Biochimica et Biophysica Acta, 470 (1977) 104—112

BBA 77800

ISOLATION OF NON-MYELIN PLASMA MEMBRANES UNIQUE TO WHITE MATTER

SWAPAN K. GHOSH * and EDWARD KOENIG **

Division of Neurobiology, Department of Physiology, State University of New York at Buffalo, Buffalo, N.Y. 14214 (U.S.A.)

(Received March 14th, 1977)

Summary

A procedure is described for isolating two membrane fractions from rabbit spinal-cord white matter enriched with 5′-nucleotidase, a nonspecific plasma membrane marker, 2′,3′-cyclic nucleotide phosphohydrolase, an oligodendroglial plasma membrane marker, and acetylcholinesterase, an axonal plasma membrane marker. While the two membrane fractions exhibited similar enrichments with respect to cyclic nucleotide phosphohydrolase, enrichments of 5′-nucleotidase and acetylcholinesterase were significantly greater in the heavier membrane fraction. Selected enzyme markers for cyto- and mitochondrial membranes were not detected. Moreover, gray matter did not yield homologous membrane fractions in the gradient when subjected to the identical procedure, indicating that the two membrane fractions were unique to white matter. While electronmicroscopic examination revealed that both membrane fractions were contaminated with myelin, the heavier fraction was least contaminated and exhibited a fair degree of homogeneity with respect to single membrane vesicular profiles. It was concluded that both membrane fractions were enriched with oligodendroglial and axonal plasma membranes, with the heavier fraction containing significantly more axolemma.

Introduction

The interactions between the membranes of the axon and the neurolemma cell (i.e., ensheathing cell) are very specific and must involve surface recognition and intercellular affinity reactions during the period anteceding myelina-

* Present address is: Department of Immunology, Roswell Park Memorial Hospital, Buffalo, New York 14203, U.S.A.

** To whom reprint requests should be sent.

tion. After myelination, the relationship of the neurolemma cell to the axon is characterized by a unique structural arrangement of enzymically inert compact myelin lamellae that form the bulk of the ensheathment at the internode. However, there are also circumscribed regions of the ensheathment at the internode in which compact myelin is not present; these include non-meylin, limiting membranes of the paranodal loops and of a tongue of glial cytoplasm that extends the full length of the internode in juxtaposition to the axon (see ref. 1). The cytoplasm in the tongue is continuous with that in the paranodal loops, and ultimately, with that in the glial cell perikaryon [1,2]. At sites of surface contact between the axon and the non-myelin limiting membranes of the glial cell, there are junctional specializations [3]. It is reasonable to infer that this glial-axonal junction may be responsible for the contamination of myelin preparations by axolemma [4]. While the presence of 2′,3′-cyclic nucleotide phosphohydrolase in myelin preparations has been regarded as a specific myelin membrane marker [5], it is more likely that it is a marker for the non-compact membrane associated with myelin [6—9].

As a first step in characterizing the surface components involved in glial-axonal interactions, it is necessary to isolate the membranes of interest. A procedure is described in this communication that yields unique membrane fractions from spinal-cord white matter of the rabbit, not obtained from dissected gray matter, highly enriched with respect to 5′-nucleotidase, a general plasma membrane marker, 2′,3′-cyclic nucleotide phosphohydrolase, an oligodendroglial membrane marker and acetylcholinesterase [10], an axonal membrane marker.

Materials and Methods

Preparation of membrane fractions. Adult rabbits (2—4 kg) were anesthesized with sodium pentobarbitol and killed by exsanguination. The spinal cord was excised and placed in a petri dish, containing ice-cold 10% Ficoll. The meninges were dissected away, and the spinal cord was bisected longitudinally. Long fiber tract bundles of white matter were stripped away from the cord with a watchmaker's tweezers under low magnification stereomicroscopy and collected. After blotting, approximately 2—3.5 g of white matter was homogenized at 4°C in 10 ml of 0.3 M Tris hydroxymethyl methylglycine (Tricine) and 0.5 mM $MnCl_2$ pH 5. Using a loose fitting conical teflon pestle-glass homogenizer, 6 up-and-down strokes (650 rev./min) were completed in 30 s; after a 30-s interval, another 6 strokes were applied. The homogenate was diluted to a final concentration of 4—5% (w/v) and centrifuged at 26000 rev./min for 15 min in a SW 27 rotor (Spinco). The supernatant was decanted and further centrifuged at 26000 rev./min for 45 min. The supernatant was discarded and the pellet was resuspended in 6.5—8 ml 0.4 M sucrose by homogenization (2 strokes) and layered over a discontinuous sucrose gradient, consisting of 6.0 ml of 0.9 M sucrose, 15 ml of 0.6 M sucrose and 10 ml of 0.5 M sucrose. The preparation was centrifuged in a SW 27 rotor at 26000 rev./min for 120 min.

The discontinuous gradient yielded three bands and one scanty pellet. The distribution of bands was as follows: (1) a light, diffuse band in 0.4 M sucrose ("M" fraction); (2) a band at the top of 0.6 M sucrose ("N" fraction); and (3)

an interface band between 0.6 M and 0.9 M sucrose ("O" fraction). The suspended fractions were recovered by syringe, and each was diluted with 3 volumes of ice cold, distilled water and sedimented at 26000 rev./min for 20 min. Pellets were resuspended by hand homogenization in a minimum volume of tricine-Mn (TM) medium and stored frozen. A schematic flow diagram of the procedure is given in Fig. 1.

Enzymic assays. 5′-Nucleotidase (EC 3.1.3.5) was assayed according to Tanaka, et al. [11] while 2′,3′-cyclic nucleotide phosphohydrolase was assayed by the method of Olafson, et al. [12]. The inorganic phosphate released was determined according to Lowry and Lopez [13]. Acetylcholinesterase (EC 3.1.1.7) was assayed according to the method of Ellman [14], as modified by Klingman et al. [15]. Acid and alkaline phosphatases (EC 3.1.3.2 and EC 3.1.3.1) were measured by the method of Cotman and Mathews [16], while NADH- and NADPH-cytochrome *c* oxidoreductases (EC 1.6.99.-) were assayed within 6 h after death of the animal, according to Sottocasa et al. [17]. Protein was analyzed by the method of Lowry, et al. [18], using bovine serum albumin as standard.

Electronmicroscopy. Pellets of selected membrane fractions were fixed in cold 3% glutaraldehyde, buffered with 0.1 M sodium phosphate (pH 7.4), postfixed in 2% OsO_4, buffered with 0.1 M sodium phosphate (pH 7.4) and embedded in Epon. Silver sections were counterstained with uranyl acetate and lead citrate, and were viewed in a Hitachi electronmicroscope.

Results

Dissection of the fiber tract bundles from spinal cord was best carried out in unbuffered 10% Ficoll. The crude homogenate in the TM medium had a pH of

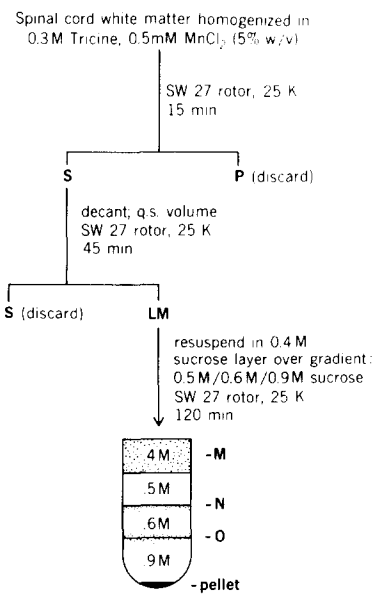

Fig. 1. Flow diagram showing preparative procedure.

TABLE I

DISTRIBUTION OF SELECTED MARKER ENZYMES IN CRUDE LIGHT MEMBRANE FRACTION FROM SPINAL-CORD WHITE MATTER

	Cytochrome *c* oxidoreductases *		Phosphatases **		$(Na^+ + K^+)$ ATPase ***
	NADH-	NADPH-	Acid	Alkaline	
Homogenate	5.56	3.0	133	44	1.97
Light membrane fraction (LM)	18.4	11.3	54	100	4.02

* nmol substrate reduced/mg protein per h.
** nmol substrate consumed/mg protein per h.
*** nmol P_i liberated/mg protein per h.

5.5, and 2 cycles of differential centrifugation yielded a crude light membrane fraction (LM) (see Fig. 1).

The LM fraction exhibited no detectable rotenone-sensitive NADH- and NADPH-cytochrome *c* oxidoreductases. These enzymes are known to be markers of inner mitochondrial membranes. Rotenone-insensitive NADH- and NADPH-cytochrome *c* oxidoreductases, however, which were markers of outer mitochondrial and microsomal membranes, respectively, [19] were enriched over homogenate in this fraction (Table I). The marker for lysosomes, acid phosphatase, was present, indicating some contamination, but there was a significant loss compared to homogenate. The plasma membrane markers alkaline phosphatase and $(Na^+ + K^+)$ATPase, on the other hand, were enriched.

Further subfractionation of LM on a discontinuous sucrose gradient yielded M, N and O fractions (Fig. 1). In terms of protein mass, the M fraction was preponderant and constituted 81% of the LM, while N, O and pellet fractions were 6, 5 and 8%, respectively. The proportions were crude estimates because only 69% of the protein placed on the gradient was recovered.

The distribution for the three major marker enzymes, 5'-nucleotidase, 2',3'-cyclic nucleotide phosphohydrolase and acetylcholinesterase, was primarily to the N and O fractions, as reflected in their preferential enrichments in these two fractions (Table II). The O fraction was the most enriched over homogenate, ranging from 11—18-fold for 5'-nucleotidase, 6—18-fold for cyclic nucleotide phosphohydrolase and 3—6-fold for acetylcholinesterase. The corresponding enzymic enrichments for the N fraction ranged 4—9-fold, 6—10-fold, and 2—3-fold, respectively. The enzyme showing the least difference in average enrichment between the two fractions was cyclic nucleotide phosphohydrolase, while that of 5'-nucleotidase and acetylcholinesterase was 40 and 55% greater, respectively, in the O fraction. The O fraction exhibited no detectable NADH- or NADPH-cytochrome *c* oxidoreductase activity (Table III).

While the M fraction was not significantly different from starting homogenate with respect to the three major marker enzymes, "washing" the recovered M pellet twice by resuspension in 0.32 M sucrose did yield significant reductions (M fraction floats in 0.32 M sucrose); i.e. M : homogenate ratios for 5'-nucleotidase, cyclic nucleotide phosphohydrolase and acetylcholinesterase were 0.2, 0.8 and 0.1, respectively. When compared with purified myelin fraction prepared in parallel from the same animal by the method of Norton and

TABLE II

SPECIFIC ACTIVITIES OF PLASMALEMMAL MARKER ENZYMES OF CRUDE AND PURIFIED LIGHT MEMBRANE SUBFRACTIONS PREPARED FROM SPINAL-CORD WHITE MATTER

Fraction	5'-Nucleotidase		2',3'-cyclic nucleotide phosphohydrolase		Acetylcholinesterase	
	Spec. act. ± S.E. **	Ratio *	Spec. act. ± S.E. **	Ratio *	Spec. act. ± S.E. **	Ratio *
Homogenate	2.26 ± 1.38	—	16.40 ± 2.12	—	52.43 ± 8.38	—
LM	6.51 ± 0.94	2.9	36.25 ± 12.51	2.2	25.15 ± 8.68	0.5
M	2.77 ± 0.29	1.2	25.86 ± 2.86	1.6	26.47 ± 4.56	0.5
N	15.86 ± 2.44	7.0	121.00 ± 27.35	7.4	134.93 ± 31.62	2.6
O	27.04 ± 4	12.0	140.26 ± 28.20	8.5	217.13 ± 35.29	4

* Ratio = subfraction/homogenate.

** μmol P_i/mg protein per h.

*** μmol acetylthiocholine/mg protein per h.

TABLE III

DISTRIBUTION OF ORGANELLE SPECIFIC ENZYMES IN CRUDE AND PURIFIED LIGHT MEMBRANE FRACTIONS PREPARED FROM SPINAL-CORD WHITE MATTER

Enzyme marker	Homogenate	LM	LM/homogenate	M, N, O
Acid phosphatase **	133	54	0.4	n.d. *
Alkaline phosphatase **	44.2	100.1	2.3	n.d.
NADH-cytochrome *c* *** oxidoreductase	5.56	18.4	3.3	n.d.
NADPH-cytochrome *c* *** oxidoreductase	3.0	11.3	3.8	n.d.

* n.d., not detectable.

** μmol *p*-nitrophenol/mg protein per h.

*** μmol cytochrome *c* reduced/mg protein per h.

Poduslo [20], the myelin fraction : homogenate ratios for the three enzymes were 0.5, 3.0, and 0.4, respectively.

Representative electronmicrographs of the several membrane fractions are shown in Fig. 2. The LM fraction shows three major classes of membraneous structures that tended to distribute somewhat preferentially in each of the subfractions. These comprised fragments of compact myelin, enriched in fraction M (Fig. 2b); large, alveolate membrane components, enriched in fraction M and to some extent, fraction N (Fig. 2b,c); and small, single membrane vesicular components, enriched in fraction O and to some extent in N. Fig. 2b is that of an "unwashed" M fraction (see above).

Owing to the selection of spinal-cord fiber tracts as starting material in the preparation of these membrane fractions, it was of interest to determine whether gray matter would yield membrane fractions of equivalent isopycnic densities. Tissues used for the two experiments conducted were cerebral cortex, from which underlying white matter had been scraped away, and both caudate nuclei. Tissues were pooled for homogenization and the entire procedure was

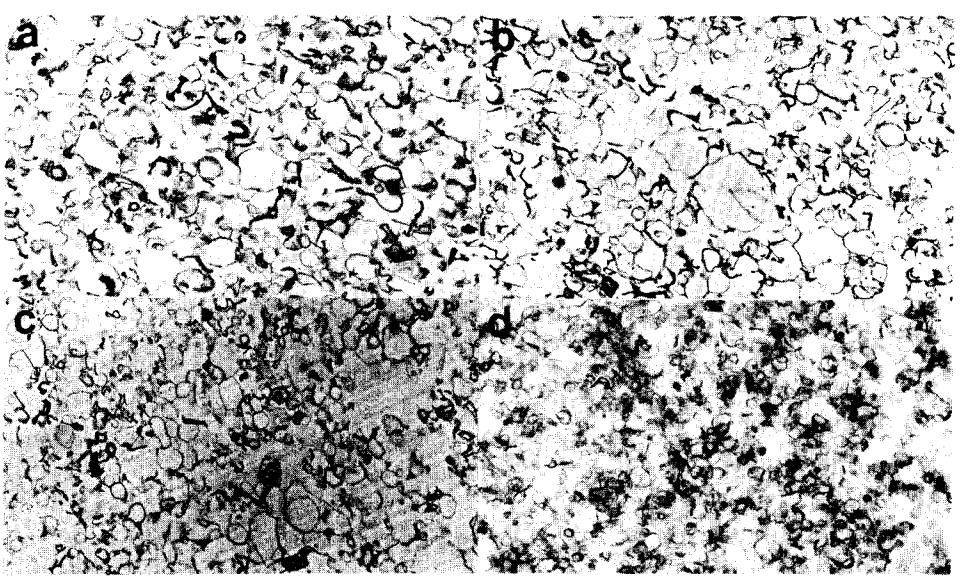

Fig. 2. Representative electronmicrographs at same magnification of (a) LM, (b) M, (c) N, and (d) O membrane fractions (see Fig. 1 for nomenclature). Magnification, 9,000 X.

identical to that used for spinal-cord white matter. The gray matter preparation yielded no discernible N, or O fractions.

Discussion

The surface membranes associated with the myelinated axon and its ensheathing cell are regionally specialized and distinct in many respects. It is possible on the basis of ultrastructural analysis to identify at least three membrane types that are either continuous or contiguous with one another; namely, compact myelin, non-myelin glial membrane and axolemma. From the standpoint of characterizing surface components mediating glial-axonal interactions, the membranes of interest are the non-myelin oligodendroglial membrane and the axolemma. The isolation of the latter membranes and their separation from myelin poses a major problem, owing to the overwhelming preponderance of myelin.

Our approach to the problem utilizes a strategy of homogenization under conditions that have been employed in this laboratory in the past to destabilize meylin for the purpose of manually translating axons out of their sheaths; i.e. 0.3 M tricine and 0.5 mM $MnCl_2$ (pH 5). The exposure of nerves to this medium causes separation and "ruffling" of myelin lamellae along the intraperiod planes (unpublished observations). Homogenization of white matter in sucrose alters drastically the density characteristics of the particulate components and is incompatible with yielding fractions equivalent to those described after homogenization in tricine-Mn.

Brief, high speed centrifugation of the TM homogenate produced a pellet constituting the bulk of the tissue mass, including most of the myelin. The turbid supernatant remaining yielded a pellet containing the crude, low density membrane fraction (LM) after extended high speed centrifugation. This fraction exhibited some enrichment of enzymes regarded to be general markers of plasma membranes (i.e. 5′-nucleotidase, $(Na^+ + K^+)$ATPase, alkaline phosphatase), as well as an enzyme regarded to be specific for oligodendroglial plasmalemma (i.e. 2′,3′-cyclic nucleotide phosphohydrolase, 6, 7, 8, 9). While the crude LM fraction showed, in addition, enrichment of NADH- and NADPH-rotenone sensitive and insensitive cytochrome *c* oxidoreductases, which were considered enzymic markers of outer mitochondrial and smooth membranes of the endoplasmic reticulum, respectively, [19], it was reduced with respect to acid phosphatase, a lysosomal enzyme, and acetylcholinesterase, a marker for axolemma. The reduction of acetylcholinesterase at this stage is curious in view of the several-fold enrichment achieved in subfractions N and O (see below); however, these subfractions represent only a small proportion of the LM mass.

Subfractionation of LM on a discontinuous sucrose gradient yielded three fractions of interest; viz., M, N and O. Fractions N and O were enriched substantially over that of homogenate with respect to 5′-nucleotidase, 2′,3′-cyclic nucleotide phosphohydrolase and acetylcholinesterase. The highest enrichment was obtained in the O fraction, which averaged almost twice that of N for 5′-nucleotidase and acetylcholinesterase, but did not exceed enrichment of 2′,3′-cyclic nucleotide phosphohydrolase significantly. The O fraction also exhibited no detectable enzymatic activities indicative of cyto- or mitochon-

drial membranes. These results suggest that both N and O fractions are relatively rich in membranes derived primarily from oligodendroglial and axonal plasma membranes, with the O fraction being richer in axolemma content.

On the other hand, the lack of alkaline phosphatase and ($Na^+ + K^+$)ATPase (data not shown) in the O fraction may indicate that this fraction contains membranes derived from specialized regions of the axolemma. Cotman and Mathews [16] observed that ($Na^+ + K^+$)ATPase and alkaline phosphatase did not co-enrich with 5′-nucleotidase and acetylcholinesterase during the isolation of synaptic membranes. Freeze-fracture studies [3] have indicated that the density of intramembranous particles of the axolemma is high in the region of the node of Ranvier, while the region of the internode is low, possibly reflecting an unequal distribution of oligomers representing ionic channels and sodium pump components. Moreover, electronmicroscopists have long recognized the existence of a subaxolemmal density limited to the nodal region [20] which may be instrumental in determining the non-random distribution of intramembranous particles. In any case, these considerations would lead to the expectation that the nodal axolemma would not distribute with membrane derived from the internode.

The foregoing inference is further supported by the findings that gray matter, which is rich in neuronal membranes, including synaptic membranes, does not yield homologous N or O fractions. These experiments indicate that the preparative procedure yields plasma membrane fractions that are apparently unique to white matter.

Electronmicroscopic examination of the LM fraction, and M, N and O subfractions revealed the presence of three principal membrane components; i.e. fragmented compact myelin, alveolate myelin membranes, and vesicular components. The short, myelin fragments, consisting generally of one major period line and fused lateral loops, were distributed mainly to M, and, to some extent, to N; occasional fragments were also seen in O. The alveolate membranes appeared to concentrate in M and N, but occasional profiles were also seen in O. Close inspection of the alveolate components revealed common boundaries between alveoli containing multiple membrane lamellae, indicating that they were probably derived from myelin. The vesicular components were largely concentrated in the O fraction, and to a lesser extent, to the N fraction.

In conclusion, the preparative procedure after homogenization in tricine-Mn medium, yields unique membrane fractions from white matter that appear to be derived from specialized portions of the axon and ensheathing cell that make up the glial-axonal membranous complex. While the findings indicate also that there is some contamination by myelin, the heavier fraction is least contaminated, and appears most enriched with respect to oligodendroglial and axonal plasma membranes. It would seem that further subfractionation of the O fraction into separate glial and axolemmal fractions will require an approach that does not rely on isopycnic density differences for separation.

Acknowledgements

This work was supported by U.S. Public Health Grant No. 04656 from the NINCDS. We thank Mr. Peter Poliszczuk for his technical assistance in preparing the material for electronmicroscopy.

References

1 Bunge, R.P. (1968) Physiol. Rev. 48, 197—251
2 Webster, H. deF. (1971) J. Cell Biol. 48, 348—367
3 Livingston, R.B., Pfenninger, K., Moor, H. and Akert, K. (1973) Brain Res. 58, 1—24
4 McIlwain, D.L. (1974) Brain Res. 69, 182—187
5 Kurihara, J. and Tsukada, Y. (1968) J. Neurochem. 15, 827—832
6 Poduslo, S. (1975) J. Neurochem. 24, 647—654
7 Mandel, P., Nussbaum, J.L., Neskovic, N.M., Sarlieve, L.L. and Kurihara, R. (1972) in Advances in Enzyme Regulation (Weber, ed.) 10, p. 113, Pergamon Press Oxford
8 Zanetta, J.P., Benda, P., Gombos, G. and Morgan, I.G. (1972) J. Neurochem. 19, 881)883
9 Volpe, J.J., Fujimoto, K., Marasa, J.C. and Agrawal, H.C. (1975) Biochem. J. 152, 701—703
10 Silver, A. (1974) The Biology of Cholinesterases, pp. 110—120, North-Holland Publishing Co., Amsterdam
11 Tanaka, R., Morita, H. and Teruya, A. (1973) Biochim. Biophys. Acta 298, 842—849
12 Olafson, R.w., Drummond, G.I. and Lee, J.F. (1969) Can. J. Biochem. 47, 961—966
13 Lowry, O.H. and Lopez, J.A. (1946) J. Biol. Chem. 162, 421—428
14 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
15 Klingman, G.I., Klingman, J.D. and Poliszczuk, A. (1968) J. Neurochem. 15, 1121—1130
16 Cotman, C.W. and Mathews, D.A. (1971) Biochim. Biophys. Acta 249, 380—394
17 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415—438
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Smoley, J.M., Kuylenstierna, B. and Ernster, L. (1970) Proc. Natl. Acad. Sci., U.S. 66, 125—131
20 Norton, W.T. and Poduslo, S.E. (1973) J. Neurochem. 21, 749—757
21 Peters, A. (1966) Quart. J. Expl. Physiol. 51, 229—236