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PREPARATION OF PURIFIED MYELIN FROM OX INTRADURAL SPINAL ROOTS BY RATE-ISOPYCNIC ZONAL CENTRIFUGATION

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SUMMARY

1. Ox peripheral nerve myelin membranes were isolated by rate-isopycnic zonal centrifugation. A method is described for the Beckman size 14 zonal rotor. The isolated membranes had the same isopycnic banding density as 15.2 % sucrose (w/w). On the basis of studies with twelve marker enzymes and electron microscopy, at least 99 % of the fraction consisted of myelin membranes. The electron micrographs showed the typical myelin lamellar rings and vesicles derived by peeling off from these lamellae.

2. The enzyme, 2',3'-cyclic nucleotide-3'-phosphohydrolase, reported to be closely associated with central nervous system myelin, was shown not to be associated with the peripheral nerve myelin.

3. A membrane fraction which had the same isopycnic banding density as 17.6 % sucrose (w/w) was shown to have high specific activity of 5'-nucleotidase and 2',3'-cyclic nucleotide-3'-phosphohydrolase. A similar fraction was isolated from ox spinal cord. This fraction of the spinal cord membranes banding at 17.6 % (w/w) contained no basic protein.

4. The disc electrophoresis pattern of the peripheral nerve myelin proteins was different from that of the spinal cord myelin.

INTRODUCTION

The myelin sheath has been the subject of extensive investigation. In the past two years considerable interest has been focused on the study of isolated myelin membranes from brain[1-4], spinal cord[5] and peripheral nerve[6-8]. The earlier published procedures of preparing myelin were long and involved repeated centrifugations, usually in a single solvent or in discontinuous gradients. Since in these methods the sample was collected as a pellet or as a cohesive condensed layer at the interface, contamination with other material could not be avoided[9]. Osmotic shock of myelin was utilized to free the myelin from axoplasmic contamination; unfortunately, shock procedures present the possibility of losing enzymes bound to the membrane by bonds which are readily ruptured due to the reduction in ionic strength[7,10].

The recovery of pure myelin membranes prepared by these methods is usually low and, in addition, the methods are time consuming. With rate-isopycnic zonal

centrifugation as reported in this paper, a myelin preparation from peripheral nerve is recovered which appears to be of high purity and high yield, in a minimal time after the death of the animal. Also a "myelin-like" fraction reported by Agrawal *et al.*[25] is partially purified.

MATERIALS AND METHODS

The isolation of myelin membranes by rate-isopycnic zonal centrifugation

Myelin membranes were isolated from ox spinal root sheats and ox spinal cord, with the Beckman Ti 14 rotor. The nerves were removed about 10 min after the death of the animal and kept in ice. For each preparation 1–4.5 g of nerves or 1 g of spinal cord was homogenized with a Servall mixer run at top speed (15500 rev./min) for 60 s in 0.1 M isotonic phosphate buffer pH 7.2, *plus* 0.1 mM EDTA (22.2 ml of 1.0 M $NaH_2PO_4 \cdot H_2O$ and 354 ml of 0.25 M $NaHPO_4 \cdot 2H_2O$, final volume 1 l). The nerve to buffer ratio was 1 g to 10 ml (w/v). The homogenate was filtered through eight layers of cheese cloth and the filtrate was brought to a final volume of 50 ml with the isotonic buffer and used directly for zonal centrifugation.

With a Beckman 141 High Capacity gradient pump, a 300-ml concave density gradient ranging from 5 to 17 % sucrose (w/w) was delivered into a Ti 14 zonal rotor rotating at 3000 rev./min in a Beckman L2-65K centrifuge. The gradient was introduced into the rotor, followed by 200 ml of 25 % sucrose (w/w) and then 150 ml of 35 % sucrose (w/w) (Table I). The overlay and cushion solutions used were 0.1 M isotonic phosphate buffer, pH 7.2, containing 0.1 mM EDTA and 35 % sucrose (w/w), respectively. Following the injection of 50 ml of sample material and 50 ml of overlay into the rotor core, the rotor was accelerated to 5000 rev./min to achieve a rate separation. After 20 min, the rotor speed was reduced to 3000 rev./min and 15 ml of overlay were injected through the rotor core in order to displace trapped air. Eight to ten 20-ml fractions were removed from the rotor by pumping in 35 % sucrose (w/w). The outflow transmittance was continuously monitored at 254 nm with a LKB uvicord II recorder. The rotor speed was increased to 40000 rev./min for 1 h to achieve isopycnic banding. After the rotor was decelerated to 3000 rev./min the remaining gradient was unloaded.

Refractive indices were measured at 20°C in a Bausch and Lomb Abbe-32 refractometer. Turbidity of the fractions was measured at 400 nm with a Unicam SP

TABLE I

RATE-ISOPYCNIC CENTRIFUGATION SYSTEM FOR CENTRAL AND PERIPHERAL MYELIN PREPARATION ROTOR Ti-14

Stage 1: Rate separation at 5000 rev./min for 20 min; eluted: 180–200 ml at 3000 rev./min.
Stage 2: Isopycnic banding at 40000 rev./min for 60 min; eluted at 3000 rev./min.

Component	*Solution*	*Volume (ml)*
Overlay	0.1 M isotonic phosphate buffer, pH 7.2, 0.1 mM EDTA	50
Sample	0.1 M isotonic phosphate buffer, pH 7.2, 0.1 mM EDTA	50
Gradient	5 %–17 % sucrose (w/w)	300
First interface	25 % sucrose (w/w)	200
Cushion	35 % sucrose (w/w)	150

500 spectrophotometer. The myelin-containing fractions and other fractions to be studied were diluted 1:1 (v/v) with cold distilled water and sedimented at 105000 × *g* for 60 min in the Beckman No. 30 rotor. Collected pellets were stored at −20°C. The recovery of myelin was approx. 12.5–20 mg protein per g wet tissue, and that of the fraction banded at 17.6 % sucrose (w/w) approx. 0.5–2 mg protein per g wet tissue.

Marker enzymes

The following enzymes were used as markers and assayed as described in the references indicated: succinate: cytochrome *c* reductase[11] (EC 1.3.99.1); monoamine oxidase[12] (EC 1.4.3.4, substrate kynuramine); rotenone insensitive NADPH: cytochrome *c* reductase[13] (EC 1.6.2.3); acid deoxyribonuclease[14] (EC 2.1.4.6); acid phosphatase[15] (EC 3.1.3.2) substrate β-glycerophosphate; 5'-nucleotidase (EC 3.1.3.5, substrate 5'-AMP)[16]; (Na^+, K^+, Mg^{2+})ATPase[16] (EC 3.6.1.4); glucose-6-phosphatase[17] (EC 3.1.3.9); L-leucyl-β-naphthylamidase (EC 3.4.1.1, leucine aminopeptidase)[18]; acetylcholinesterase (EC 3.1.1.7)[19]; and 2',3'-cyclic nucleotide-3'-phosphohydrolase[20]. Spectrophotometric measurements were carried out in a Unicam SP 500 spectrophotometer and in a Hitachi–Perkin Elmer 365 double-wavelength spectrophotometer. Protein was measured according to the method of Lowry[21], organic phosphorus according to that Böttcher *et al.*[22], and inorganic phosphorus according to that Olafsen *et al.*[20].

Electron microscopy

The membranes were fixed with OsO_4, embedded in Araldite 512 and stained with uranylacetate followed by staining with lead citrate[23]. The specimens were examined in a Siemens Elmiskop I electron microscope.

Disc electrophoresis

Acrylamide electrophoresis was carried out as described by Takayama[24] at pH 2.0. The gels were fixed with 7.5 % acetic acid, stained with 1% solution of amido black in 7.5 % acetic acid. Gels were destained electrically.

Chemicals

ATP, kynuramine·2HBr, glucose-6-phosphate and adenosine 2'-3'-cyclic phosphate were purchased from Sigma (U.S.A.), AMP from Merck (Germany), cytochrome *c* from Fluka (Switzerland), NADPH and NADH from Boehringer (Germany), rotenone from Aldrich (U.S.A.), DNA from Koch–Light (G.B.), *Escherichia coli* alkaline phosphate from Worthington (U.S.A.), L-leucyl-β-naphthylamide·HCl and β-naphthylamine from Fluka. All other chemicals used were of analytical grade.

RESULTS

Sedimentation properties of spinal root sheet myelin

Typical sedimentation profiles of myelin are shown in Figs. 1 and 2. The average banding density for 40 runs was approx. 1.066 g/ml, 15.2 % sucrose (w/w). The membrane fraction with a banding density the same as that of 17.6 % sucrose (w/w) was also present in the spinal cord (Fig. 3). By contrast, the myelin derived from the spinal cord was resolved into three peaks with banding densities equivalent to those of 11.0 %, 14.0 %, and 16.0 % sucrose (w/w) (Fig. 3).

The peripheral nerve and the spinal cord myelin were found to have a phospholipid to protein ratio of 1.8 (w/w) while the fraction banding at 17.6 % sucrose (w/w) had a ration of 0.9 (w/w). The recovery of myelin was approx. 12.5–20 mg protein per g wet tissue and that of the fraction banded at 17.6 % sucrose (w/w) approx. 0.5–2 mg protein per g wet tissue.

Distribution of marker enzymes

Table II shows the specific activities of enzymes present in the total homogenates of peripheral nerve, in the peripheral nerve myelin, and in the 17.6 % sucrose

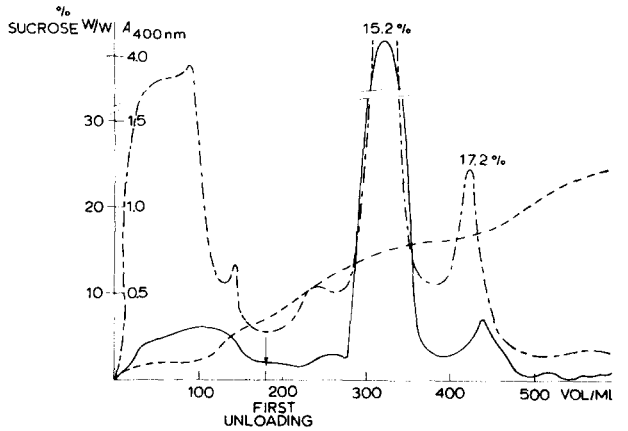


Fig. 1. Rate-isopycnic zonal separation of myelin membranes and the "myelin-like" membrane fraction of ox intradural spinal roots, in the Ti 14 rotor. The myelin banded at 15.2 % sucrose (w/w), and the "myelin-like" fraction at 17.6 % sucrose (w/w). The specific activity of 2',3',-cyclic nucleotide-3'-phosphohydrolase and 5'-nucleotidase in the 17.6 % fraction were 1580 and 162 nmoles/min per mg protein, respectively. ———, $A_{400\ nm}$; —·—, $A_{254\ nm}$; – – – –, % sucrose (w/w).

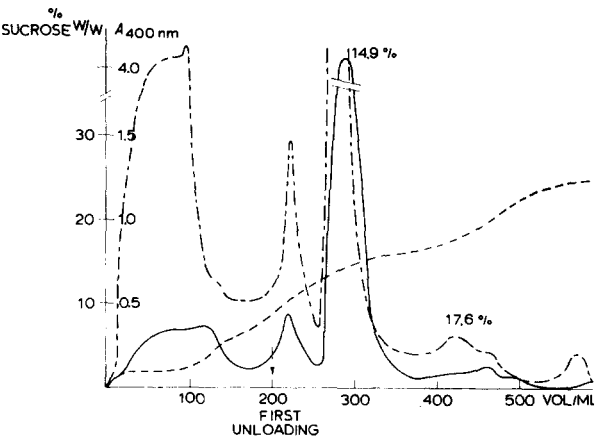


Fig. 2. Rate-isopycnic zonal separation of myelin membranes and the "myelin-like" membrane fraction of ox intradural spinal roots in the Ti 14 rotor. The myelin banded at 14.9 % sucrose (w/w) and the "myelin-like" fraction at 17.6 % sucrose. The specific activity of 2',3'-cyclic nucleotide-3'-phosphohydrolase and 5'-nucleotidase in the 17.6 % sucrose fraction were 3370 and 235.4 nmoles/min per mg protein, respectively. ———, $A_{400\ nm}$; —·—, $A_{254\ nm}$; – – –, % sucrose (w/w).

(w/w) membrane fraction. The activities of mitochondrial, microsomal, lysosomal and plasma membrane marker enzymes are reduced to almost zero in the myelin fraction. In the peripheral nerve 2',3'-cyclic nucleotide-3'-phosphohydrolase was found not to be associated with the myelin fraction and a 5–9-fold increase in the specific activity of this enzyme was found in the 17.6% sucrose (w/w) membrane fraction, which was also enriched in 5'-nucleotidase activity. Spinal cord total homogenate (Table III) was found to have 4-fold higher activity of 2', 3'-cyclic nucleotide-3'-phosphohydrolase than peripheral nerve total homogenate; the central nerve

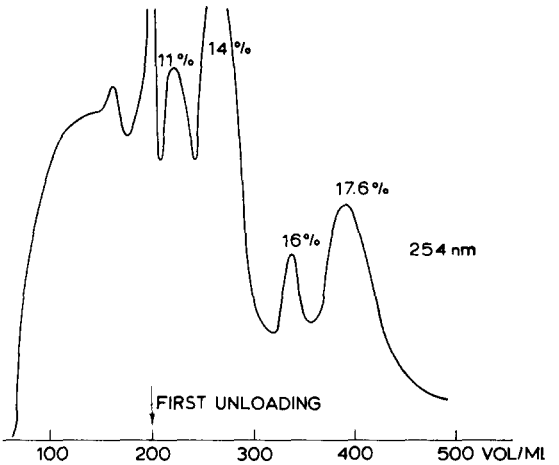


Fig. 3. Rate-isopycnic zonal separation of myelin membranes and the "myelin-like" membrane fraction of ox spinal cord, in the Ti 14 rotor. The myelin banded at 11%, 14% and 16% sucrose (w/w) and the "myelin-like" fraction at 17.6% sucrose (w/w).

TABLE II

SPECIFIC ACTIVITIES* OF MARKER ENZYMES IN PERIPHERAL NERVE MYELIN FRACTIONS

Marker enzyme	*Total homogenate*	*Myelin*	*17.6% sucrose (w/w) fraction*
Glucose-6-phosphatase	27.0	0	not determined
Rotenone-insensitive NADPH:cytochrome *c* reductase	51.0	0.05	not determined
Acid DNAase	58.6	0	not determined
Acid phosphatase	6.5	0	not determined
Succinate:cytochrome *c* reductase	273	3.6	not determined
5'-Nucleotidase	28.6	0	162**–235.4***
(Na^+, K^+, Mg^{2+})-ATPase	22.6	0	0
Acetylcholinesterase	—	0	
Leucine aminopeptidase	6.8	0	not determined
Monoamine oxidase	—	0	not determined
2',3'-Cyclic nucleotide-3'-phosphohydrolase	370–500	200–420	1580**–3370***

* The specific activities are expressed as nmoles substrate or product broken down or formed per min per mg protein.

** Zonal profile as shown in Fig. 1.

*** Zonal profile as shown in Fig. 2.

TABLE III

2′,3′-CYCLIC NUCLEOTIDE-3′-PHOSPHOHYDROLASE* AND 5′-NUCLEOTIDASE** ACTIVITIES IN OX SPINAL CORD AND PERIPHERAL NERVE FRACTIONS

	Total homogenate	*Supernatant*	*Myelin*	*Fraction 18 (16 % sucrose)*	*Fraction 17 (6 % sucrose)*
Peripheral nerve	18–20 (28.6)	0.16	0.2–0.4 (0)		1.58–3.37 (162–235.4)
Spinal cord	79 (7.3)	—	6.65 (0)	6.37 (0)	8.2 (51.3)

* The activity of the 2′,3′-cyclic nucleotide-3′-phosphohydrolase is expressed in μmoles substrate hydrolysed/min per mg protein, except for the total homogenate where the activity is in μmoles/min per g wet wt.

** 5′-Nucleotidase activity is given in parentheses and is expressed in nmoles/min per mg protein.

myelin fractions had 20-fold higher activity of this enzyme than the peripheral nerve myelin fractions. The membrane fraction purified from the spinal cord with isopycnic banding at 17.6 % sucrose (w/w) was enriched in 2′,3′-cyclic nucleotide-3′-phosphohydrolase and had 7-fold higher specific activity of 5′-nucleotidase as compared to the activity of the total homogenate of the spinal cord.

Electron microscopy of isolated myelin

Electron micrographs of the isolated peripheral nerve myelin showed no other recognizable cellular organelles. Most of the myelin was present entirely as lamellar rings and vesicles derived from layers detached from the myelin (Fig. 4 A and B). The split occurs between intraperiodic lines (Fig. 4C). The thinnest structure being one major dense line and two intraperiodic lines, no fragments of axoplasma were seen embedded within the myelin rings. The fraction banding isopycnically at 17.6 % sucrose (w/w) (Fig. 5) showed myelin-like fragments and a membrane sheet which resembles the myelin-like fraction reported by Agrawal *et al.*[25].

Disc electrophoresis

When the different subcellular fractions, the peripheral myelin fraction, the spinal cord myelin fraction, and the 17.6 % fractions were solubilized in 2 M urea in phenol–acetic acid–water (2:1:1, w/v/v) and subjected to electrophoresis in 10 % (v/v) acetic acid as described by Takayama[24], a very simple electrophoretic pattern was observed. The peripheral nerve myelin (Fig. 6, tube 2) showed only three major proteins while the spinal cord myelin (Fig. 6, tube 3) was characterized by the presence of the basic proteins, also present in the peripheral nerve myelin, and by the absence of the major protein which appears in peripheral nerve myelin. This demonstrates the difference between the protein composition of central and peripheral nervous system myelin (Fig. 6). The "myelin-like" fraction derived from the spinal cord banding isopycnically at 17.6 % sucrose (w/w) (Fig. 6, tube 4) showed, except for the absence of the basic protein, a higher content of high molecular weight proteins as compared to that of spinal cord myelin (Fig. 6, tube 3). The similar fraction derived from the peripheral nerve showed almost the same electrophoretic pattern as the myelin fraction, probably because of contamination with myelin fragments.

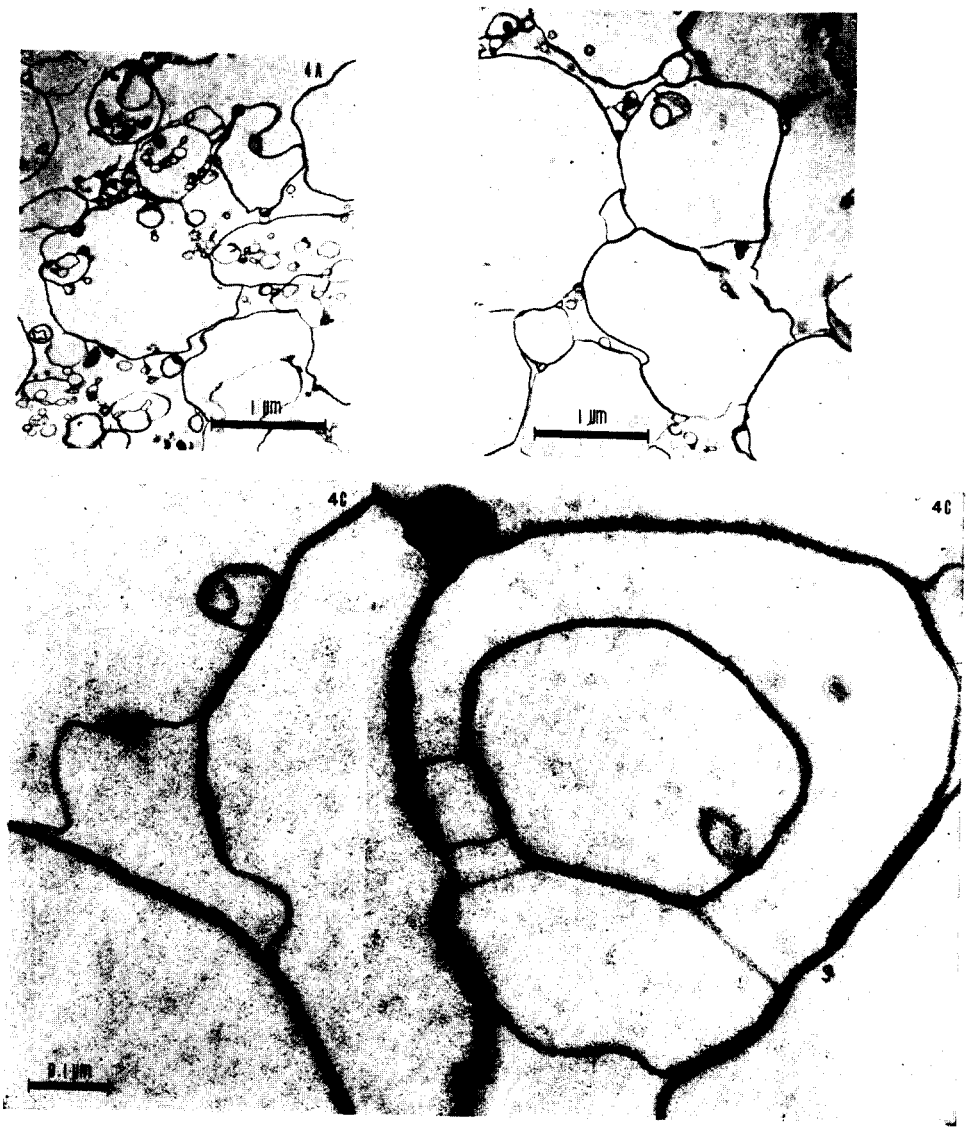
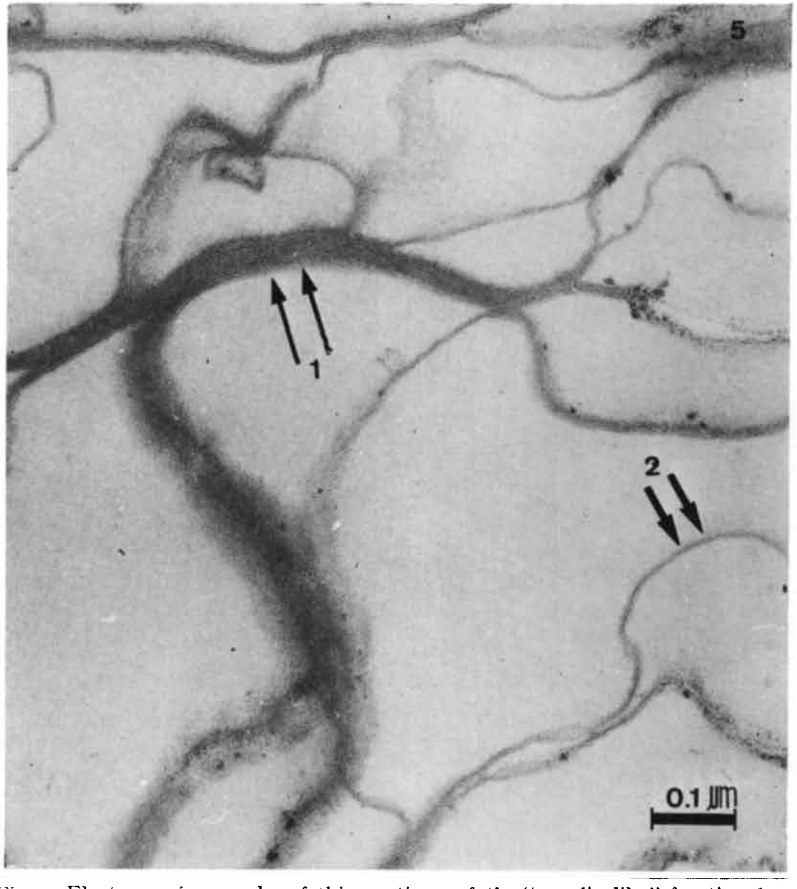


Fig. 4. Electron micrographs of thin sections of intradural spinal root myelin membranes. A. Low magnification survey micrography showing myelin membranes and the absence of mitochondria and trapped axoplasma. B and C. High magnification micrographs of myelin membranes showing vesicles peeling off and splitting off at the intraperiodic line.

DISCUSSION

Only a few reports have appeared on the isolation of myelin membranes by zonal centrifugation[26–28], and these only dealt with central nervous system myelin. The reports concerning purification of peripheral nerve myelin use the classical method of Autilio *et al.*[1] and the recovery is low[7]. The yield of our preparation is



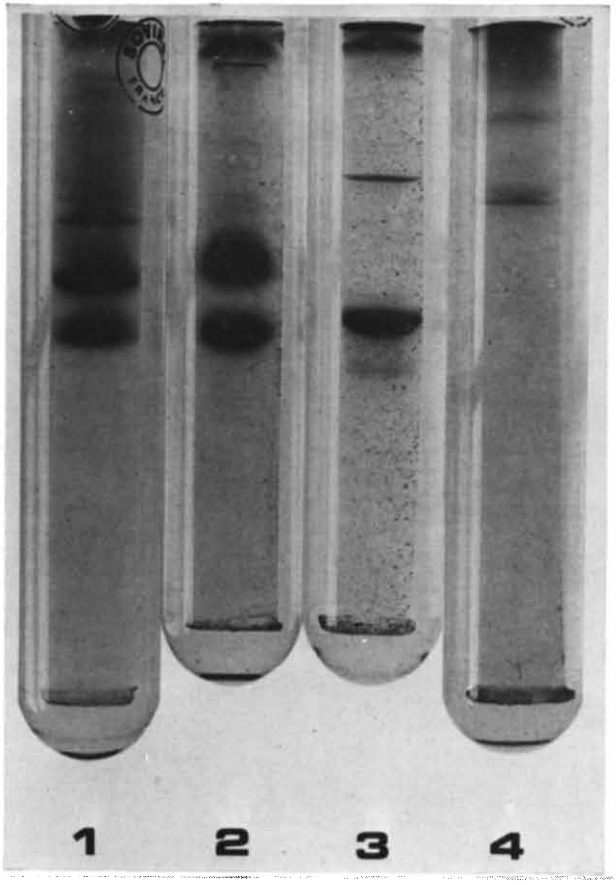


Fig. 5. Electron micrographs of thin sections of the "myelin-like" fraction from ox intradural spinal roots. Low magnification survey micrograph showing heavy dense myelin (1) and sheets of membranes, which are associated with the myelin (2).

Fig. 6. Disc electrophoretic patterns of the intradural spinal root and spinal cord fractions. The membrane fractions were solubilized in a solution of phenol–acetic acid–water (2:1:1, w/v/v) containing 2 M urea. Disc electrophoresis was performed according to the method of Takayama[24] in 7.5% gels (see Materials and Methods). 1. Intradural spinal root, total homogenate (50 μg). 2. Intradural spinal root, myelin fraction (50 μg). 3. Spinal cord, myelin fraction (50 μg). 4. Spinal cord, the "myelin-like" fraction (50 μg).

higher than any other previously reported[7,8] and recovers between 20 and 25 % of the total protein content of the nerve homogenate. The results of the marker enzymes tests, the phospholipid to protein ratio of 1.8, and the electron microscopic appearence clearly indicate that there is no significant contamination of our myelin preparation with any other organelles or with axoplasma. The isolated peripheral nerve myelin showed a banding density equivalent to that of 15.2 % sucrose (w/w), far from that of other nervous tissue organelles.

Autilio *et al.*[1] obtained their most dense and most concentrated fraction of myelin at 1.094 g/ml (23 % sucrose, w/w). When myelin was prepared from spinal cord (central nervous system), the myelin formed three isopycnic bands at 11 %, 14.0 % and 16 % sucrose (w/w), depending on the gradient steepness. Myelin did not form bands beyond these densities. When the purified myelin was banded isopycnically on a linear gradient between 5 % and 35 % sucrose (w/w), no new myelin bands were seen. These results are in good agreement with the results of Day *et al.*[28] who showed in their zonal centrifuge profiles of rat brain homogenates the same banding densities for myelin. The enzyme 2',3'-cyclic nucleotide-3'-phosphohydrolase was reported by Kurihara and Tsukada[29] and others[20,30,-33] to be exclusively located or strongly associated with the myelin sheath of the central nervous system. It was found that the total activity of the 2',3'-cyclic nucleotide-3'-phosphohydrolase in the peripheral nerve homogenate was only one-fourth of the total activity in the spinal cord homogenate. In contrast to the spinal cord myelin, the activity in the peripheral nerve myelin was very low, about one-twentieth of the activity in our spinal cord myelin preparation.

There have been reports[8,34] of proteinase and β-naphtylaminidase activity in peripheral nerve myelin. In our peripheral myelin preparation we could not show any significant proteinase activity and no β-naphthylaminidase activity. The membrane fraction banding at 17.6 % sucrose (w/w) seems to be identical to the "myelin-like" fraction reported by Agrawal *et al.*[25] and Morgan *et al.*[35] and are shown here also to have high 2',3'-cyclic nucleotide-3'-phosphohydrolase activity, high activity of 5'-nucleotidase, absence of the basic protein and a phospholipid to protein ration of 0.9. The disc electrophoresis patterns reveal the pronounced differences between the peripheral and central nervous system myelin preparation; differences in lipid[6] and protein composition[36] have already been reported.

As has been shown, the method described in this paper is applicable for the preparation of myelin membranes from central and peripheral nerves with high purities and high recoveries, and can be used for purification of myelin membranes from individual nerves or small specific parts of the brain.

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