebrosides,					
non-OH FA	25.1	21.7	18.9	21.9	4.5†
α-OH FA	60.3	59.9	69.0†	64.3	76.6†
Sulfatides,					
non-OH FA	9.2	9.4	5.0†	7.9	2.0
α-OH FA	6.2	8.9	7.8	5.9	16.5†
Sphingolipids,					
mg/100 mg lipid extract					
Cerebrosides,					
non-OH	5.3	1.0	0.8	1.1	0.1†
α-OH	14.4	2.7	2.9	3.4	1.6†
Sulfatides,					
non-OH	1.9	0.4	0.2†	0.4	0.05†
α-OH	1.3	0.4	0.3	0.3	0.3
Alkanes,					
μg/mg protein	9.6	4.8	0.4†	2.9†	2.4

*Each value is the average of triplicate analysis on three or four separate samples.
†Means significantly different from the value found in the control fraction A ($p < 0.01$) (Student's *t*-test).

Sphingolipid analysis showed that the relative quantities (%) of cerebrosides and sulfatides (formed either with unsubstituted or hydroxylated fatty acids) were similar in control A and B fractions. However, the absolute amounts were much lower in the A premyelin fraction. The relative amount of sphingolipids were normal in mld, but not in shi mutants, especially in cerebrosides formed with α-hydroxylated fatty acids and sulfatides formed with unsubstituted fatty acids. The absolute amounts of sphingolipids were normal in both shi and mld.

In fraction A from the qk mutant, both relative and absolute amounts of sphingolipids were drastically altered. The percentage of cerebrosides and sulfatides formed with nonhydroxyfatty acids was dramatically reduced, but cerebrosides and sulfatides formed with hydroxyfatty acids increased. In terms of absolute amount, only cerebrosides and sulfatides formed with α-hydroxyfatty acids were reduced.

Fraction B (myelin) in the control contained a larger amount of sphingolipids than any fraction A and compared favorably with previously published results on purified myelin.

Fatty Acid Analysis

Fraction B (myelin) contained much more very-long-chain saturated and monounsaturated fatty acids compared to any fraction A. Conversely, fraction A contained more palmitic and stearic acid. Polyunsaturated fatty acids were increased in shi and mld, but decreased in qk. In the qk mutant, lignoceric (24:0) and nervonic (24:1) acids were drastically reduced, behenic acid (22:0) was less reduced, and arachidic acid (20:0) was normal (Table 2).

Alkanes

The alkane analysis revealed quantitative and qualitative differences among all the fractions studied (*see* Fig. 1). The alkane content was higher in fraction B (myelin) than in any fraction A, in good agreement with our

TABLE 2
Fatty Acid Profile of the Premyelin Fraction A in Normal and Dysmyelinating Mutant Mice: Comparison with the Myelin Fraction B

	Control		A		
Fatty acids	B	A	shi	mld	qk
16:0	18.8	28.2	31.5	35.3	37.2
16:1	0.7	0.5	0.6	0.9	0.8
17:0	0.4	0.7	0.7	0.6	0.7
18:0	30.2	37.3	36.5	30.3	36.8
18:1 (*n*–9)	28.5	25.2	21.1	23.8	20.7
18:2 (*n*–6)	0.4	0.3	0.3	0.4	0.3
18:3 (*n*–3)	0.3	0.4	0.3	0.3	0.3
20:0	1.1	0.5	0.3	0.3	0.3
20:1 (*n*–9)	3.7	1.3	1.0	0.9	0.6
20:4 (*n*–6)	2.1	1.1	1.9	2.1	1.0
22:0	1.7	0.5	0.3	0.3	0.2
22:1 (*n*–9)	0.5	0.4	0.2	0.4	0.1
23:0	0.5	0.3	0.3	0.3	0.1
22:4 (*n*–6)	0.8	0.4	1.0	0.6	0.2
22:6 (*n*–3)	0.8	0.9	2.6	1.4	0.5
24:0	3.3	0.6	0.4	0.5	0.1
24:1 (*n*–9)	6.2	1.3	0.6	1.3	0.1

Each value is the average of duplicate analysis on three separate samples.

Fatty acids are abbreviated by the usual conventions. The values are in percent of the identified fatty acids.

Means significantly different with the value found in the normal fraction A ($p < 0.01$) (Student's *t*-test).

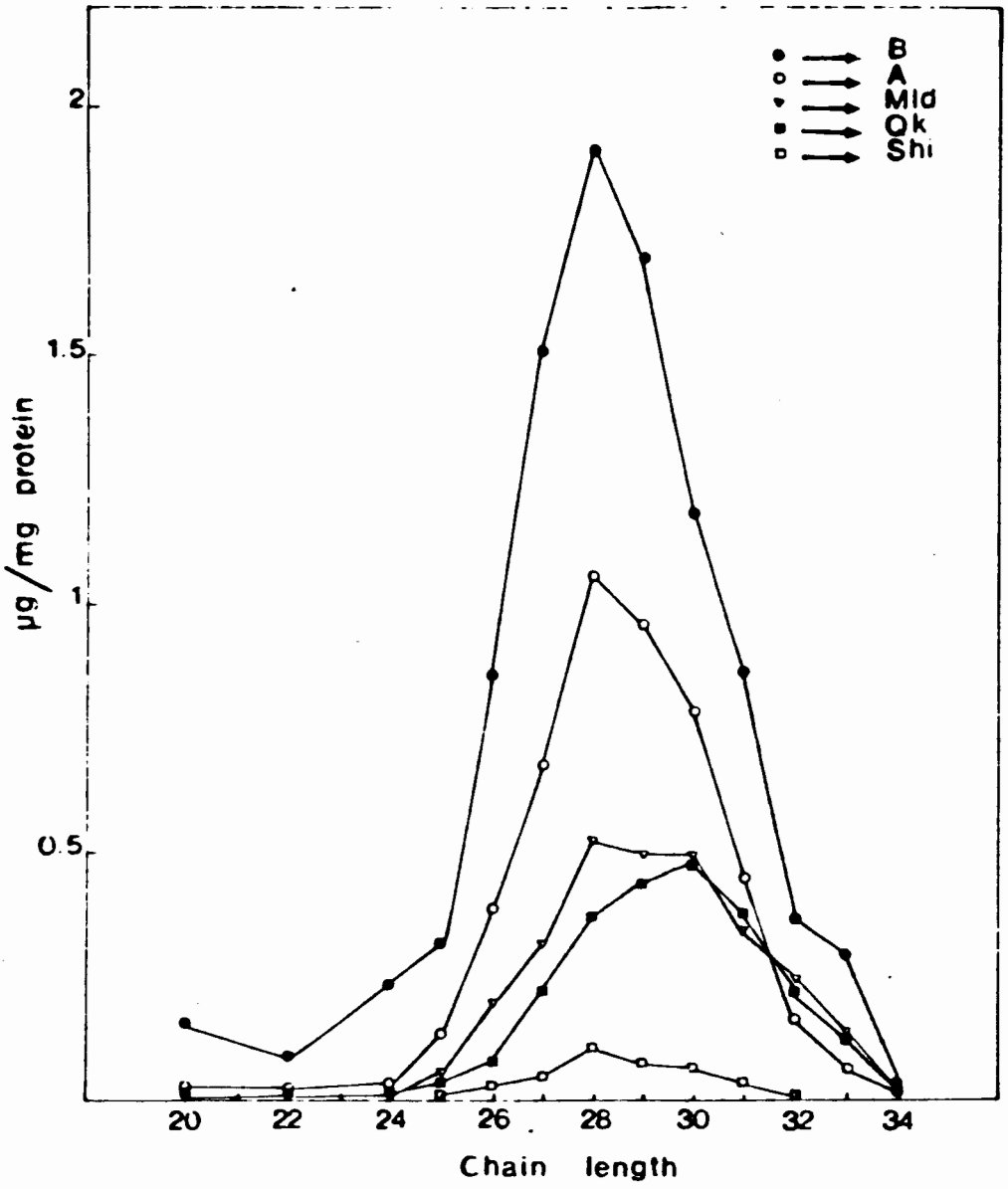

Fig. 1. Amount and distribution of alkanes in the premyelin fraction A in normal and dysmyelinating mutant mice, compared with the myelin fraction B. Results are given in µg alkanes/mg membrane protein of each fraction (mean of duplicate analysis on three separate samples).

previous demonstration of their accumulation in myelin (Bourre et al., 1977b).

In premyelin material (fraction A) the amount of alkanes was reduced by 50% of the myelin fraction B. In the mutants, mld and qk presented decreased absolute amounts (approximately 50%) and the shi fraction A was nearly free of alkanes. Quantitatively, each fraction exhib-

TABLE 3
Maximal Apparent Coupling Constant (2 T_{II} Hyperfine Splitting Constant) and Rotation Frequency of the Marker (V_c)

		Control		A		
	Temp., °C	B	A	shi	mld	qk
2 T_{II}, gauss	20	58.2	55.8	55.2	55.8	55.9
	35	52	51	49.7	49.8	50.4
10^7 Vc, H	35	77.8	78.3	75.7	78	90.5

[a] V_c was not determined at 20°C, as in the formula of Henry and Kei. This is not valid in the range of the low frequency.

ited a nearly-equal level of even and and odd chains, with a maximum in the 28-carbon atom range, except for qk, in which a shift to 30 carbon atoms was noted, as already observed in the peripheral nervous system (Darriet et al., 1978b). The mld and qk presented a similar abnormal distribution pattern, whereas that of the shi was nearly normal.

Electron-spin Resonance

Whatever the temperature, the maximal apparent coupling constant (hyperfine splitting constant, 2 T_{II}) was significantly lower in fraction A than in fraction B by 5%. This constant was not affected in the mld and qk mutants, but was slightly reduced in the shi mutant (*see* Table 3).

The frequency of rotation, V_c, was dramatically increased in qk, slightly decreased in shi, and close to control fraction A in mld.

In many fractions, adding alkanes to the membranes decreased the order parameter (by 5% at 20°C and 6% at 35°C) when using a mixture of alkanes ranging between 18 and 32 carbon atoms and at the physiological concentration. This effect varied according to the alkane concentration and the chain length. Adding purified basic protein increased the order parameter under the same conditions (up to a ratio of basic protein–total membrane protein equal to 0.4).

DISCUSSION

Biochemistry

In the normal animal, the fraction B (myelin) contained more lipids, more sphingolipids, more alkanes, and more very-long chain saturated and mono-unsaturated fatty acids than the premyelin fraction A. This agrees with our previous results (Bourre et al, 1980a), showing that fraction B is formed of typical myelin figures and contains large amount of basic protein.

Quaking

Cerebrosides and sulfatides formed with unsubstituted fatty acids were drastically reduced in the qk mutant demyelin fraction A. In contrast, cerebrosides and sulfatides formed with hydroxyfatty acids were not quantitatively affected. Indeed, in this mutant, very-long-chain saturated and mono-unsaturated fatty acid biosynthesis is reduced (Bourre et al., 1973, 1980c), but hydroxyfatty acid biosynthesis is also reduced (Murad and Kishimoto, 1975) and hydroxylated fatty acids are synthesized from unsubstituted homologs (Murad and Kishimoto, 1975; Singh and Kishimoto, 1981). Thus, the defect observed in the qk mutant could be a result of a defective synthesis of ceramides formed with nonhydroxylated fatty acids (Zalc et al., 1974). However, the synthesis of ceramides formed with hydroxylated fatty acids could be normal. In addition, the reduced synthesis of cerebrosides from ceramides (Costantino–Ceccarini and Morell, 1971) and sulfates from cerebrosides is to be noted (Sarlieve et al., 1971).

Since two different routes of synthesis for nonhydroxyfatty acids and hydroxyfatty acid cerebrosides have been suggested (Gibson and Brammer, 1984), a specific alteration in the synthesis of nonhydroxyfatty-acid sphingolipids could be postulated in the qk. Another speculation could be a defective assembly of the myelin at the level of the unsubstituted fatty acid sphingolipids. The reduction of very-long-chain fatty acids in fraction A in the qk compared to the normal fraction A is in good agreement with the decrease in sulfatides and cerebrosides formed with unsubstituted fatty acids. The mutant presents a reduction of approximately 50% in the alkane content; the shift into longer chains observed in the qk mutant in fraction A was already observed in the peripheral nervous system (Darriet et al., 1978a).

Shiverer and Myelin Deficient

The quantities of cerebrosides and sulfatides in the premyelin material A were similar in normal shi and mld. This would suggest that these compounds are normally synthesized, showing dissociation between myelin synthesis and myelin lipid biosynthesis similar to the proposed dissociation of myelin synthesis and myelin-associated enzyme activities in the shi mouse (Bird et al., 1980).

The shi and mld mutants are two allelic mutations (Bourre et al., 1980a), but only the shi mutant presented an almost total absence of alkanes in fraction A; in the mld mutant, fraction A presented a reduction of approximately 50% in the alkane content.

The alkane content was higher in fraction B (myelin) compared to any fraction A, in good agreement with our previous demonstration of alkane occurrence in the myelin (Bourre et al., 1977b; Darriet et al., 1978b).

Moreover, in fraction A, although apparently detected in nearly normal amounts by electrophoresis, basic protein is hardly detected by radioimmunoassay in shi and largely reduced in mld (Bourre et al., 1980d). Thus, altered myelination could be a result of immunologically abnormal basic protein in shi and mld, explaining the absence of any major dense line (Privat et al., 1979; Matthieu et al., 1980) and the apparent lack of basic protein determined by immunofluorescence (Dupouey et al., 1979). However, in the shi mutant, the absence of alkanes in fraction A is to be noted and raises the question of the role of these molecules at the level of the major dense line and their alteration in the shi mutant.

Biophysics

It would appear that the results concerning 2 $T_{\perp}$ may be interpreted by an increase in the disorder within the paraffinic chains of the A fraction in comparison with the B fraction (the normal pure myelin). This increase in disorder is probably a result of the relative increase in $C_{16:0}$, which shows a higher degree of flexibility.

The same explanation may be given for qk (37.2% of 16:0), mld 35.3% of 16:0), and shi (31.5% of 16:0) mutants. It can be observed that the shi mouse has, moreover, a larger proportion of 22:6; the unsaturation of the chains causes further disorder, which could explain the lower level of 2 $T_{\perp}$ observed in the latter mutation.

The results concerning the rotation frequency in the marker are usually consistent with those relative to the 2 $T_{\perp}$, the rotation frequency being increased according to the degree of disorder within the chains. The V_r frequency value of 75.7×10^7 H is, however, remarkably weak in the shi mouse: This is unexpected and not yet explained. It appears not to be a result of the basic protein content. Although this protein is less upset in mld than in shi, the lowest frequency recorded in the shi mouse should be accompanied by a higher level of 2 $T_{\perp}$, and even more so since the basic protein is located at the polar section of the membranes, a zone explored by the 5-NS marker.

It has been noted that alkanes are considerably decreased in the shi mutation. The spatial location of the alkanes in the biological membranes is not known. Their occurrence increases the fluidity of biological membranes. Their absence can, therefore, remove this fluidification and thus cause a decrease in the rate of rotation frequency of the marker. If such were the case, this result should indicate a hydrophobic repartition of the alkanes within the membrane in the bilayer depth, since their effect would be more easily observed in this region than in the polar region (where 2 $T_{\perp}$ is not increased). In any case, the alteration of the dense lines in the myelin observed in the shi and mld mutant mice would result from the absence of basic protein, rather than a change in the alkane content.

CONCLUSION

It could be speculated that the mutation in shi provokes alteration of the basic protein synthesis and/or defective synthesis of alkanes; in the qk, an abnormal myelin assembly could be the result of an alteration in the synthesis of cerebrosides and sulfatides formed with unsubstituted very-long-chain fatty acids or an alteration of their addition to the plasma membrane.

Alterations of biophysical parameters in the mutant parallel the changes in fatty acids and alkanes, since alkanes increase the fluidity and decrease the order parameter.

Moreover, basic protein increases the order parameter when added to premyelin membrane and reduces the amount of basic protein in qk, in good agreement with increased fluidity. However, fluidity is not increased in mld and shi, although basic protein is nearly absent.

Speculations on biochemical defects and alterations in myelin structure can hardly be tested: The role of alkanes and sphingolipids in membrane is unknown (increasing stability and fluidity, interdigitation of their very-long-chain fatty acids in the bilayer). Basic protein is located at the major dense line, but its role for an adhesive function in maintaining compact myelin is discussed.

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