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Density Profile and Basic Protein Measurements in the Myelin Range of Particulate Material from Normal Developing Mouse Brain and from Neurological Mutants (*Jimpy; Quaking; Trembler; Shiverer* and its mld Allele) Obtained by Zonal Centrifugation

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Abstract: The particulate material of aquaeous homogenate of forebrain was separated by zonal centrifugation in a continuous 0.4–1.2 M-sucrose gradient after sedimentation at 100,000 *g* to eliminate the soluble material. Based on the absorbance at 280 nm, four major peaks were obtained from adult normal mice corresponding to 1.1 M (A), 0.68 M (B), 0.35 M (C), and 0.12 M (D) sucrose. The two smaller and lighter peaks, C and D, were not present when purified myelin was separated by the same procedure. B consisted of pure compact myelin; A was made of vesicles, sometimes with a double membrane. Throughout development, the myelin peak shifted from 0.58 M in young animals to 0.70 M in very old ones. Moreover, the myelin peak B drastically increased during development, as compared with peak A. In the *Trembler*, the profile was close to normal, with a slightly higher yield of myelin which also peaked at a higher density. In the *quaking*, there were only two shoulders in the myelin density range at 0.68 M and 0.75 M; in the *Jimpy* only a faint shoulder was seen, at approximatively 0.67 M. In the *shiverer*, B was absent and only an A peak was present, at approximatively 0.85–0.90 M, which contained non-compact lamellar membranes and myelin figures with an abnormal major dense line. In the mld (an allele of *shiverer*) the density profile resembled the one obtained in the *shiverer* (one peak in the 0.88 M region). When considering myelin basic protein (MBP) content in the normal developing animal, it was minimum in fraction A, mainly found in the B myelin peak, but also present in the light fraction (C + D). In young animals this latter peak was prominent, in contrast to the adult. In the *Trembler* mutant, the profile was close to normal; in the *quaking*, MBP was mainly found in peak A (0.85 M), and in the *Jimpy* MBP was very low and nearly constant throughout the gradient (with faint quantities in light C + D fraction). In mld, the content was very low, with a peak in the 0.88 M region, in contrast with its *shiverer* allele, where MBP is hardly detected. More meaningful myelin studies can be carried out by using zonal centrifugation in continuous sucrose gradient, to determine the density of mutant myelin and the degree of myelin maturation in animals.

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Abbreviation used: MBP, Myelin basic protein.

All methods for myelin purification take advantage of the low density of myelin (0.85 M-sucrose, $d = 1.11$) to separate it from other membranes by discontinuous sucrose density centrifugation (Norton, 1977). This is accomplished either by overlaying the homogenate in isotonic (0.32 M) sucrose on more dense sucrose and allowing the myelin to migrate down to the interface (Adams and Fox, 1969; Norton and Poduslo, 1973) or by making the homogenate in dense sucrose and allowing the myelin to rise to the surface (Laatsch et al., 1962; Waehneldt and Mandel, 1972). The former method has been used by a number of investigators in various animals. But one must be aware that nonstandard samples (neurological mutants or very young animals) may yield results difficult to interpret.

For instance, the "myelin fraction" isolated under classical conditions from *Jimpy* mice (with absence of myelin in their brain) is scarcely myelin as shown by morphological and biochemical investagations (Matthieu et al., 1974).

Normal myelin consists of a continuum of membranes with different densities which distribute in a bell-shape mode in both discontinuous (Bourre et al., 1977) and continuous density gradients (Adams and Fox, 1969; Bourre et al., 1978; Waehneldt, 1978). But the brains of the *quaking* dysmyelinating mutant mouse (as well as that of young animals) yield very small amounts of myelin with normal density and large amounts of high-density particles, possibly related to pre-myelin material (Bourre et al., 1977). These results are in agreement with the observation that the density of *quaking* myelin is higher than that of normal myelin (Greenfield et al., 1971; Sheads et al., 1977). Zonal centrifugation of brain homogenates (Mahaley et al., 1968; Murdock et al., 1968; Shapira et al., 1970; Day et al., 1971) or purified myelin (Bourre et al., 1968; Waehneldt, 1978) produces a peak corresponding to myelin membrane. The present investigation was undertaken to determine the zonal centrifuge density profile of mouse brain homogenates in the myelin density range and to compare material obtained from adult, developing, and dysmyelinating mutant brains. The basic protein, specific for myelin, was determined in the gradient fractions as an index for the presence of myelin or myelin-related material.

MATERIALS AND METHODS

We obtained the *quaking* mutant on the C57-B6 strain, and both *Jimpy* and *Trembler* were on the B6-CBA strain. The *shiverer* on C3H-SWV strain was obtained from Washington University (Bird et al., 1978); our mld colony originated from Indiana (Doolittle and Schweikart, 1977). Allelism of *shiverer* and mld was determined by mating homozygotic *shiverer* (shi/shi) with heterozygote mld (mld/+) and vice versa. One-half of the pups presented tremors (were "shivering"), thus showing that mld and *shiverer* are actually alleles.

Zonal Centrifugations

All solutions (bidistilled water included) were adjusted to pH 7 with sodium hydroxide and kept at 4°C. Forebrains were weighed and washed with saline. Small fragments obtained with a razor blade were washed again and pelleted at 1000 r.p.m. (5 min). The pellet (approximatively 2 g fresh brain) was homogenised in 50 ml water and spun at 1000 r.p.m. for 5 min to discard unbroken material.

By water-shocking directly the brain homogenate, one reduces to a minimum the contamination of myelin by axonal fragments and by microsomes. When brain tissue was homogenised in sucrose, as others did with rabbit brain (Shapira et al., 1978), we obtained a myelin fraction containing myelinated axons. The supernatant was centrifuged for 60 min at 100,000 *g*, so as to eliminate soluble material. The pellet homogenised in 50 ml water was placed on a 0.4–1.2 M-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 rotor Al 14 of the Beckman L5-65 centrifuge for 2 h at 32,000 r.p.m. Thereafter, 10-ml fractions were collected by pumping 1.2 M-sucrose into the rotor. The absorbance was continuously monitored at 280 nm (Isco UV 4 apparatus) and subsequently checked in the 60 fractions by determination of the absorbance with the aid of a spectrophotometer. Sucrose molarity was determined by measuring its refractive index (O.P.L. Apparatus). Samples were diluted with water, pelleted at 100,000 *g* (60 min) and prepared for electron microscopy as previously described (Baumann et al., 1973). Approximately 2 g of forebrain tissue was used for each run. Each experiment was performed at least three times.

Basic Protein Determination

A direct radio-immuno assay was set up according to Cohen et al. (1975) and reassesed in order to improve its sensitivity. The detection limit was lowered to 200 pg/ml (Delassalle et al., 1980).

RESULTS AND DISCUSSION

In normal adult animals, the zonal centrifuge density profile provided a major peak (B in Fig. 1) corresponding to 0.68 M-sucrose, this molarity being the one found when submitting purified myelin to a discontinuous or continuous density gradient (Bourre et al., 1978; Waehneldt, 1978), but they are region represented a higher density; the C and D peaks (0.35 M- and 0.12 M-sucrose, respectively) were possibly related to very light myelin. These two peaks had not been found when purified myelin was subfractionated onto continuous gradients (Bourre et al., 1978; Waehneldt, 1979), but they are also present in the peripheral nervous system (Matthieu et al., 1979). The soluble material eliminated by the 100,000*g* centrifugation would have produced an absorbance peak at 0.07 M-sucrose. Thus, in our conditions peaks C and D represented true particulate material. When a collected fraction

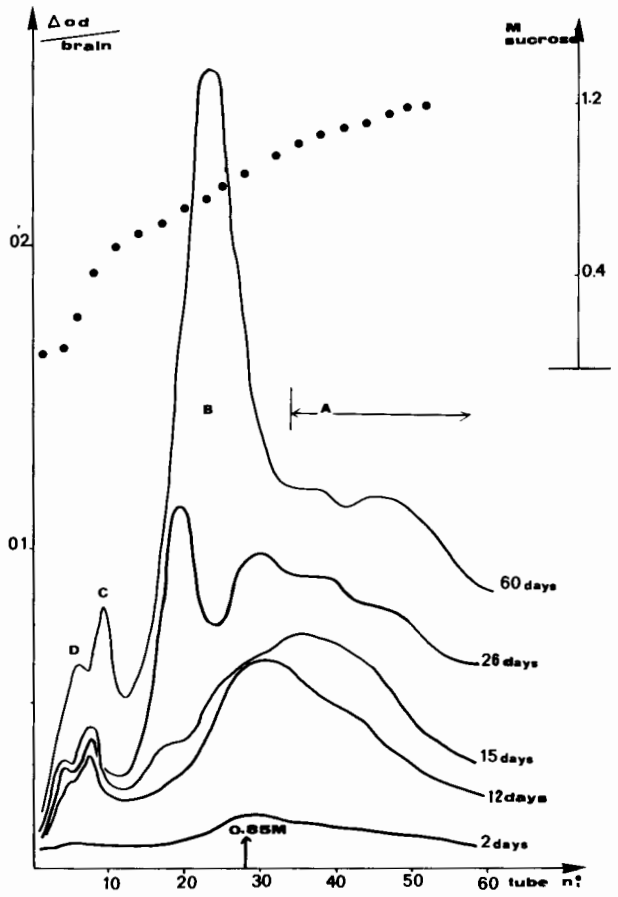

FIG. 1. Density profiles of material from the normal mouse forebrain at different ages. The sucrose molarity (●●●) was determined by measuring its refractive index. These are representative curves from at least three experiments.

was recentrifuged by the same procedure, it banded at its original density (same tube numbers) and overlapped very slightly with neighboring fractions. The electron microscope examinations of the fractions showed that B is formed of very pure myelin: in particular, tube 15 consisted of pure myelin always with many compact lamellae (Fig. 3; cf. Fig. 4). Tube 24 also contained myelin, but with only two or three lamellae. Tube 32 (A region) contained vesicles, some material resembling myelin, and a few unidentified dense bodies. According to its appearance, A was tentatively described as containing unidentified plasma membranes, but no compact myelin. All the fractions were free of mitochondria, synaptosomes, ribosomes, or nuclei. The MBP was present mainly in peak B, relatively absent in the other peaks (Fig. 5).

In the developing brain (Fig. 1), the peak position of forebrain myelin, as observed by electron microscopy, shifted from the density of 0.59 M in the young animal to that of 0.70 M in the adult as previously shown when purified myelin was applied to a continuous gradient (Waehneldt, 1978). As shown in Fig. 1, young brains yielded a relatively large amount of material from region A in the myelin peak B (between 0.4 and 0.25 M-sucrose).

During development, the MBP first appeared in the low-density fraction (peak C and D, Fig. 5); at 12 days peak B contained only 1% of its adult content in MBP. Fraction A is poor in this protein. Thus, during myelinogenesis, the MBP accumulated rapidly in peak B, whereas it remained constant in C and D.

Results from mutants are shown in Fig. 2. The *Trembler* mouse, which has defective myelination in the peripheral nervous system but an apparently normal central nervous system (Ayers and Anderson, 1973) presented a profile close to normal. However, the myelin recovery was somewhat larger in the mutant and the density profile peaked at a higher value (0.72 M-sucrose). It is very probable that CNS myelin is abnormal in this mutant, although its ultrastructure after centrifugation, as well as its MBP content (Fig. 6), was apparently normal.

In *quaking*, there were two shoulders in the curve around the myelin density range (at 0.68 M and 0.75 M) and region. A was apparently divided into two shoulders, of density 0.85 M and 1.08 M. The former

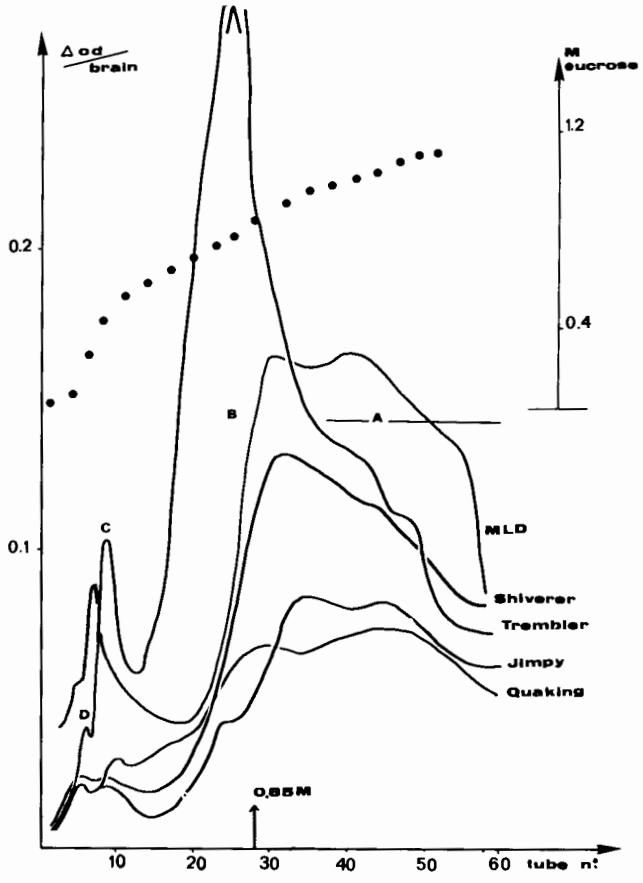

FIG. 2. Zonal centrifuge density profiles of material from different mutant strains. *Trembler, quaking, shiverer,* and mld animals were 2 months old. *Jimpy* were 23–26 days old (animals affected by this mutation die at 28 days).

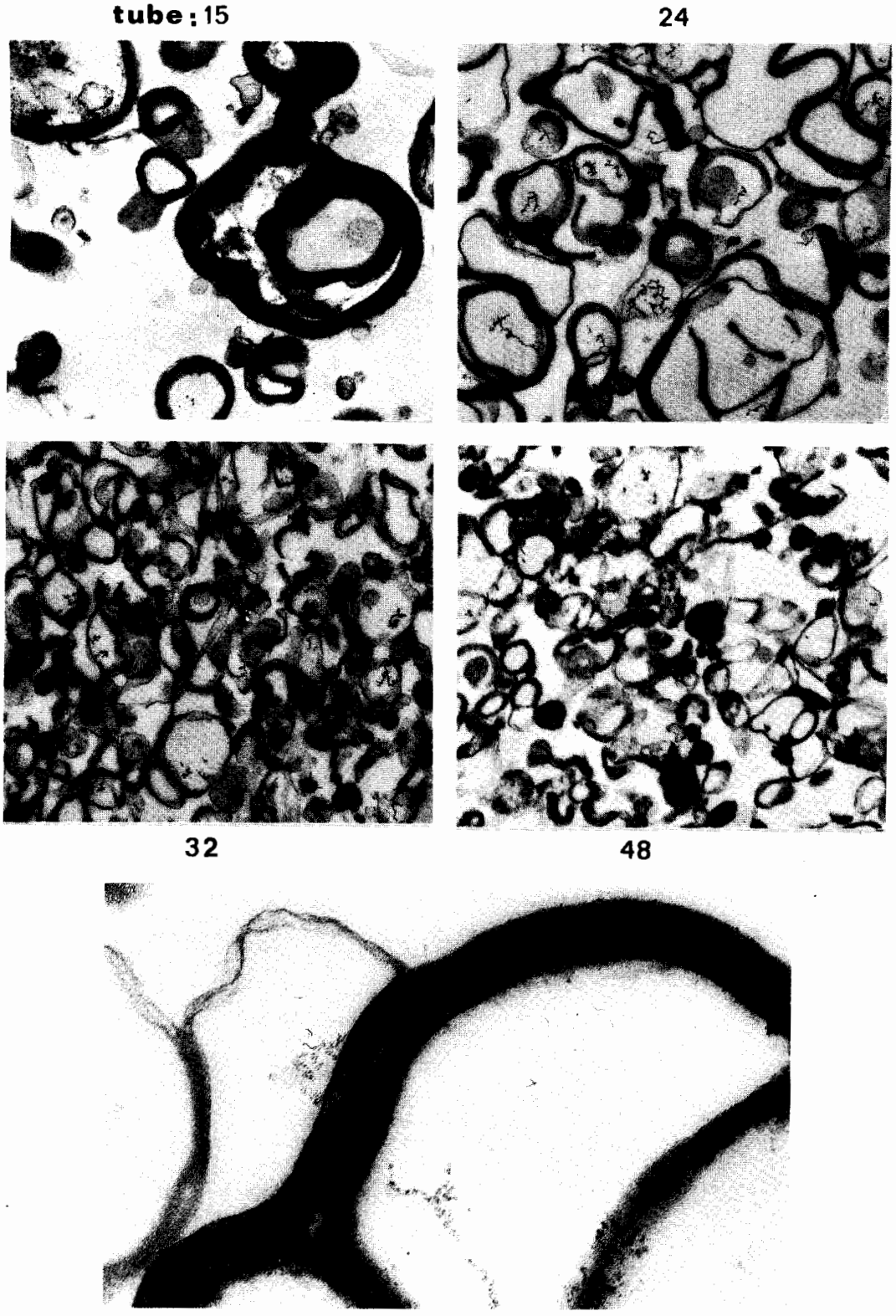

FIG. 3. Electron micrographs of various subfractions obtained in normal animal. Magnification: ×12,500 (×82,500 for the higher magnification of tube 15).

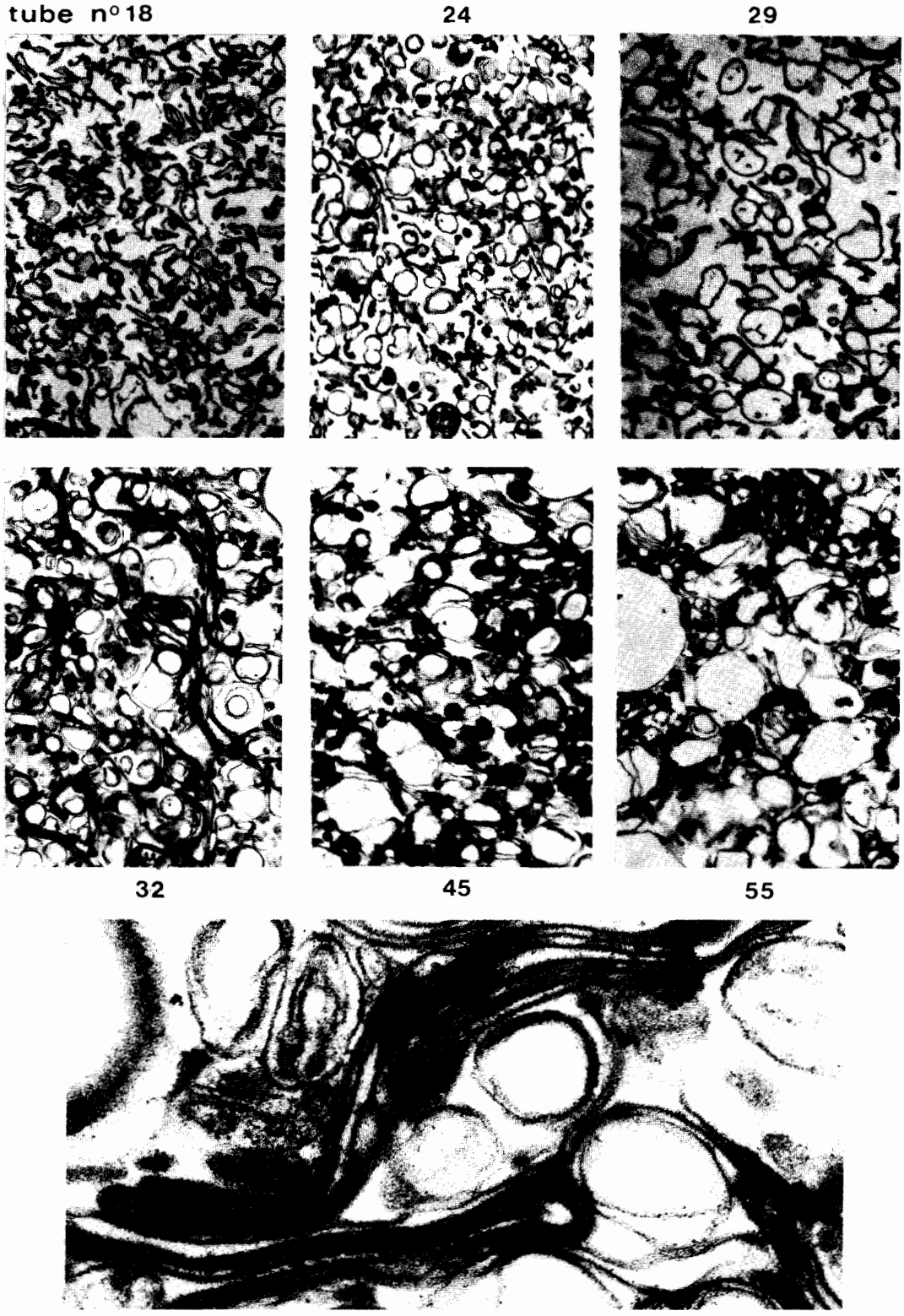

FIG. 4. Electron micrographs of various subfractions obtained from *shiverer* mutants. Magnification: ×12,500 (×82,500 for the higher magnification of tube 32).

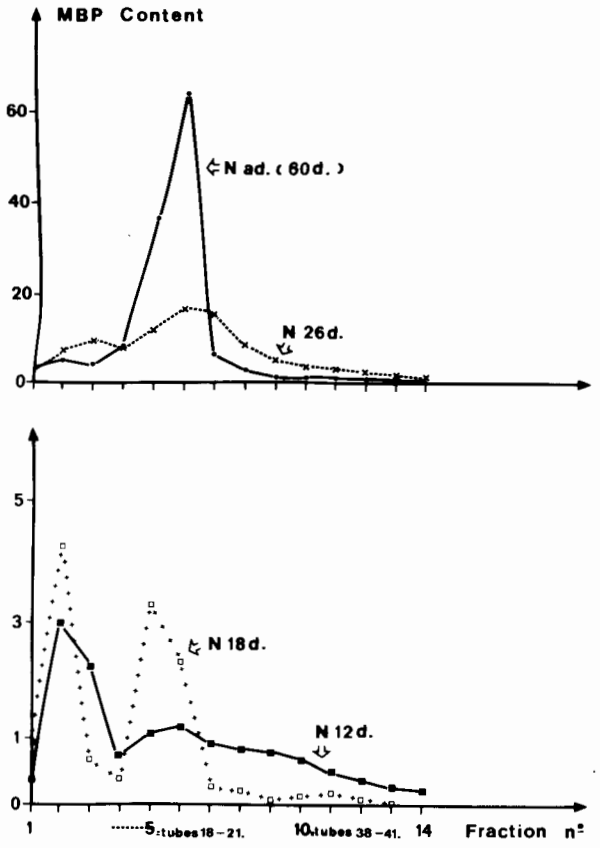

FIG. 5. Myelin basic protein content during normal development. The 14 fractions were obtained by pooling the 60 fractions (as in Fig. 1), 4 × 4. Values are given in micrograms equivalent MBP per mouse brain. N, normal; Ad, adult; 26d, 26-day-old animal.

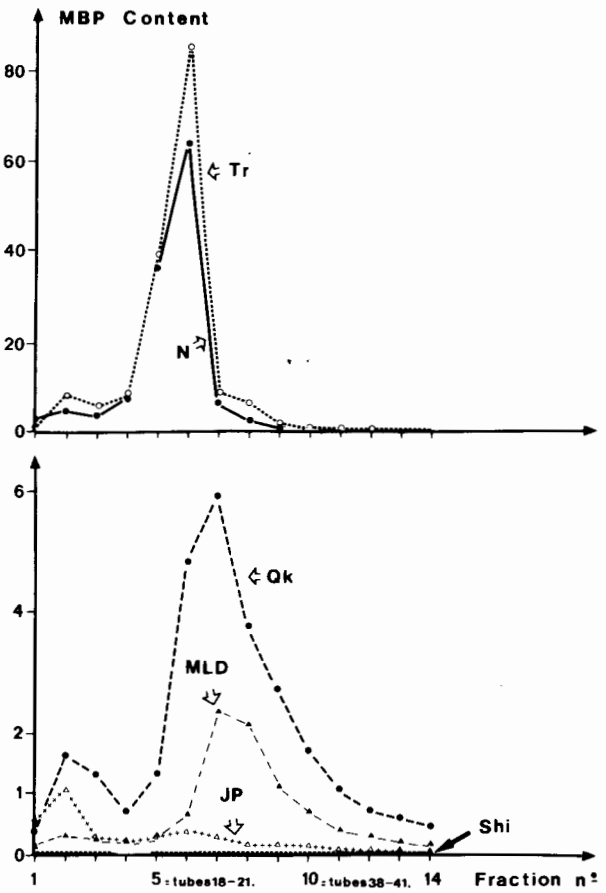

FIG. 6. Myelin basic protein content in different mutants. The 14 fractions were obtained by pooling the 60 fractions (as in Fig. 2), 4 × 4. (See remark to Fig. 5.) Tr, *Trembler;* Qk, *quaking;* Jp, *jimpy;* Shi, *shiverer.*

one probably corresponded to the ascending part of the curve found in isolated myelin (Bourre et al., 1977), and it contained a large amount of MBP (Fig. 6) and could be in agreement with the hypothesis of an arrest of myelinogenesis in this mutant.

The ultrastructural examinations of fractions in the A region showed mainly the presence of vesicles. The B fraction also contained vesicles, as well as some myelin-related material or myelin with a few lamellae. However, compact myelin with many lamellae as shown for tube 15 in normal animals was very rare in this material.

In *Jimpy* (Fig. 2), a very faint shoulder was seen at approximatively 0.68 M-sucrose, corresponding to the extremely low amount of normal myelin found in their brain (Sidman et al., 1964). It contained a small amount of MBP (Fig. 6). A was divided into two waves, at approximatively 0.32 M on 1.05 M-sucrose.

In *shiverer* (Fig. 2), a neurological mutant with little myelin, formed of few lamellae and lacking a major dense line (Jacque et al., 1978; Dupouey et al., 1979; Privat et al., 1979), there was no apparent myelin peak within the density which normally includes myelin. However, a peak was present at approximatively 0.85–0.90 M (0.88 M) sucrose; this peak was devoid of MBP (Fig. 6). From the ultrastructural point of view, both fractions in tubes 18 and 24 (in the B region) contained small vesicles and few membranes. This general appearance was maintained in tube 29, which also contained larger vesicles, as compared with previous fractions. Tube 32 consisted of some dense bodies and myelin membranes of three types: the large majority of them consisted of non-compact lamellar membranes and myelin with an abnormal major dense line, while a few cases of myelin membranes with normal periodicity could also be found. Normally whorled myelin sheaths were not present. Material present in tube 45 was similar to that of normal brain (with the exception of a higher amount of dense bodies).

In mld the profile was very similar when compared with *shiverer:* no peak in the B region, but a peak at 0.88 M. In contrast with *shiverer,* it contained a small amount of MBP (Fig. 6). However, the amount of material in mld is higher than in *shiverer* (as shown by the area under the curve in Fig. 2). In both shi and mld, minute amounts of myelin with normal periodicity were seen (11.3 ± 0.5 nm). However, in these mutants, the thickness of the

major dense line was not uniform, and the periodicity was eventually increased to 13.0 ± 0.7 nm.

Considering the amount of material recovered in fractions between 0.32 M and 0.85 M-sucrose (as determined by the area under the curves), it is shown that peaks of region A heavily contaminate myelin preparations from all mutants, although this material is possibly largely formed by "premyelin" membranes. However, this region is poor in MBP in normal adult mice as well as in the mutants. It must be taken into account that, in *shiverer* for instance, the material between 0.32 M and 0.85 M does not contain the major normal peak. This method provides a useful tool in determining the density range of the myelin in neurological mutants. Unlike other techniques, no material is discarded, because the total brain solid material is used. The large amount of material discarded during the procedure described by Norton and Poduslo (1973) peaks around 1 M-sucrose in our continuous gradient. Moreover, it is partly formed of myelin-related material (McIntyre, 1978), although poor in MBP. The absolute amount of A region is high; it is the same in all mutants (and close to normal in quantity, if not in composition), but peak B (myelin) is minimal: thus the analysis of true compact myelin can be seriously disturbed by the material present in the A region in cases of both mutant and young animals if conventional methods for myelin purification are used.

As shown by the present investigation, it is possible to carry out meaningful myelin studies by analysing the density profile of material separated by zonal centrifugation.

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REFERENCES

Adams D. and Fox M. (1969) The homogeneity and composition of rat brain myelin. *Brain Res.*, **14**, 647–661.

Ayers M. and Anderson R. (1973) Onion bulb neuropathy in the Trembler mouse. Comparison with normal nerve maturation. *Acta Neuropathol. (Berl.)* **32**, 43–59.

Baumann N., Bourre J. M., Jacque C., and Harpin M. L. (1973) Lipid composition of Quaking mouse myelin: Comparison with normal mouse myelin in the adult and during development. *J. Neurochem.* **20**, 753–759.

Bird T. D., Farell D. F., and Sumi S. M. (1978) Brain lipid composition of the Shiverer mouse (genetic defect in myelin development) *J. Neurochem.* **31**, 387–391.

Bourre J. M., Pollet S., Daudu O., Le Saux F., and Baumann N. (1977) Myelin consists of a continuum of particles with different density with varying lipid composition: Major differences are found between normal mice and Quaking mutant. *Biochimie* **59**, 819–824.

Bourre J. M., Daudu O., and Baumann N. (1978) Utilisation du rotor zonal pour montrer que la myéline humaine est formée d'un continuum de particules de densités différentes. *C.R. Acad. Sci. [D] (Paris)* **286**, 355–358.

Cohen S. R., McKhann G. M., and Guarnieri M. (1975) A radioimmuno-assay for myelin basic protein and its use for quantitative measurements. *J. Neurochem.* **25**, 371–376.

Day E., McMillan P., Mickey D., and Appel S. H. (1971) Zonal centrifuge profile of rat brain homogenate. Instability in sucrose, stability in iso-osmotic Ficoll sucrose. *Anal. Biochem.* **39**, 29–51.

Delassalle A., Jacque C., Drouet J., Raoul M., Legrand J. C., and Cesselin F. (1980) Radioimmuno-assay of the myelin basic protein in biological fluids, conditions improving sensitivity and specificity. *Biochimie (in press)*.

Doolittle D. P. and Schweikart D. K. (1977) Myelin deficient, a new neurological mutant in the mouse. *J. Heredity* **68**, 331–332.

Dupouey P., Jacque C., Bourre J. M., Cesselin F., Privat A., and Baumann N. (1979) Immunochemical studies of myelin basic protein in shiverer mouse devoid of major dense line of myelin. *Neurosci. Lett.* **12**, 113–118.

Greenfield S., Norton W., and Morell P. (1971) Quaking mouse: Isolation and characterization of myelin protein. *J. Neurochem.* **18**, 2119–2128.

Jacque C., Privat A., Dupouey P., Bourre J. M., and Baumann N. (1978) Shiverer mouse: A dysmyelinating mutant with absence of major dense line and basic protein in myelin. *2nd ESN Meeting, Proc. Eur. Soc. Neurochem., Vol. 1* (Neuhoff V., ed), p. 131. Verlag Chemie, Wiesbaden.

Laatsch R., Kies M., Gordon S., and Alvord E. (1962) The encephalo-myelitic activity of myelin isolated by ultracentrifugation. *J. Exp. Med.* **115**, 777.

McIntyre R., Quarles R., Webster M. H. de F., and Brady R. (1978) Isolation and characterization of myelin related membranes. *J. Neurochem.* **30**, 991–1002.

Mahaley M., Day E., Anderson N., Wilfong R., and Brater C. (1968) Zonal centrifugation of adult, foetal and malignant brain tissue. *Cancer Res.* **28**, 1783–1789.

Matthieu J. M., Quarles R. H., Webster H. de F., Hogan E., and Brady R. (1974) Characterization of the fraction obtained from the CNS of Jimpy mice by a procedure for myelin isolation. *J. Neurochem.* **23**, 517–523.

Matthieu J. M., Waehneldt T., Webster H. de F., Beny M., and Fagg G. (1979) Distribution of CNS myelin proteins and membrane enzymes in fractions isolated by continuous gradient zonal centrifugation. *Brain Res.* **170**, 123–133.

Murdock D., Katona E., and Moscarello M. (1969) Preparation of myelin using the zonal ultracentrifuge. *Can. J. Biochem.* **47**, 818–820.

Norton W. T. (1977) Isolation and characterization of myelin. In *Myelin* (Morell P., ed), pp 151-159. Plenum Press, New York.

Norton W. T. and Poduslo S. (1973) Myelination in rat brain: Method of myelin isolation. *J. Neurochem.* **21**, 749–757.

Privat A., Jacque C., Bourre J. M., Dupouey P., and Baumann N. (1979) Absence of the major dense line in myelin of the mutant mouse shiverer. *Neurosci. Lett.* **12**, 107–112.

Shapira R., Binkley F., Kibler R., and Wunbaum J. (1970) Preparation of purified myelin of rabbit brain by sedimentation in a continuous sucrose gradient. *Proc. Soc. Exp. Biol. Med.* **133**, 238–245.

Shapira R., Mobley W., Thiele S., Wilhelm M., Wallace A., and Kibler R. (1978) Localization of 2',3'-cyclic nucleotide 3'-phosphohydrolase of rabbit brain by sedimentation in a continuous sucrose gradient. *J. Neurochem.* **30**, 735–744.

Sheads L., Ely M, and Sampugna J. (1977) Myelin subfraction isolated from mouse brain. Studies of normal mice during development, Quaking mutant and three brain regions. *J. Neurobiol.* **8**, 67–89.

Sidman R. L., Dickie M. M., and Appel S. H. (1964) Mutant mice (Quaking and Jimpy) with deficient myelination in the central nervous system. *Science* **144**, 309–311.

Waehneldt T. and Mandel P. (1972) Isolation of rat brain myelin monitored by polyacrylamide gel electrophoresis of dodecyl sulfate-extracted proteins. *Brain Res.* **40**, 419.

Waehneldt R. (1978) Density and protein profiles of myelin from two regions of young adult rat CNS. *Brain Res. Bull.* **3**, 37–44.