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Myelin consists of a continuum of particles of different density with varying lipid composition : major differences are found between normal mice and quaking mutants.

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Summary. — Mouse brain myelin consists of a continuum of particles of different densities, as shown by sucrose density gradient centrifugation. In normal animals most of the material (65 per cent) is concentrated between 0.6 and 0.7 M sucrose (the maximum being found at 0.66 M sucrose, corresponding to 23 per cent). The density differences among various myelin fractions are related to their protein/lipid ratios, as lighter fractions contain less protein and more lipid. Lipid analysis shows a decrease in the amount of every lipid from the lightest to the heaviest fraction : the light fraction is richer in phosphatidyl-ethanolamine, phosphatidyl-serine and cerebrosides. The distribution is highly abnormal in purified myelin from Quaking mutant ; very low quantities of myelin with normal density are found, but unexpected large amount of high density particles are found, possibly related to a « pre-myelin » material (oligodendrogial) processes which are not maturing into normal myelin).

INTRODUCTION.

Density gradient centrifugation was used by number of investigators to separate myelin into subfractions [1-6] and it has been found that in ox brain two major fractions are obtained in about equal amount [4]. Recently myelin was fractionated into three [7] and four subfractions [8] and, acccording to the protein content, it was proposed that myelin in rat brain homogenates « consists of a continuum of particles » [6] ; partially purified myelin was separated into 4 subfractions and precursor products relationships between these different membrane fractions were investigated [9]. The purpose of this work was to demonstrate that myelin consists of a continuum of particles of different density with different lipid composition, and to compare the distribution in normal and Quaking mice, a neurological mutant with defective myelination [10] and abnormal myelin composition [11 - 12].

MATERIAL AND METHODS.

Myelin subfractionation.

Myelin was isolated from adult mice according to Norton and Poduslo [13] and the purity was checked by electron microscopy, marker enzymes [11, 14] and protein gel profile [15]. For Quaking animal the technique for young normal animal was used. The centrifugations were made in SW 27 rotor with the Beckman L 5-65. The purified myelin was homogenized in 0.32 M sucrose and layered (10 ml) on a new discontinuous sucrose gradient made of 0.72 M (12 ml) and 0.52 M [12]. The 2 layers at interfaces (A and B + C) and the pellet D were collected with Pasteur pipetts, diluted to 0.2 M and spun down as described below. The pellets were homogenized in 0.32 M and the corresponding upper layer (A) was layered (5 ml) over 0.48 M (7 ml), 0.45 M (7 ml), 0.47 M (7 ml), 0.38 M (7 ml). The intermediate layer (B + C) was layered (4 ml) over 0.72 M (12 ml) and 0.62 M (18 ml) thus providing B and C fraction. The pellet D was layered (5 ml) over 0.82 M (7 ml), 0.78 (7 ml), 0.75 (7 ml) and 0.72 (7 ml). B fraction was layered over 0.62, 0.58, 0.55, 0.52 M ; C fraction over 0.72, 0.68, 0.65, 0.62 M ; each fraction was 7 ml. Because of the relative differences of myelin subfractions, one gradient was made with A, 2 with D, 2 with B and 10 with C. (Myelin was obtained from 12 gradients ; A, B + C and D were obtained from 12 gradients ; B and C from B + C were obtained with 24 gradients). All gradients were run at 21 000 rpm for 30 min ; the layers were collected, diluted with water up to approximatively 0.2 M and spun 15 min at 21 000 rpm. The final fractions

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were collected (and eventually pooled) washed three times with water, lyophilized and weighed. Membranous pellets were then homogenized in known amount of distilled water and protein content was measured [**16**].

Microsomes were obtained as previously described [17]. The microsomal pellet (15 mg) was homogenized in 0.32 M sucrose (18 ml) and layered over 0.85 M (15 ml) and spun 30 min at 21 000 rpm. Upper phase, interface (M) lower phase and pellet were collected, diluted with water and spun 20 min at 65 000 rpm (75 Ti rotor) and washed twice with water under the same conditions. Pellets were lyophilized and weighed. The layers at the interface (M) « myelin like » microsomal material was eventually osmotic shocked and treated under the same conditions as myelin. Thus, after the second gradient was obtained the « myelin like » microsomal fraction.

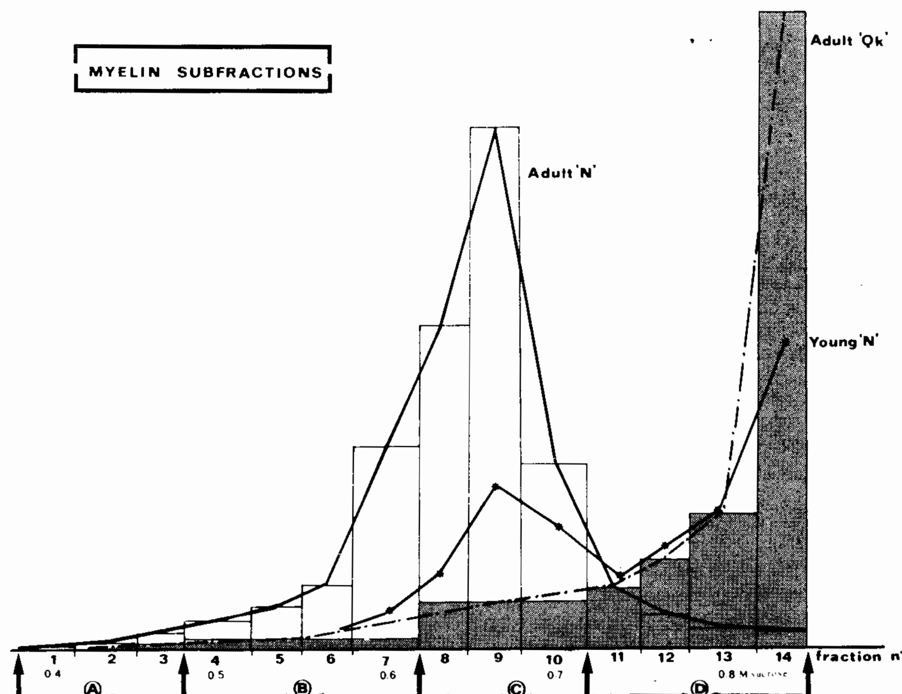

FIG. 1. — *Myelin subfractions.*
Each point was the mean value from 3 experiments in adult animals (N and Qk). For young normal animals one experiment was performed.

This study needed 191 normal mice (C 54 B) and 167 Quaking mutants.

LIPID EXTRACTION.

Lyophilized myelin samples were suspended in 5 ml chloroform-methanol-water 70:30:4 (v/v/v). Extraction was done by sonications (Sonifier B 12 Branson) 6 × 30 sec at + 4°C with 30 sec intervals between each sonication. The non extractible fraction was pelleted after centrifugation at 2 000 rpm during 10 min (International clinical centrifuge) Lipid extract was completed to a known final volume.

Separation of lipids was performed on TLC glass plates (10 × 10 cm) coated with silicagel 60 F 254 Merck. About 500 µg of total lipid extract were spotted at 2 cm from the bottom and the left of the plate. The solvents used in succession were the following :

1) chloroform-methanol-water 70:30:4 (v/v/v) migrating from the bottom to the top of the plate.
2) at right angle to the first, chloroform-methanol 2:8 (v/v) on 2/3 of the plate.
3) in the same direction, chloroform-methanol 2:1 (v/v).
4) finally, chloroform-methanol 2:1 (v/v) in the same direction as 1 from the bottom to the top of the plate.

Lipids were detected by iodine vapors. After complete sublimation of the iodine, the areas of

individual lipids were scraped off and submitted to quantification.

For quantification of lipids, so as to eliminate interferences, each assay has been performed with a constant amount of lipid to which is added increasing amounts of the standard substance to be determined. The slope of the curve obtained is compared to one consisting only of the standard scale. This method is called « standard additions method » [**18**]. Most of the methods used for cholesterol [**19**], galactolipid [**20**], phosphorus [**21**], NANA [**22**] determination are classical.

RESULTS AND DISCUSSION.

The figure 1 shows that normal mice myelin from central nervous system consists of a continuum of particles of different density. Most of the material (65 p. cent) is concentrated between 0.6 and 0.7 M ; the optimum is found at 0.66 M (corresponding to 23 per cent sucrose). Thus no evidence is found of a biphasic distribution, nor any discrete separation into light or heavy components. Obviously it is possible to separate arbitrarily myelin in two (heavier and lighter) or more fractions, by making the appropriate gradients. The density difference of various myelin fractions is related to their protein/lipid ratio : it is shown

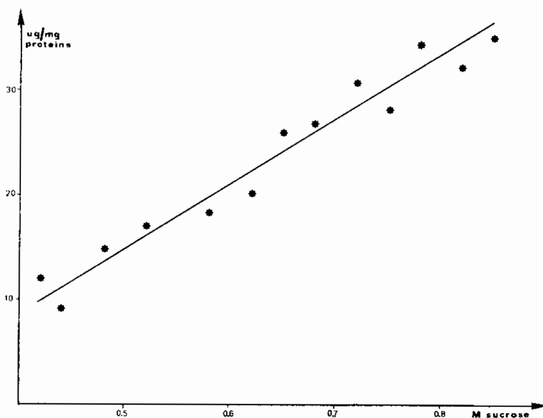

FIG. 2. — *Protein content in each individual subfraction.*

Each point was the mean value from 3 experiments (6 measurements).

on figure 2 that the protein content is regularly increasing from the lightest fraction to the heaviest (conversely the lipid content is decreasing). But other influence may also play a role, for example the change in proportion of the different myelin proteins throughout the density range, as heavy and light myelin have different protein composition [**23**].

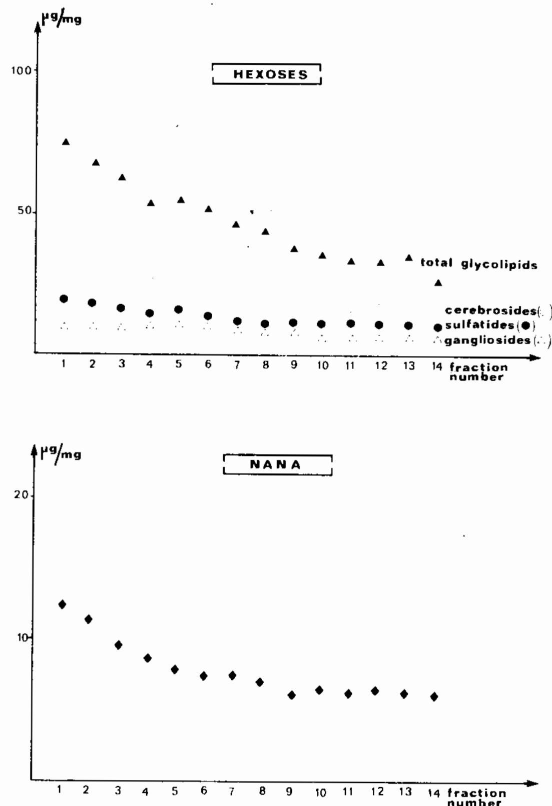

FIG. 3. — *Cholesterol and phospholipid content in each individual subfraction.*

Total P : total phosphorus PC, PE, PS, PI mean phosphorus from respectively phosphatidyl-choline, phosphatidyl-ethanolamine, phosphatidyl-serine, phosphatidyl-inositol. Sph : phosphorus from sphingomyelin.

Results are expressed in µg per mg lyophilized myelin.

Unexpected is the curve obtained with myelin from Quaking animals : we have found very low quantities of light fractions, and very important densest fraction. As shown (in table I) 97.2 per cent of the normal myelin is found between 0.38 M and 0.78 M, only 21.9 per cent in the mutant. These 0.32-0.78 M fractions are much more diminished if the results are expressed in ug/brain (instead of mg/100 mg myelin) thus Quaking is 1.9 per cent of the normal value (the myelin yield per brain is 8.4 per cent of the normal value). As shown in figure 1 the peak at 0.67 M does not appear in the mutant, and the densest fraction (table 1) 0.82 - 0.85 M is multiplied by 50

Table I.

Myelin subfractions in normal and Quaking brain.

raction number		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		A			B				C			D			
Sucrose fraction (M)	0.32–0.38	0.42	0.45	0.48	0.52	0.55	0.58	0.62	0.65	0.68	0.72	0.75	0.78	0.82	0.85
mg/100 mg myelin															
Normal	0	0.31	0.44	1.05	2.3	2.5	3.9	15.8	19.2	31.0	14.6	3.5	2.0	1.5	1.3
Quaking		0.6			2.4				8.3			4.4	6.2	11.8	65.6
µg/brain															
Normal	0	22	31	73	161	175	273	1106	1344	2170	1022	245	364	105	91
Quaking		4			14				50			26	37	71	394
mg/100 mg myelin															
Normal	97.2													2.8	
Quaking	21.9													77.4	
µg/brain															
Normal	6985													196	
Quaking	131													465	

Mean value from 3 experiments.

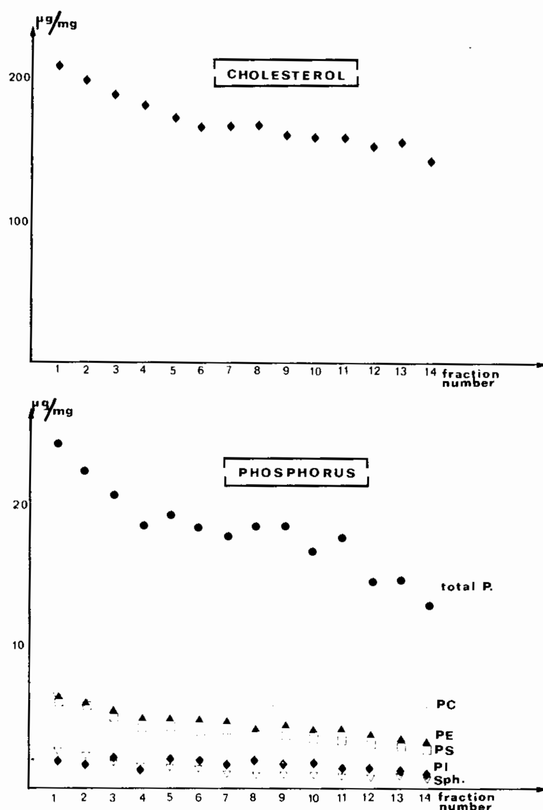

Fig. 4. — *Hexose and NANA content in each individual fraction.*
NANA : N-acetyl neuraminic acid.
Results expressed in µg per mg lyophilized myelin.

when expressed in mg/100 mg myelin. Moreover, even when considering the results in µg/brain (absolute amount) although myelin yield is largely reduced in the mutant, this densest fraction is multiplied by 4 (394 µg in the Quaking, 91 µg in the normal) ; thus Quaking brain contains abnormally large amount of this dense fraction, possibly related to a pre-myelin membrane (oligodendroglial membrane ?).

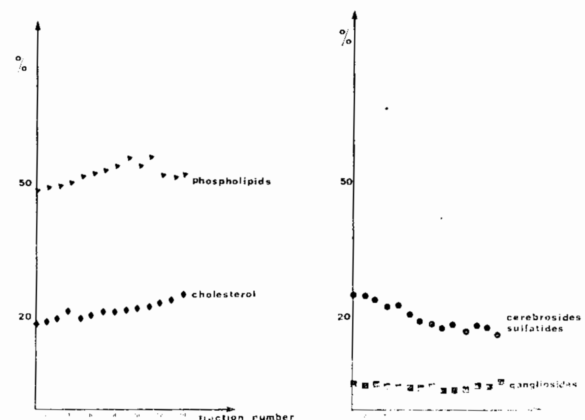

Fig. 5. — *Relative percentage of each individual lipid in myelin subfractions.*

The lipid analysis shows a decrease of the amount of all lipids (cholesterol, phospholipids, galactolipids and gangliosides) from the lightest

to the heaviest fraction in normal myelin (figures 3 and 4). The decrease in phospholipids seems to be due essencially to phosphatidyl-ethanolamine (PE) and phosphatidyl-serine (PS), the decrease in galactolipids is due to cerebrosides (and to sulphatides to a lesser extend). Thus normal light fractions are enriched in PE, PS and cerebrosides. When considering the relative percentage of various lipids (figure 5) as expressed in mg of lipids per 100 mg lipid extract in each fraction, gangliosides content is constant, phospholipids present a peak, cholesterol slightly decrease from the heaviest fraction to the lightest and galactolipids (cerebrosides + sulphatides) increases ; indeed, taking into account the figure 4, cerebrosides are largely concentrated in the light fractions. The heaviest Quaking fraction (N° 14) has nearly the same lipid content as the corresponding normal fraction (Table 2) with however higher content in phospholipids, mainly at the level of phosphatidyl-choline and sphingomyelin. The content in total galactolipids is normal but the profile is somewhat disturbed.

Thus the defect in myelinogenesis in the Quaking is possibly due to a deficiency in the synthesis of the lightest subfractions of myelin from the heaviest myelin fraction. The heavy subfraction (0.82-0.85 M) of myelin may be composed of membranes in transition from the oligodendroglial membrane to myelin. Taking in account that microsomes contain myelin lipids [17] these results support the hypothesis of a membranous precursor of myelin (microsomes from oligoglial cells) which is first converted to the heaviest myelin fractions which are in turn converted to the lighter fractions.

The abnormal high content of densest fraction in the Quaking is not due to a microsomal material but probably to a defect in the maturation of oligoglial process into myelin. When pure microsomes are homogenized in 0.32 M and layered over 0.85 M, approximatively the same amount of material is found at the interface. If this material is osmotically shocked and further resuspended and layered over 0.85 M (as for myelin preparation), the amount obtained in normal and mutant mice is in the same order of magnitude. In mutant microsomes there is no accumulation of material : the abnormal high amount of heavy material found in mutant myelin is not directly related to microsomes.

Myelin « pre-membrane » are probably synthesized in microsomes (subfraction containing endoplasmic reticulum and some plasma membrane), then mature in oligodendroglial process which yields the loose and further on to compact myelin (the former probably being the oligoglial plasma membrane wrapping around the axon). The Quaking myelin is probably heavy myelin possibly related to oligoglial process which do not mature normally.

TABLE II.

Lipid composition of myelin fraction n° 14 in normal and Quaking animals.

	Normal	Quaking
Cholesterol	14.1	12.4
Phosphorus	12.8	19.2
PE	2.8	3.6
PC	5.3	7.7
PS	3.2	3.8
PI	0.7	0.5
sphingomyelin	0.7	3.6
Hexose	25.5	25.4
cerebrosides	11.2	11.9
sulphatides	9.2	3.0
gangliosides	5.1	10.6
NANA	0.6	0.7

Results are expressed in µg/mg lyophilized myelin.

This view is supported by myelin subfractionation in young normal animals (13 day old, table I). The animals presents a « myelin » made of normal myelin and also large amount of the densest fraction, possibly due to the high content of oligodendroglial processes in the way of being transformed into myelin.

As the synthesis of myelin proteins (basic proteins as well as proteo-lipids) in the whole brain of Quaking mouse proceeds at normal rate [24, 25], it is suggested that the mutant presents a defect at the step of assembly of myelin components into a final membrane product due to defective lipid biosynthesis.

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RÉSUMÉ.

La myéline pure isolée par gradient de centrifugation est formée d'un continuum de particules ; chez l'animal normal 65 p. cent des membranes sont retrouvés dans les fractions de sucrose comprises entre 0,6 M et 0,7 M (le maximum se situe à 0,66 M, correspondant à 23 p. cent de sucrose). La différence de densité entre les diverses sous-fractions est due, au moins partiellement, au rapport entre les quantités de protéines et de lipides : les fractions légères contiennent moins de protéines et plus de lipides. L'analyse des lipides montre que tous les lipides sont quantitative-

ment plus importants dans les fractions de plus en plus légères mais de plus ces derniers sont très enrichies en cérébrosides, ainsi qu'en phosphatidyl-éthanolamine et phosphatidyl-sérine. Le profil de densité de la myéline est très anormal chez le mutant Quaking : les fractions légères sont extrêmement réduites, mais il y a une fraction lourde qui est très accrue : cette fraction est probablement liée à un matériel prémyélinique (prolongement oligodendroglial qui n'évolue pas vers une myéline normale).

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