

ISOLATION AND CHARACTERIZATION OF PLASMA  
MEMBRANE FRACTIONS FROM GARFISH  
*LEPISOSTEUS OSSEUS* OLFACTORY NERVE

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ABSTRACT

Garfish *Lepisosteus osseus* olfactory nerve, because of its large size and the unusually high concentration of axonal membrane, is an excellent source of axonal membrane. A procedure is described for the isolation of two types of plasma membranes from the nerve which are obtained in yields of about 20 mg (fraction I) and 1.5 mg (fraction II) per g of wet nerve. Both membrane fractions consist mostly of rounded membrane vesicles, with a unit membrane thickness of ~7.5 nm. The two membrane fractions are different in their lipid to protein ratios, Na-K ATPase activities, polypeptide patterns on sodium dodecyl sulfate (SDS) gel electrophoresis, and fatty acid compositions. They have similar phospholipid composition. On the basis of the relative concentration of axonal and Schwann cell plasma membranes in the nerve, the Na-K ATPase activities of the two membrane fractions and a comparison of the properties of the membrane fractions to those of squid and lobster nerve membrane preparations, fraction I seems to be the axonal membrane and fraction II the Schwann cell plasma membrane. Fraction I has a low protein to lipid ratio. Its polypeptide pattern on SDS gel appears to be much more complex as compared to that of fraction II membrane.

INTRODUCTION

To achieve a satisfactory understanding of the mechanism of excitation in a nerve cell the detailed molecular composition and structure of the axonal membrane must be known. At present, little information has been accumulated about the molecular composition of axonal membrane, although numerous types of other plasma membranes have been the subject of study for years (1).

A major problem has been the identification of a natural source rich in axonal membrane readily separable from contaminating membranes. However, reports have appeared recently on the isolation of membranes from squid stellar nerves (2), squid retinal nerves (3, 4), and lobster walking leg nerves (5, 6).

Because of the large size and unusually high concentration of axonal membrane, the garfish *Lepisosteus osseus* olfactory nerve (7) is an excel-

lent source of axonal membrane relatively free of other cellular membranes and has already been the subject of several reports on the chemical (8-12) and physiological (13) properties of excitable membrane. In this nerve, the axonal fibers occur in bundles of several hundreds, each bundle surrounded by a single layer of Schwann cell. Besides

axonal and Schwann cell membranes, the only cellular components seen in electron micrographs of the nerve are mitochondria, neurofilaments, and microtubules. We have isolated a membrane fraction from this nerve in high yield which appears to be axonal membrane as judged from the yield of the membrane, based on morphological and chem-

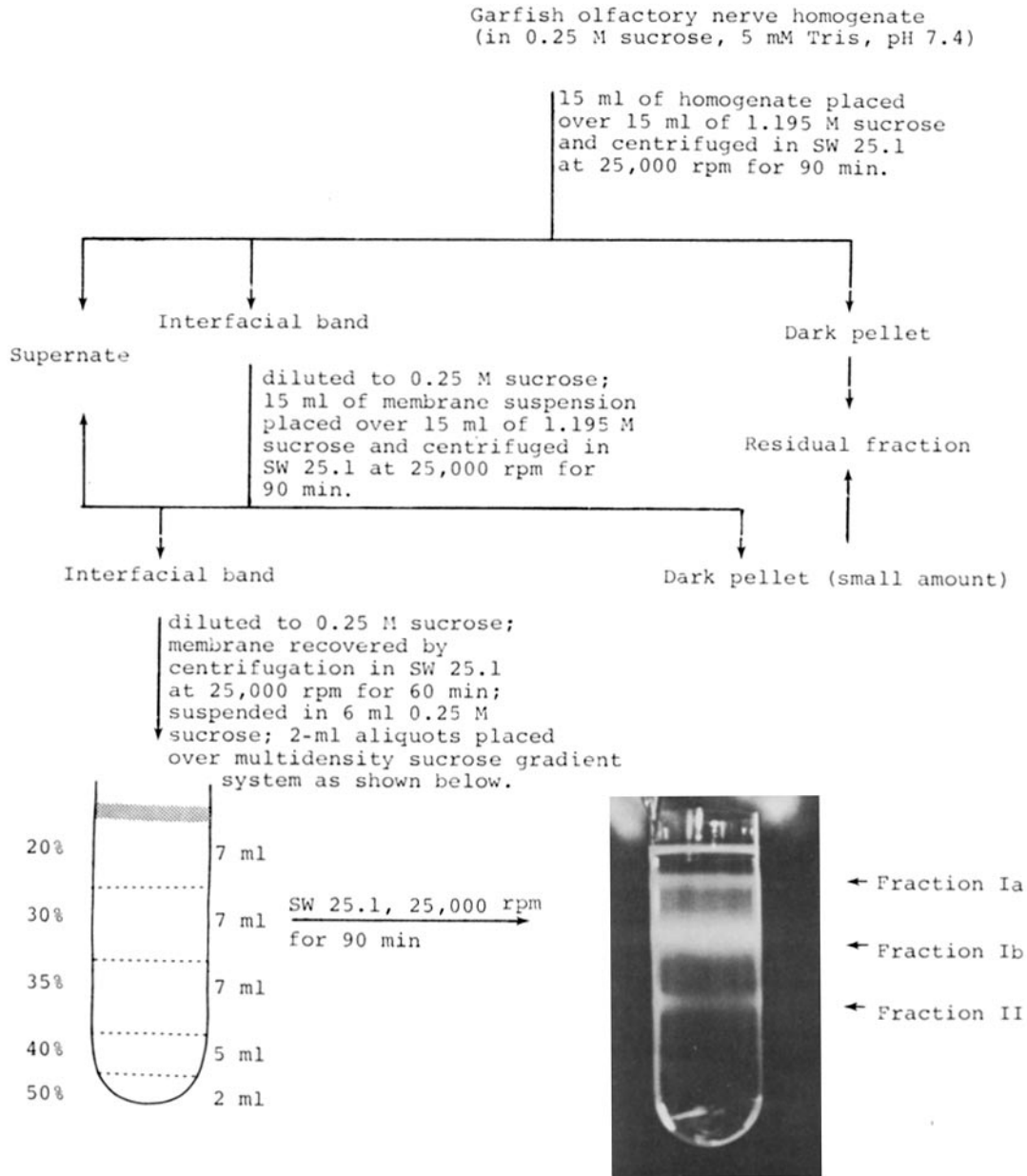


FIGURE 1

ical studies. This paper details the procedure for the isolation of the membrane preparation and describes some of its chemical, enzymatic, and morphological properties. The properties of another membrane fraction which seems to be the Schwann cell membrane are also described and discussed. A preliminary report of this work has appeared elsewhere (14).

## MATERIALS AND METHODS

### *Isolation of Olfactory Nerve*

Fresh garfish heads or the upper snouts, packed in ice and shipped by air, were obtained from Gulf Specimen Co. (Panacea, Fla.). These were received within 8 h after the animals were killed. All the operations, thereafter, were carried out in a cold room at 0°–4°C. The nerve bundles were taken from the snouts, as described by Easton (13) and placed in the gar Ringer solution (13). The olfactory nerve was separated from the trigeminal nerve and accompanying blood vessels. About 5 g of wet nerve (blotted dry between filter papers) could be obtained from 25 snouts (15–20 cm long). The yield of nerve varies considerably depending on the size of the snouts.

### *Isolation of Membrane Fractions*

The procedure used for the isolation of the membrane fractions is shown in Fig. 1. The nerve (4–5 g) was first rinsed twice with 25 ml of 0.25 M sucrose, 5 mM Tris, pH 7.4. The nerve was cut into small pieces and homogenized in 0.25 M sucrose, 5 mM Tris, pH 7.4 (15 ml/g wet tissue), in a Teflon-glass Duall grinder (0.01–0.015 cm clearance between pestle and tube) using a total of about 15 up and down passes of the pestle. The homogenate was filtered through four layers of cheesecloth to remove unhomogenized nerve and gelatinous material. 15-ml aliquots of the homogenate were placed over 15 ml of 1.195 M sucrose in tubes of Beckman rotor SW 25.1 and centrifuged in a Beckman model L refrigerated (2°C) centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 90 min at 25,000 rpm. The interfacial membrane material was recovered from all the tubes by means of a Pasteur pipette and the combined membrane suspension diluted to 0.25 M sucrose with 5 mM Tris buffer (refractive index was used to adjust the molarity). Centrifugation of the membrane suspension over 1.195 M sucrose was repeated. After the first centrifugation a dark-colored residual pellet occurred at the bottom of the tube while only a small similar pellet was present in the second centrifugation step. The interfacial membrane material was recovered, diluted to 0.25 M sucrose (5 mM Tris, pH 7.4), and spun in a SW 25.1 rotor at 25,000 rpm for 60 min. The membrane pellet was resuspended in 6 ml of 0.25 M sucrose (5 mM Tris, pH 7.4) and 2-ml aliquots were placed over freshly made discontinuous gradients pre-

pared from 2, 5, 7, 7, and 7 ml, respectively, of 50, 40, 35, 30, and 20% sucrose all containing 5 mM Tris at pH 7.4 and centrifuged for 90 min in the SW 25.1 rotor at 25,000 rpm. Three major membrane bands were obtained as shown in Fig. 1. Fraction Ia, which contained about 25% of the total membrane protein, sedimented just above the 20–30% sucrose interface; fraction Ib, which contained about 65% of the total membrane protein, sedimented at the 20–30% sucrose interface. On doubling the centrifugation time both Ia and Ib bands appeared as a single band at the 20–30% sucrose interface. Fraction II, about 10% of the total membrane protein, was found at the 30–35% sucrose interface in either case. Only small amounts (less than 1%) of material were found at the 35–40% and 40–50% sucrose interfaces. The membrane fractions were recovered by forcing a 60% sucrose solution containing 5 mM Tris at pH 7.4 from the bottom of the tube and monitoring the effluent by scanning at 280 nm. The combined fractions were diluted to 0.250 M sucrose (5 mM Tris, pH 7.4) and the membrane was recovered by centrifugation at 25,000 rpm for 60 min in the SW 25.1 rotor. The yields of membranes, fractions Ia and Ib and fraction II, were about 20 and 1.5 mg, respectively, per g of wet nerve.

### *Electron Microscopy*

The whole nerve in small pieces and membrane fractions, as thin pellets in the centrifuge tubes, were fixed in the cold (0°–4°C) for 2 h in a fixative (15) which contains 3.6 ml of 70% glutaraldehyde, 2 g of paraformaldehyde, and 25 mg anhydrous calcium chloride in 50 ml cacodylate buffer, 0.1 M, pH 7.5. These were postfixed in 1.33% osmium tetroxide in 0.067 M *s*-collidine or 0.033 M cacodylate for 2 h, stained in 2% aqueous uranyl acetate for 2 h, dehydrated in a graded series of acetone (50, 70, and 100%) and propylene oxide, and finally embedded in Epon-Araldite. Thin sections were cut on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and were examined after staining with a saturated solution of uranyl acetate followed by lead citrate (16) in a Hitachi HU-11C electron microscope.

### *Enzyme Assays*

Na-K activated ATPase and NADH-ferricyanide oxidoreductase were assayed according to procedures of Wallach and Kamat (17, 18). The assays used for cytochrome *c* oxidase, monoamine oxidase, and lactate dehydrogenase were those of Wharton and Tzagoloff (19), Tabor et al. (20), and Kornberg (21), respectively. The procedure of Ochoa (22) modified by Caplan and Greenawalt (23) was used for malate dehydrogenase assay. The procedure used for assaying glucose-6-phosphatase was that of Hubscher and West (24); that for acetylcholinesterase was that of Ellman et al. (25), and that for 5'-nucleotidase was that of Widnell and Unkeles (26).

### Analytical Procedures

Protein concentrations were determined by the procedure of Lowry et al. (27) after solubilization with deoxycholate (0.16% final concentration) and heating on a steam bath for 30 s (28), using bovine serum albumin as the standard.

Lipids were extracted by the technique of Bligh and Dyer (29). Lipid phosphorus was determined by the micromethod of Rouser et al. (30), and cholesterol by the procedure of Zlatkis et al. (31). The phospholipid composition was determined by two-dimensional thin-layer chromatography followed by phosphorus analysis (30). Fatty acid methyl esters were prepared with the  $\text{BF}_3$ -methanol reagent, as described by Morrison and Smith (32). These were analyzed by gas-liquid chromatography after purification by preparative thin-layer chromatography. Details on the analytical procedures on lipids are given in an earlier paper on the lipid composition of garfish olfactory nerve (9).

Sodium dodecyl sulfate-polyacrylamide gel (SDS) electrophoresis was carried out at 5.6% polyacrylamide gel concentration in 1% SDS, 0.1 M Tris buffer, pH 7.4, according to Fairbanks et al. (33). The gels were 7 cm long and 0.5 cm diameter. The membranes are solubilized in a solution (about 2 mg protein containing

membrane in 1 ml) containing 1% SDS, 0.6% dithiothreitol, 10% sucrose, 1 mM EDTA, and 0.01% bromophenol blue in 0.01 M Tris, pH 8.0, followed by heating in a boiling water bath for 15 min (34). Approximately 30–40  $\mu\text{g}$  protein (in 15–20  $\mu\text{l}$  of solution) was used for each gel. The electrophoresis was carried out at a constant current of 4 mA per gel for about 2.5 h; the tracking dye, bromophenol blue, moved to about 1 cm from the bottom of the gel. Gels were removed from the tubes, the position of the tracking dye marked by India ink, and stained with Coomassie blue essentially according to Fairbanks et al. (33).

### RESULTS

#### *Morphology of the Nerve*

Fig. 2 illustrates a cross section of the garfish olfactory nerve at low magnification. The axons are separated only by extracellular space from one another in groups of several hundreds, and each group is surrounded by a single layer of Schwann cell cytoplasm and membrane. Thus, a high ratio of axonal to Schwann cell membrane is extant in this nerve. Mitochondria where present almost fill

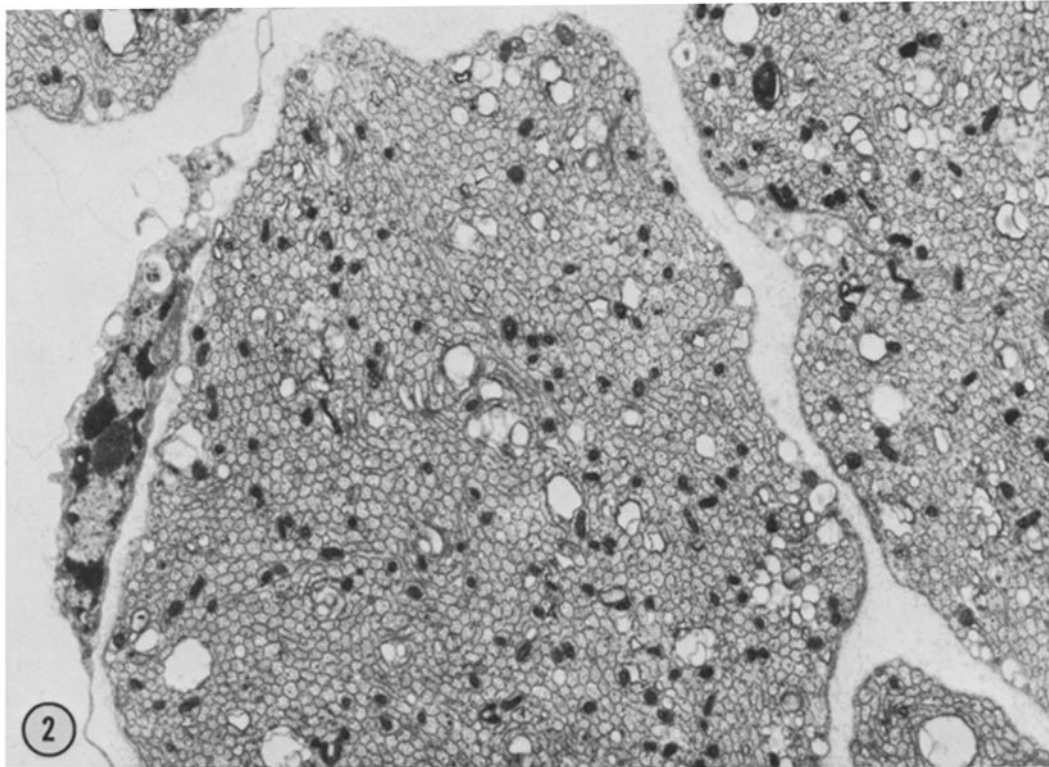


FIGURE 2 Electron micrograph of garfish olfactory nerve. Axons occur in bundles surrounded at the periphery by a single layer of Schwann cells. Swollen axons are probably a result of fixation.  $\times 9,000$ .

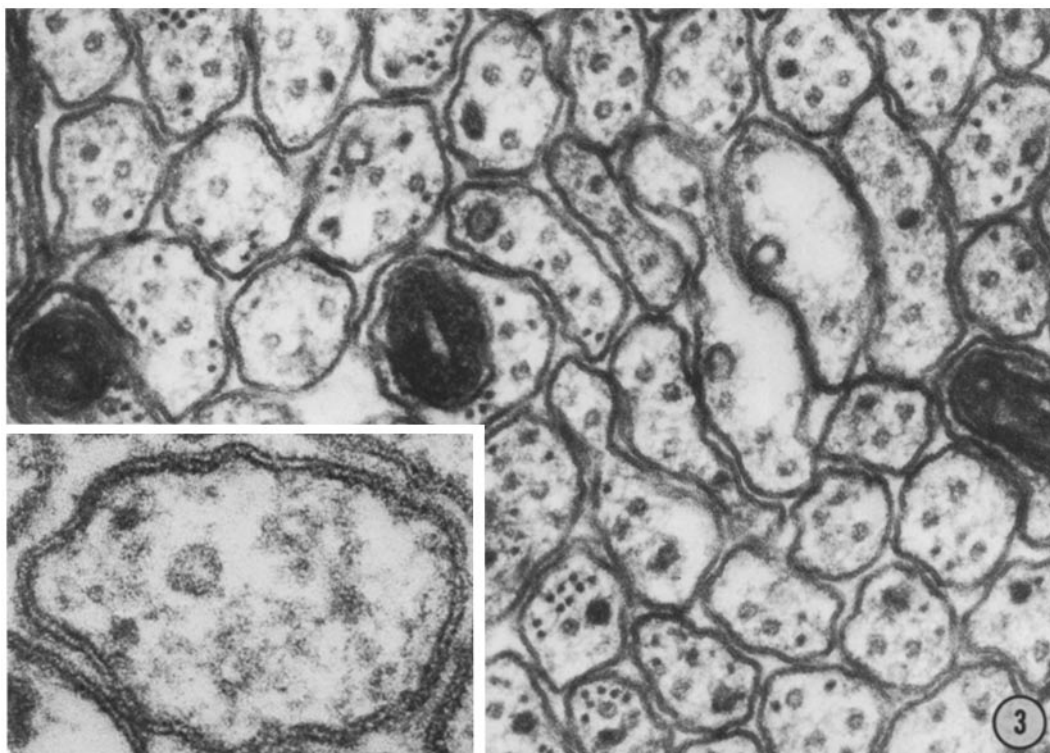


FIGURE 3 Electron micrograph of portion of a nerve bundle. Only extracellular space separates axons. Axons contain microtubules, microfilaments, and occasional mitochondria. Axolemma is triple layered and  $\sim 7.5$  nm in thickness.  $\times 85,000$ . *Inset*,  $\times 255,000$ .

the whole cross section of the axon (Fig. 3). From the observed concentration (1.2%) of cardiolipin in the total lipids of olfactory nerve (9) it can be estimated that the contribution of mitochondrial membranes to the total membrane of the nerve is about 6%, assuming that the cardiolipin content of the lipids of garfish olfactory nerve mitochondria is also about 20% (29) of the total phospholipid. Besides axon, Schwann cell, and mitochondria, the only other organelles seen in electron micrographs are microtubules and neurofilaments.

#### *Isolation of the Membrane Fractions*

The rationale (Fig. 1) used for isolation of axonal membrane from garfish olfactory nerve is to first separate the plasma membranes, axonal, and Schwann cell, from mitochondria and other cellular components by a two-density sucrose gradient system (2) and then to subject the plasma membrane preparation to the centrifugation in a multidensity gradient system to separate the axonal membrane from Schwann cell membrane. This second step also removes additional contami-

nants from the membrane preparations. Three major membrane fractions are obtained on centrifugation of the plasma membrane preparation on the multidensity gradient system (Fig. 1). From the results of enzymatic and compositional studies to be described, it appears that, of the three major membrane fractions, fractions Ia and Ib together constitute one type of membrane and fraction II another type of membrane. On a continuous sucrose density gradient between 20 and 50% sucrose, fractions Ia and Ib appear together as a single band at a density corresponding to about 23% sucrose, and fraction II appears as a single band at a density corresponding to about 33% sucrose (Fig. 4).

#### *Morphology of Membrane Fractions*

All three membrane fractions consisted of membrane-bounded vesicles which varied considerably in diameter and apparent purity. Fraction Ia (Fig. 5) is the most homogeneous as judged from electron micrographs of the three preparations. Most vesicles appear nearly empty, and little

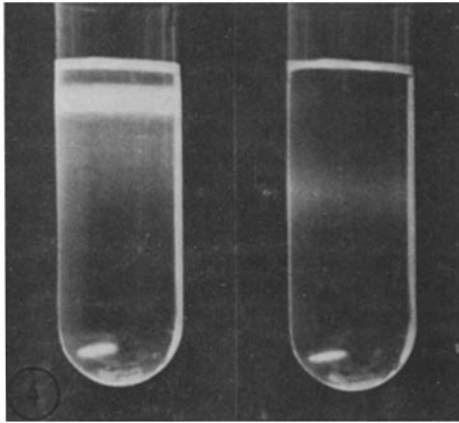


FIGURE 4 Centrifugation of fraction I (left) and fraction II (right) membranes on a linear sucrose density gradient. Gradient was made from 14 ml each of 50 and 20% sucrose solutions. 2 ml of membrane suspension in 0.25 M sucrose were placed over the gradient and centrifuged in a SW 25.1 rotor at 25,000 rpm for 4.5 h.

intervesicular material is present. Fraction Ib (Fig. 6) contains vesicles with some luminal contents which cannot be identified by structural criteria except for an occasional dense mitochondrion. Some dense material appears between vesicles. Fraction II (Fig. 7) appears even less homogeneous than Ib, with more intravesicular material and some amorphous intervesicular debris. In all cases the membranes of the vesicles appear trilaminar and have a thickness of  $\sim 7.5$  nm.

The additional contaminants in fraction Ib are probably responsible for the separation of Ia and Ib in the discontinuous gradient centrifugation (Fig. 1). The slightly higher protein to lipid ratio observed in Ib (Table III) is in agreement with this interpretation.

#### *Distribution of Protein and Enzyme Activities*

To assess the purity of the membrane fractions and to differentiate the axonal membrane from the

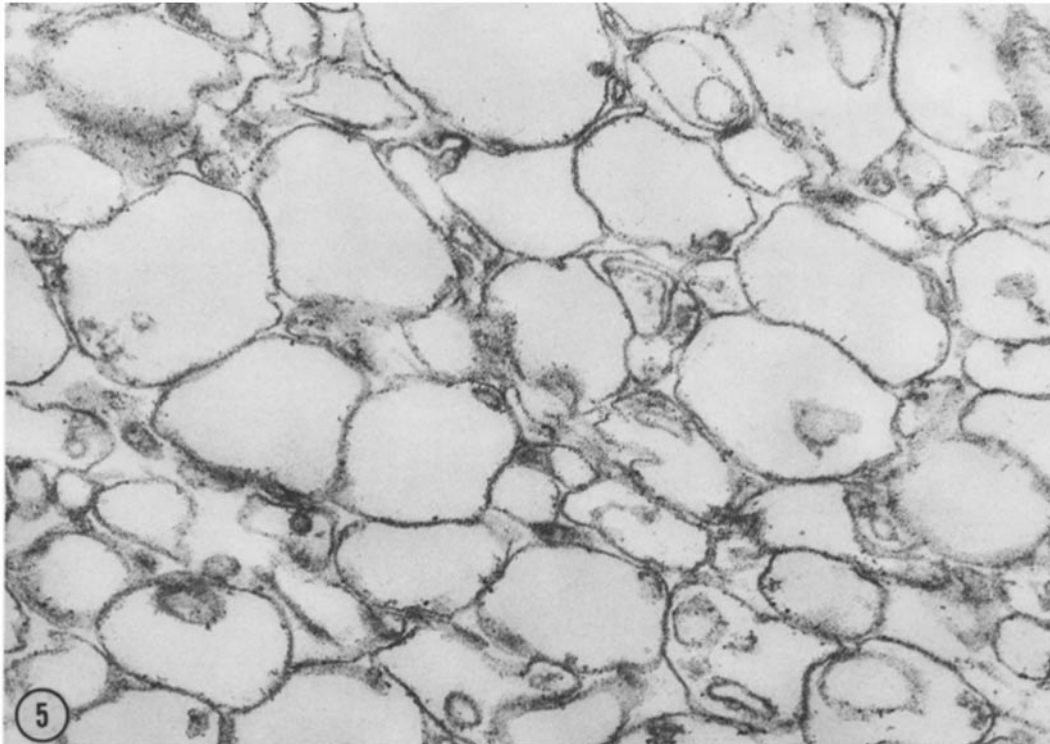


FIGURE 5 Electron micrograph of fraction Ia. Vesicles as large as  $0.3 \mu\text{m}$  are present and appear nearly empty. Little intervesicular material is present. Vesicular membranes are triple layered and  $\sim 7.5$  nm in thickness.  $\times 75,000$ .

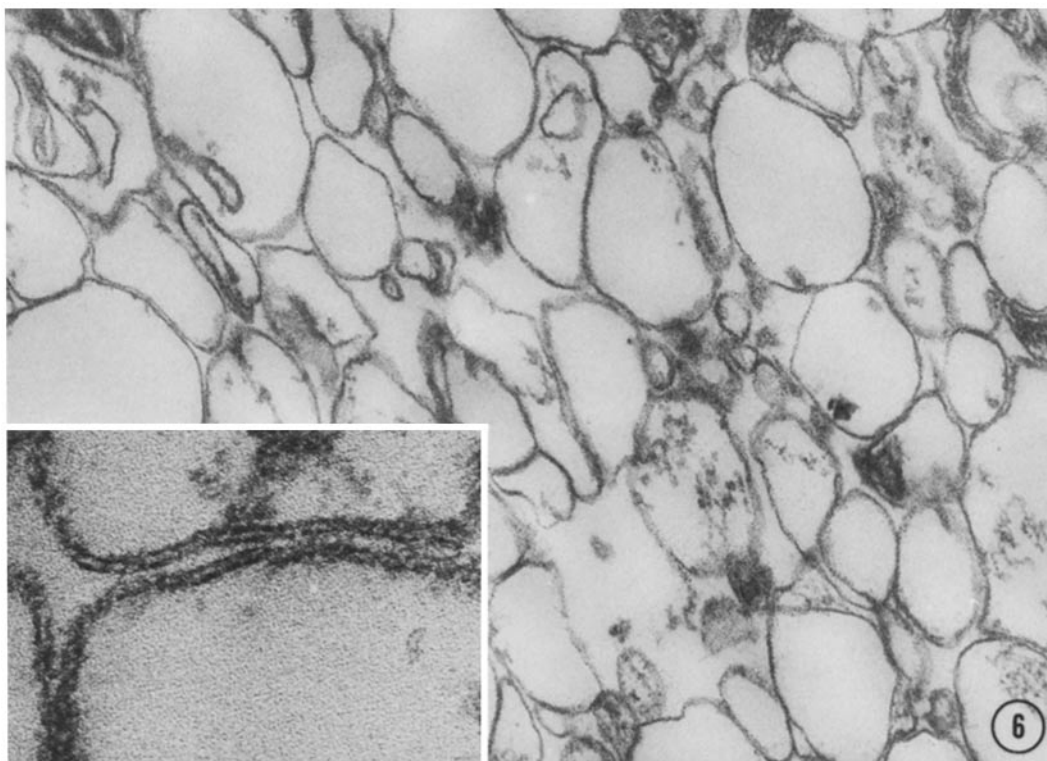


FIGURE 6 Electron micrograph of fraction Ib. Vesicles as large as  $0.5 \mu\text{m}$  are present and many contain a granular or fibrillar material. Little intervesicular material is present. Vesicular membranes are triple layered and  $\sim 7.5 \text{ nm}$  in thickness.  $\times 75,000$ . Inset,  $\times 300,000$ .

Schwann cell membrane, several membrane-specific enzymes were assayed in membrane fractions obtained at various stages of the isolation procedure. These results, together with the concentration of protein in each membrane fraction, are given in Table I. Together fractions I and II contain about 18% of the total protein of the nerve homogenate. Cytochrome *c* oxidase activity found in fractions I and II is about 4 and 6%, respectively, of the total nerve homogenate activity. Only a negligible amount of cytochrome *c* activity (0.2%) was found in membrane fraction Ia. Since, in garfish olfactory nerve, mitochondrial membrane probably accounts for about 6% of the total nerve membranes, and since the membrane fractions I and II contain only about 4 and 6%, respectively, of the total nerve homogenate cytochrome *c* oxidase activity, the mitochondrial membrane contamination in the membrane fractions, especially fraction I, is not significant. Malate dehydrogenase activity of the membrane fractions also indicated a small amount of mitochondrial

contamination, and monoamine oxidase activity could not be detected in the membrane fractions. Assay for lactate dehydrogenase did not show any contamination from axoplasmic contents. About half of the total nerve homogenate Na-K ATPase activity was recovered in the two membrane fractions. A fourfold increase in Na-K ATPase specific activity (Table II) was found in the fraction I membrane; however, no such increase was observed in fraction II. The ATPase specific activity was a little higher in the fraction Ia membrane than in the fraction Ib membrane. No specific enrichment was found for 5'-nucleotidase or NADH-ferricyanide oxidoreductase activities in the membrane fractions (Tables I and II).

#### *Chemical Composition*

The chemical composition of membrane fractions is given in Table III. The protein to lipid ratio is unusually low in both fractions Ia and Ib. The protein content is a little higher in fraction Ib than in Ia (34.1% vs. 25.8%). A much higher

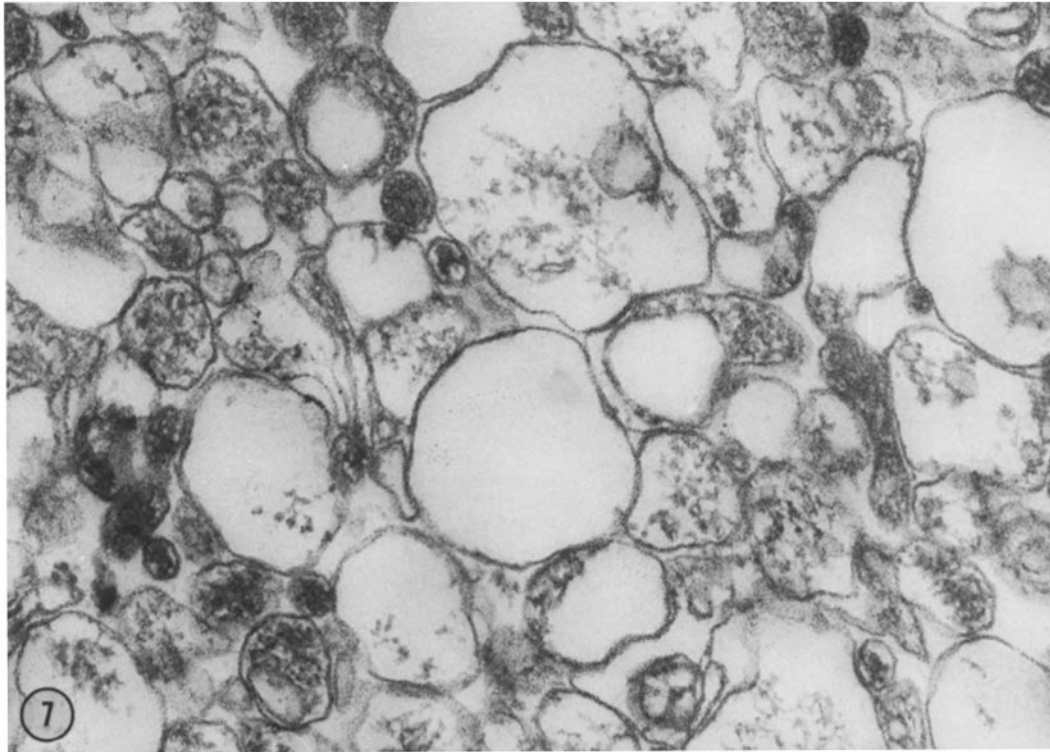


FIGURE 7 Electron micrograph of fraction II. Vesicles as large as  $0.5 \mu\text{m}$  are present and nearly all contain considerable granular or fibrillar material. Some intervesicular material is also present. Vesicular membranes are triple layered and  $\sim 7.5 \text{ nm}$  in thickness.  $\times 75,000$ .

TABLE I  
Distribution of Protein and Enzyme Activities Among Fractions Obtained in the Isolation of Axonal Membrane from Garfish Olfactory Nerve

	Protein	Na-K ATPase	NADH-Fe(CN) <sub>6</sub> oxidoreductase	5'-Nucleotidase	Cytochrome <i>c</i> oxidase	Malate dehydro- genase
	%	%	%	%	%	%
Total homogenate	100	100	100	100	100	100
0.25-1.195 M sucrose interface membrane	$22.4 \pm 2.0$ (5)	$59.2 \pm 5.0$ (5)	$21.7 \pm 1.5$ (5)	$32.9 \pm 4.0$ (3)	$16.1 \pm 4.0$ (5)	$5.9 \pm 1.1$ (5)
Residual fraction	$9.2 \pm 0.6$ (5)	$2.7 \pm 1.7$ (5)	$24.9 \pm 1.4$ (5)	$14.2 \pm 1.6$ (3)	$56.7 \pm 7.5$ (5)	$26.8 \pm 5.3$ (5)
Membrane fraction Ia	$4.4 \pm 1.1$ (5)	$14.8 \pm 1.8$ (5)	$2.4 \pm 0.2$ (5)	$4.8 \pm 1.6$ (3)	$0.2 \pm 0.1$ (5)	$0.1 \pm 0.04$ (5)
Membrane fraction Ib	$11.6 \pm 2.0$ (5)	$27.0 \pm 6.1$ (5)	$8.2 \pm 0.3$ (5)	$14.3 \pm 2.1$ (3)	$3.9 \pm 0.8$ (5)	$1.7 \pm 0.2$ (5)
Membrane fraction II	$2.2 \pm 0.2$ (5)	$2.6 \pm 0.9$ (5)	$3.6 \pm 0.3$ (5)	$2.4 \pm 0.3$ (3)	$6.2 \pm 1.5$ (5)	$2.0 \pm 0.4$ (5)
Supernate	$77.1 \pm 2.6$ (3)	$32.9 \pm 10.9$ (3)	$52.3 \pm 2.6$ (3)	$67.6 \pm 2.0$ (3)	$22.9 \pm 3.4$ (3)	$76.5 \pm 3.1$ (3)
Total recovery	104.5	80.0	91.4	103.2	89.9	107.1

Values given are mean  $\pm$  SEM. Number of experiments is shown in parentheses. Enzyme activities are represented as: Na-K ATPase, micromoles Pi per hour per milligram protein; NADH-Fe(CN)<sub>6</sub> oxidoreductase, nanomoles NADH oxidized per minute per milligram protein; 5'-nucleotidase, nanomoles Pi per minute per milligram protein; cytochrome *c* oxidase and malate dehydrogenase, as units per minute per milligram protein as defined in references 19 and 22.



TABLE II  
Specific Activities for Na-K ATPase, NADH-Fe(CN)<sub>6</sub> Oxidoreductase, and 5'-Nucleotidase  
in Membrane Fractions Isolated from Garfish Olfactory Nerve

	Protein, mg/g wet wt	Na-K ATPase, μmol Pi/h/mg protein	NADH-Fe(CN) <sub>6</sub> oxidoreductase, nmol/min/mg protein	5'-Nucleotidase, nmol Pi/min/mg protein
	%	%	%	%
Total homogenate	48.7 ± 2.2 (3)	1.7 ± 0.2 (5)	402 ± 32 (5)	18.8 ± 2.5 (3)
0.25-1.195 M sucrose interface	10.2 ± 0.3 (3)	4.1 ± 0.2 (5)	487 ± 60 (5)	25.8 ± 9.0 (3)
Fraction Ia	1.4 ± 0.5 (3)	6.7 ± 0.4 (5)	351 ± 22 (5)	34.4 ± 14.9 (3)
Fraction Ib	4.8 ± 0.7 (3)	4.6 ± 0.4 (5)	396 ± 37 (5)	25.6 ± 9.6 (3)
Fraction II	0.8 ± 0.2 (3)	1.4 ± 0.5 (5)	661 ± 62 (5)	17.8 ± 4.0 (3)
Residual fraction	4.4 ± 0.2 (3)	0.5 ± 0.2 (5)	1,194 ± 54 (5)	40.6 ± 9.0 (3)

Values given are mean ± SEM. Number of experiments is shown in parentheses.

TABLE III  
Chemical Composition of the Membrane Fractions Isolated from Garfish Olfactory Nerve

	Protein	Total lipid	Protein to lipid weight ratio	Phospholipid, % of total lipid	Cholesterol, % of total lipid	Phospholipid to cholesterol, molar ratio
	%	%				
Membrane frac- tion Ia	25.8 ± 1.8 (2)	74.2 ± 1.8 (2)	0.35 ± 0.03 (2)	74.2 ± 0.7 (5)	25.9 ± 0.7 (5)	1.42 ± 0.04 (5)
Membrane frac- tion Ib	34.1 ± 0.1 (3)	65.9 ± 0.1 (3)	0.51 ± 0.00 (3)	73.8 ± 0.3 (5)	26.2 ± 0.3 (5)	1.41 ± 0.02 (5)
Membrane frac- tion II	54.7 ± 1.2 (3)	45.3 ± 1.2 (3)	1.21 ± 0.05 (3)	80.7 ± 1.9 (4)	19.3 ± 1.9 (4)	2.17 ± 0.27 (4)

Values given are mean ± SEM. Number of experiments is shown in parentheses.

concentration of protein (54.7%) is found for fraction II. Of the lipids in both fractions Ia and Ib, about 75% are phospholipids and about 25% are cholesterol. A somewhat higher phospholipid to cholesterol ratio is found in fraction II. The phospholipid composition, expressed as the percentage distribution of phosphorus in the lipid components of the various membrane fractions, is given in Table IV. The phospholipid compositions of fractions Ia and Ib membranes are identical and similar to that of the whole nerve (9), with the exception of cardiolipin which is present only in negligible amounts. Thus, the major phospholipid components in membrane fraction I are phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and sphingomyelin. About 4% of the total lipid phosphorus is found in phosphatidylinositol. Membrane fraction II also has a

phospholipid composition similar to that of fraction I but the concentration of cardiolipin is high (3.1%) probably due to contamination by mitochondrial membranes, which agrees with the results of enzyme assays (Table I). As would be expected (35), the residual fraction which is rich in mitochondria has a high concentration (9.4%) of cardiolipin and low concentrations of phosphatidylserine (3.4%) and sphingomyelin (4.3%).

The percentage distributions of fatty acids in the lipids of fractions Ia and Ib membranes are similar and resemble that of the whole nerve lipids (Table V). This is characterized by the presence of long-chain polyunsaturated fatty acids, namely arachidonic and docosahexaenoic acids, in relatively high concentrations. The lipids of the residual fraction have a fatty acid composition similar to that of the whole nerve and the fraction I

TABLE IV  
*Phospholipid Composition of the Membrane Fractions Isolated from Garfish Olfactory Nerve*

Components	Fraction I	Fraction II	Residual fraction	Whole nerve*
Phosphatidylethanolamine	33.6 ± 0.7 (6)	34.0 ± 0.1 (2)	37.3 ± 0.9 (3)	32.3
Phosphatidylcholine	42.2 ± 0.5 (6)	42.2 ± 1.2 (2)	42.3 ± 0.6 (3)	41.3
Phosphatidylserine	12.0 ± 0.2 (6)	9.2 ± 0.4 (2)	3.7 ± 0.5 (3)	11.5
Phosphatidylinositol	4.0 ± 0.3 (6)	3.6 ± 0.2 (2)	2.7 ± 0.7 (3)	4.3
Sphingomyelin	7.5 ± 0.3 (6)	6.7 ± 0.1 (2)	4.3 ± 0.5 (3)	9.2
Phosphatidic acid	Trace	Trace	0.2 ± 0.1 (3)	0.2
Diphosphatidyl glycerol	Trace	3.1 ± 0.1 (2)	9.4 ± 0.8	1.2

Fraction I refers to membrane fractions Ia and Ib together obtained on separation of the total plasma membrane preparation on the multi-density sucrose gradient system as shown in Fig. 1. Fractions Ia and Ib were analyzed separately and were found to have similar phospholipid composition.

Values given are mean ± SEM. Phospholipid composition is expressed as the percentage of total lipid phosphorus.

\* Values taken from reference 9.

TABLE V  
*Composition of the Major Fatty Acids\* of the Total Lipids of the Membrane Fractions*

Fatty acid	Whole nerve	Fraction I	Fraction II	Residual fraction
16:0	28	27	25	24
18:0	15	18	14	15
18:1	11	11	9	11
22:1/20:3?‡	5	5	9	6
20:4‡	11	10	6	11
22:6	31	29	37	33

The values are expressed as the gas-chromatographic peak-area percent. Fraction I refers to membrane fractions Ia and Ib together obtained on separation of the total plasma membrane preparation on the multidensity sucrose gradient system as shown in Fig. 1. Fractions Ia and Ib were analyzed separately and were found to have similar fatty acid composition.

\* Only the major fatty acids are given. For complete fatty acid composition of the garfish nerve lipids, see reference 9. The relative concentration of the fatty acids, especially 22:6, 20:4, and 22:1/20:3, appears to vary depending on the natural source from where they are obtained; however, the phospholipid composition is found to be constant.

‡ Since the separation of 20:4 and 22:1/20:3 fatty acids from one another was not complete in our gas-chromatographic analysis, the concentrations of these fatty acids given are only approximate, but the results clearly show a much higher ratio of 20:4 to 22:1/20:3 fatty acids in the lipids of fraction I (and whole nerve and crude mitochondrial) membrane lipids than in the lipids of fraction II membrane lipids.

membrane. The fraction II membrane lipids have a higher concentration of docosahexaenoic and docosamonoenoic acids and a lower concentration of arachidonic acid than fraction I. This difference in the fatty acid composition of total lipids also suggests that fraction I and fraction II are different types of membrane. The results of the SDS-polyacrylamide gel electrophoresis (Fig. 8) also indicate a difference between the fraction I and II membranes and, at the same time, a similarity between fractions Ia and Ib. The polypeptide patterns of both fractions Ia and Ib are similar and are composed of numerous polypeptides with a wide range of molecular weight (22,000–240,000 daltons, estimated by comparing the mobilities with those of a mixture of standard proteins). However, a different, less numerous, polypeptide pattern is found for fraction II membrane.

## DISCUSSION

We have isolated three major plasma membrane fractions from garfish olfactory nerve (Fig. 1) using a relatively mild isolation procedure which consists essentially of homogenization of the nerve in buffered 0.25 M sucrose followed by repeated centrifugation, first, over a two-density sucrose gradient and, then, over a multidensity gradient. Two of these membrane fractions (Ia and Ib) are essentially free from mitochondrial membranes and axoplasmic contents as judged from the results

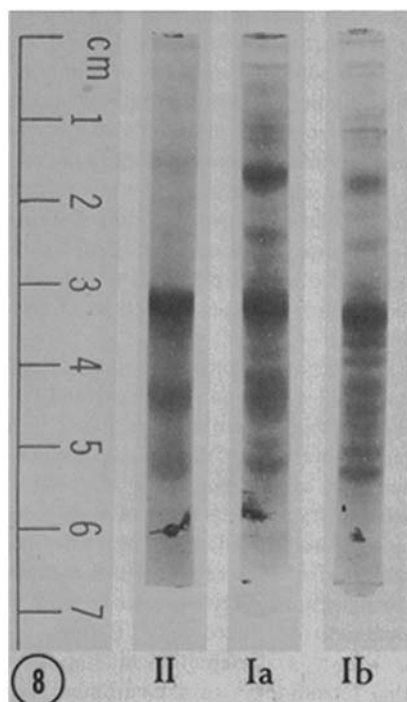


FIGURE 8 Comparison of the gel patterns of membrane proteins of fractions Ia, Ib, and II, stained with Coomassie blue.

of assays for marker enzymes and electron micrographs. The third fraction (II) is contaminated with mitochondrial membranes and exhibits a maximum of 6% of cytochrome *c* oxidase activity. The results of marker enzymes studies (Table II) and the results of compositional studies (Table III) strongly suggest that the fractions Ia and Ib membranes together constitute one type of membrane and fraction II another type of membrane. Thus, fractions Ia and Ib have very similar Na-K ATPase activity which differs from that of fraction II (Table II). Both fractions Ia and Ib have more or less similar lipid to protein ratios, although the lipid content of fraction Ia is slightly higher than that of fraction Ib (Table III). They have an identical phospholipid to cholesterol ratio (Table III), phospholipid composition (Table IV), fatty acid composition (Table V), and similar polypeptide patterns on SDS-polyacrylamide gel electrophoresis (Fig. 8). Membrane fraction II, however, has a much different lipid to protein ratio, fatty acid composition, polypeptide pattern, and somewhat higher phospholipid to cholesterol ratio.

Furthermore, with centrifugation on a continuous sucrose density gradient between 20 and 50% sucrose, fractions Ia and Ib appear as a single band and fraction II another single band. A mixture of fraction I and II will also separate into corresponding bands on a continuous gradient (Fig. 4).

The possibility that fraction II is of mitochondrial origin, especially outer mitochondrial membrane, is clear; however, several lines of evidence suggest that it is not. Fraction II is obtained in much higher concentration (about 10% of membrane protein) than would be expected from a mitochondrial source, since in garfish olfactory nerve the latter accounts for less than 10% of the total membrane. Further, a maximum of only 6% of the total mitochondrial enzyme activity was found in the membrane fraction. No monoamine oxidase activity could be detected in fraction II. The phospholipid composition, the protein to lipid ratio, and the phospholipid to cholesterol ratio are different from those of mitochondrial membranes (35). Furthermore, the total fatty acid pattern (Table V) of the lipids of fraction II is different from that of the mitochondrial-rich residual fraction.

Taking into consideration the relative concentrations of the axonal and the Schwann cell membranes in the nerve and their Na-K ATPase activities, fraction I is likely of axonal origin and fraction II of Schwann cell membrane. These assignments are in general agreement with the results of Fischer et al. (3, 4) and Camejo et al. (2) on squid nerve membranes with respect to density properties and the ATPase activities of the two membrane fractions concerned. In the discussion to follow, fraction I will be referred to as axonal membrane and fraction II as Schwann cell membranes.

The separation of fraction I into two subfractions on discontinuous gradient in the initial stage of centrifugation (Fig. 1) may be due to either the small difference in the concentration of protein resulting from loss of membrane-associated protein in the case of Ia or entrapment of axoplasmic content or microtubular protein in the case of Ib. Preliminary studies on the polypeptide pattern of the two membrane subfractions by SDS-polyacrylamide gel electrophoresis (Fig. 8) have not shown any indication of such a loss or entrapment of a particular component. The appearance of two subfractions during the isolation of plasma membranes from neuron (36) and bovine corpus luteum

(37) by centrifugation on discontinuous sucrose gradient has been reported.

A major characteristic property of the axonal membrane of garfish olfactory nerve, as compared with other types of plasma membranes (38), appears to be a low concentration of protein (25.8–34.1%) in the membrane (Table III), and in this respect this membrane resembles myelin more than other plasma membranes. A similar low protein content was reported for the axonal membrane preparations from squid stellar nerve (29.5%) (2) and from lobster walking leg nerve (24.0%) (6).

The composition of the lipids of garfish olfactory nerve axonal membrane appears to be relatively simple. The only neutral lipid present in the membrane is cholesterol. The fatty acid and the lysophospholipids found in the axonal membrane preparations from squid stellar nerve and retinal nerve (2, 39) may be formed by the degradation of membrane phospholipids during the isolation of membrane. The glycolipid which was found in the garfish whole olfactory nerve (9) was not detected in the axonal membrane. The phospholipid to cholesterol ratio is very similar to that of the squid nerve axonal membranes. The absence of phosphatidylinositol in squid nerve membrane preparations (39) may be due to incomplete separation of components in the chromatographic system used for separation of the phospholipids. As was found for squid stellar nerve membranes (2), both the axonal and the Schwann cell membrane fractions have similar phospholipid composition but there is a difference in the fatty acid composition of the total lipids of the two membrane fractions. Lipids of both membrane fractions are rich in long-chain polyunsaturated fatty acids, but a smaller concentration of arachidonic acid is found in the Schwann cell membrane lipids. The presence of a high concentration of polyunsaturated fatty acids has also been reported in the axonal membrane preparation from squid retinal nerve (39).

The axonal membrane from garfish olfactory nerve is obtained in high yield (more than 40%), on the basis of the recovery of Na-K ATPase. The contamination of the membrane preparation by other possible membrane types, namely mitochondrial and Schwann cell membranes, appears to be very small.

X-ray diffraction studies by Blasie et al. (40) indicate that the electron density profile at 12 Å resolution of fraction I membrane (fraction II was

not studied) corresponds closely to that obtained on whole garfish olfactory nerve. Also, comparative diffraction measurements on whole nerve, on the membrane fraction, and on lipid bilayer vesicles made from the total lipids of the membrane suggest an arrangement of lipids in the membrane as a bilayer with a thin protein layer occurring in the lipid polar head group region. Because of the preponderance of axonal membrane in the nerve, the pattern observed on the whole nerve may be considered to be essentially that of the axonal membrane.

It should be emphasized that the identification of fraction I as axonal membrane and fraction II as Schwann cell membrane is based on indirect evidence obtained in the present studies and by comparison of the properties of garfish plasma membrane fractions to those of squid nerve (2–4) and lobster nerve (6). Unequivocal identification of membrane fractions requires further studies such as the detection of tetrodotoxin binding sites and immunoelectron microscopy. Barnola et al. (6) have shown a preferential binding of tritium-labeled tetrodotoxin to a membrane preparation isolated from lobster nerve. Further studies are also needed to explain why the fraction I membrane separates into two subclasses, Ia and Ib, with a small but significant difference in protein to lipid ratio. It is possible that the delay of about 8 h between the sacrifice of the animal and the isolation of the membrane material produces a loss of some membrane protein.

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