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CHARACTERIZATION OF TWO DIFFERENT MEMBRANE FRACTIONS ISOLATED FROM THE FIRST STELLAR NERVES OF THE SQUID *DOSIDICUS GIGAS*

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SUMMARY

1. Two membrane fractions were isolated from homogenates of the first stellar nerve of *Dosidicus gigas* by a sequence of differential, discontinuous and gradient centrifugations in sucrose solutions. Membrane Fraction I has an apparent density of 1.090 g/ml and membrane Fraction II a value of 1.140 g/ml.

2. At low and high magnifications in the electron microscope, both fractions appear as rounded membrane profiles similar to the plasma membrane observed in the unfractionated tissue. No other subcellular component was observed. Fraction I has a thickness of 95–110 Å and that of Fraction II is 72–100 Å.

3. The percentage lipid composition of the two membranes was established. The following lipid classes were tentatively identified: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, cholesterol, free fatty acids and the hydrocarbon *n*-pentacosane. The relative distribution of the fatty acids present in the phospholipids of the two membrane fractions was different.

4. The morphological appearance, the yield of the two membranes and the distribution of (Na^+-K^+)-dependent, ouabain-sensitive ATPase has led us tentatively to relate Fraction I with the axolemma and Fraction II with the Schwann cell plasma membrane.

INTRODUCTION

The excitable membrane of the squid nerve axon has been a preferred subject for biophysists and electrophysiologists for many years[1]. The experimental results obtained with this preparation forms a large portion of the body of evidence on which are based the theories proposed to explain the excitability and conduction of nerves. Morphological studies show that nerve fibers as used in neurophysiological experiments are made of axons wrapped by the Schwann cells and, depending on the dissecting technique, a layer of connective tissue of variable thickness[2]. The multicellular arrangement on nerve fibers introduces difficulties into the interpretation of excitability and conduction in terms of the chemical constituents of the excitable membrane.

To accomplish this interpretation a direct knowledge of the chemical composition of the excitable membrane is necessary, and obviously the first step for achieving this goal is the isolation of the excitable membranes from nerve fibers.

Attempts to characterize the components of the axon membrane have been carried out using the "axon envelopes" obtained by extruding the axoplasm from nerve fibers. It is assumed that what remains as the envelope after this procedure are the Schwann cell and the axolemma.

CONDREA AND ROSENBERG[3] have studied the phospholipids present in these envelopes. However, in order to evaluate directly the chemical nature of the axolemma, isolation of the different membrane fractions of nerve fibers is required. FISHER *et al.*[4] have recently reported work in this direction using the optic nerves of the squid *Dosidicus gigas*. From our laboratory preliminary notes on the composition of membrane fractions isolated from the first stellar nerve of the same animal have been published[5,6]. The present paper is a detailed account of the methodology used to prepare these membranes and of their morphological and chemical properties.

METHODS

Membrane isolation

The first stellar nerve of giant squids (*D. gigas*), captured in the Peruvian Pacific, were dissected from the living animal, cleaned of most adjacent connective tissue, packed into polyethylene bags and placed in glass vacuum bottles with dry ice. The containers were air-mailed from Peru to Venezuela and the tissue stored frozen at $-15°$ until used.

40 g of frozen nerve were processed each time. The tissue was allowed to thaw in 200 ml of 0.33 M sucrose prepared in 5 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM $MgSO_4$ (Sucrose A). The nerves were placed in a Buchner funnel without filter paper and the sucrose solution allowed to drain. The tissue was washed twice more with 200-ml portions of Sucrose A, then 120 ml of Sucrose A were added and the tissue minced with a Virtiss homogenizer with stainless steel blades, at 16000 rev./min for 2 min. The minced tissue was then homogenized with a Teflon pestle homogenizer (clearance: 0.15–0.22 mm) attached to an electrically driven motor at approx. 500 rev./min. Ten up-and-down strokes were used to homogenize each portion. The homogenate was placed in tubes of the Beckman rotor No. 30 and centrifuged for 30 min at $70000 \times g$ at 2° (the relative centrifugal force is referred to the centre of the tubes). The supernatant was discarded and the white compact pellet suspended in 90 ml of Sucrose A. The suspension was homogenized with the same Teflon–glass homogenizer, this time using three strokes. Small portions of this fraction, labeled P-1, were stored for enzymatic analysis and light and electron microscopy studies. Portions of 30 ml of the same preparation (P-1) were carefully layered on 30 ml of 1.195 M sucrose prepared in the same buffer as Sucrose A. The upper layer of this discontinuous gradient had a density of 1.045 g/ml and the lower one 1.140 g/ml. The tubes containing the gradient were centrifuged at $74000 \times g$ for 60 min at 2° in the Beckman rotor SW 25.2. The well-defined white band at the interface was collected. The supernatant and infranatant as well as the heavy pellet at the bottom were discarded. The collected fractions consisting of the interfacial

bands, and with a total volume of 30 ml, were diluted to 90 ml with 2 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM $MgSO_4$ (Buffer I).

Refractive index measurements of the diluted suspension showed that after this 3-fold dilution its density corresponded closely to that of 0.33 M sucrose (1.045 g/ml). Portions of 30 ml of the diluted suspensions were relayered on top of 1.195 M sucrose and this discontinuous gradient centrifuged in the SW-25.2 rotor at 74000 × *g* for 60 min at 2°. The sharp white band at the interface was collected and the regions above and below it discarded. No pellet is observed in this step. The white interfacial suspension was diluted 3-fold with Buffer I, distributed in tubes for the No. 30 rotor and centrifuged at 70000 × *g* for 30 min at 2°. The supernatant was discarded, the white pellet collected in 6 ml of Buffer I and homogenized by hand with a Teflon pestle homogenizer with a clearance of 0.10–0.15 mm. 2-ml aliquots of this suspension were placed on top of SW-25.2 rotor tubes containing a linear gradient from 0.66 to 1.195 M sucrose prepared in Buffer I. The tubes were centrifuged at 74000 × *g* during 12 h at 2°. After centrifugation, two well-defined white zones at regions corresponding to densities of 1.090 and 1.140 g/ml were observed. The lighter band was labeled Fraction I (Membrane I) and the heavier Fraction II (Membrane II). These fractions were harvested by puncturing the bottom of the tube and collecting 3-ml aliquots. 0.1-ml portions of the 20 fractions collected from each tube were diluted to 1 ml with water and analyzed for absorption at 220 and 280 mμ. Fractions I and II exhibiting a strong absorption at these wavelengths were collected, diluted 3-fold with Buffer I and centrifuged for 30 min at 70000 × *g* at 2°. Fractions I and II, collected as white pellets, were suspended in 36 ml of Buffer I and centrifuged at 70000 × *g* at 2° for 15 min. The pellets of the purified membrane fractions were resuspended in Buffer I, usually 1 ml for Fraction I, and 2 ml for Fraction II. For electron microscopy, portions of the pellets were incubated overnight in artificial sea water. The whole of the fractionation procedure was carried out at approx. 4°.

Electron microscopy

Pellets corresponding to Fractions I and II were fixed by immersion for 18 h in ice-cold artificial sea water containing 0.6 % $KMnO_4$ and buffered at pH 8.0–8.1 with veronal acetate. After dehydration in an ethanol series, the pellets were embedded in Epon. Fine sections obtained in a LKB ultramicrotome were placed on Formvar-coated copper grids, double-stained with uranyl acetate[7] and lead citrate[8] and examined in a Siemens Elmiskop IA electron microscope.

Lipid extraction

1-ml portions of the membrane suspensions were extracted using the procedure of FOLCH *et al.*[9]. The washed total lipid extracts were evaporated under reduced pressure, dissolved in 1 ml of chloroform and stored in amber-colored bottles with Teflon-lined screw-caps. Lipid solutions were kept at −15° until analyzed. Dry weight determinations of the extracts were performed by placing aliquots in aluminium pans, evaporating the solvent at approx. 60° and weighing the pans in a Cahn RG recording balance with a sensitivity of 0.004 mg.

Lipid fractionation and analysis

Total lipid extracts were fractionated by thin-layer chromatography. 1–8 mg of lipids were applied to silica gel H plates (Merck-Darmstadt) and the plates developed

with light petroleum (b.p. 60–70°)–ethyl ether–acetic acid (85:15:1, by vol.). The different bands containing polar lipids (origin) and the nonpolar lipids were visualized by spraying the plates with water. Appropriate standards were run in each plate in order to identify the bands. Wet zones of the silica gel, containing polar lipids, cholesterol, fatty acids and hydrocarbons, were scrapped off the plate and quantitatively transferred to micro-columns the bottoms of which were plugged with glass wool. The nonpolar lipids were eluted with at least twenty bed volumes of chloroform–methanol (1:1, by vol.). In order to elute the polar lipids, two bed volumes of methanol were passed through the columns after the chloroform–methanol. The proportion of chloroform–methanol in the eluates was adjusted to 1:1, and 0.2 vol. of water was added. The tubes containing the eluates were shaken and centrifuged for 5 min at 2000 rev./min. The upper phase containing any residual silica gel was discarded, and the chloroform phase containing the lipids was evaporated to dryness under reduced pressure. The dry lipid residues were dissolved in 0.5 ml of chloroform and the dry weight measured as previously described. The homogeneity of the fractions isolated was checked by analytical thin-layer chromatography. The polar lipids were fractionated by thin-layer chromatography in two dimensions using the solvent systems of CONDREA *et al.*[10], and ABRAMSON AND BLECHER[11]. The proportion of the different phospholipids was established by phosphorus determinations[12], after $HClO_4$ digestion of the silica gel containing the lipids as visualized by exposure to I_2 vapors. The tentative identity of the different phospholipids was obtained by comparing their mobility in one- and two-dimensional thin-layer chromatography against that of standard lipids purchased from Applied Science Laboratories (Pa., U.S.A.) and Supelco (Pa., U.S.A.). The phosphatide spray of DITMER AND LESTER[13], and ninhydrin (0.5 % in methanol) were used as aids in this identification. The following anthrone reagent for glycolipids was found very specific and sensitive: 10 ml of 2 % anthrone in concentrated H_2SO_4, 20 ml of absolute ethanol, 2 ml of *o*-phosphoric acid and 1 ml of water. The plates were sprayed and heated at 100°; in 15 min, glycolipids show as blue-green spots. Cholesterol was identified by its chromatographic behaviour and reaction with specific reagents[14]. Fatty acids were identified by their chromatographic mobility in thin-layer chromatography and by that of their methyl esters in thin-layer and gas–liquid chromatography with the use of appropriate standards. The identity of the hydrocarbon present in the membrane lipid extracts was also tentatively established by gas–liquid and thin-layer chromatography.

Gas–liquid chromatography

Hydrocarbons. A Varian-Aereograph 2100-40 chromatograph was used equipped with flame ionization detectors. The stainless steel columns (150 cm × 0.47 cm internal diameter) were packed with 5 % SE-30, 70/80 mesh. A temperature program from 160 to 325° was used with a program rate of 6° per min. N_2 was used as carrier gas with a flow rate of 50 ml/min.

Fatty acid methyl esters. Chromatography of these compounds was carried out in glass columns (150 cm × 0.32 cm) packed either with 12 % HI-EFF-2BP in acid-washed gas-chrom W 100/120 mesh, or with HI-EFF-8BP in gas-chrom Q (Applied Science). The temperature was kept at 180° and the carrier gas (N_2) flow was 40 ml/min. Preparation of methyl esters for chromatography: Free fatty acids

and phospholipids (0.5–5.0 mg), separated by one-dimensional thin-layer chromatography, were visualized under ultraviolet light after spraying the plates with fluorescein. The silica gel containing the spots was scraped off and transferred to tubes with Teflon-lined screw-caps. 5 ml of methanol and 0.25 ml of concentrated H_2SO_4 were added and the capped tubes placed in an oven at 100° for 3 h. The reaction was interrupted by addition of 0.5 ml. of water, and the solutions were extracted with 0.5 ml of hexane which was then washed twice with distilled water. Aliquots of the hexane extract were examined by thin-layer chromatography and only fatty acid methyl esters were observed.

Analytical procedures

Protein was measured by the method of LOWRY *et al.*[15], and ATPases (EC 3.6.1.3) according to the following procedure: 0.05 ml of tissue fraction were preincubated for 10 min at 37° with 1.0 ml 1 M imidazole–HCl (pH 7.4), 0.05 ml 0.1 M $MgSO_4$, 0.1 ml 0.1 M KCl, 0.1 ml 0.1 M NaCl and 0.58 ml water. In other samples 0.5 ml of water was substituted for 0.5 ml of 2 mM ouabain. A third incubation mixture contained neither NaCl nor KCl but 0.78 ml of water. After preincubation, 0.02 ml of 0.1 M Tris–ATP (Sigma, Mo., U.S.A.) were added to all samples including a blank without tissue fraction. All samples were incubated at 37° for 30 min. The reaction was stopped by adding 1 ml of 6 % $HClO_4$, the precipitated protein centrifuged down and 0.5-ml aliquots of the supernatant used for measurements of liberated phosphate[16]. The activity of "diaphorases" (E.C. 1.6.99) was measured by the absorbance changes at 340 mμ of a mixture containing 0.4 ml of 0.02 M Tris–HCl buffer (pH 7.4), 0.4 ml of NADH (0.2 mg/ml), 0.2 ml of 0.0033 M $K_3Fe(CN)_6$ and 0.05 ml of the tissue fraction[17]. The RNA content of the membrane fractions was estimated according to the following procedure: 0.2 ml of membrane suspension, containing 300–500 μg of protein, was treated with 2.5 ml of methanol. After 30 min at 0°, the precipitate containing proteins and nucleic acids was collected by centrifugation and 1.0 ml of 0.5 M $HClO_4$ was added. The suspension was incubated at 37° for 3 h, centrifuged and the spectrum of the supernatant established.

The lipid-free precipitated protein present in the upper phase of Folch's extracts was used to measure the amount of reducing carbohydrates in the protein moiety of the membrane fractions. The upper phase was evaporated to dryness and washed with methanol–water (1:1, by vol.) until no reducing sugars could be detected in concentrates of the washings. The precipitated proteins, collected by centrifugation, were suspended by sonication into 0.5 ml of water and aliquots used for quantitative determinations[18] using as standard an equimolar mixture of glucose, mannose and galactose.

RESULTS

Isolation procedure

The used sequence of differential centrifugations and discontinuous gradients yielded a membrane fraction that was easily separated into two components by centrifugation in a linear sucrose gradient (Fig. 1). The differences in density that allow the fractionation are caused by the dissimilar protein to lipid ratios in Fraction I and II. Three factors were found critical in order to obtain good yields and sharp

separation of the fractions: the pH of the sucrose solutions, the type of homogenizer used and the extent of homogenization. With the conditions described, the average yield was 4 mg of Membrane I and 15 mg of Membrane II (protein + lipid) per g of nerve protein present in the first homogenate.

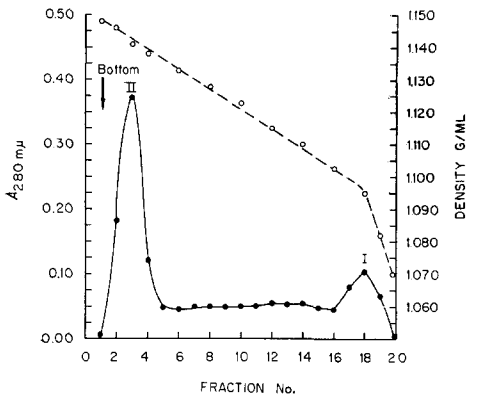

Fig. 1. Separation of Membrane I and II obtained after 12 h centrifugation at 74000 × *g* in a linear sucrose gradient from 0.66 to 1.195 M. The sharp fall of density after Fraction 18 is caused by dilution of the gradient with the buffer in which the membranes are initially suspended.

Ultrastructure

At low magnification, both fractions appear to be composed exclusively of rounded membrane profiles. Other cell components such as mitochondria, multivesicular bodies and other organelles were not observed (Figs. 2 and 3).

At higher magnification, the structures observed in Fraction I consist of single membranes, 95–110 Å thick, showing the characteristic trilaminar pattern (Fig. 4) and also, in certain regions, a globular arrangement (Fig. 5) as described before in the intact squid giant fiber[2]. Fraction II membranes appear clean at both their surfaces and form rounded single-walled profiles of variable sizes. A small amount of a medium-dense material could be observed sometimes attached to the membrane surfaces. Often this material forms an intervening dense layer between two apposed membranes (Fig. 4). The membranes of Fraction I also tend to form multilaminated structures like myelin figures (Fig. 5).

The membranes observed in Fraction II are 72–100 Å thick and also show the alternated trilaminar and globular substructures (Fig. 6). The membranes form single-walled rounded profiles which are more numerous and with smaller average diameters than those forming Fraction I (Fig. 3). Also, a larger amount of the medium-dense, non-homogenous material appears attached to both membrane surfaces, being more abundant at one of the surfaces. Two adjacent membranes may coalesce and form a five-layered component. However, more frequently the attached dense material is interposed between both adjacent membranes as a dense layer separating them (Fig. 6): myelin-like structures were not observed.

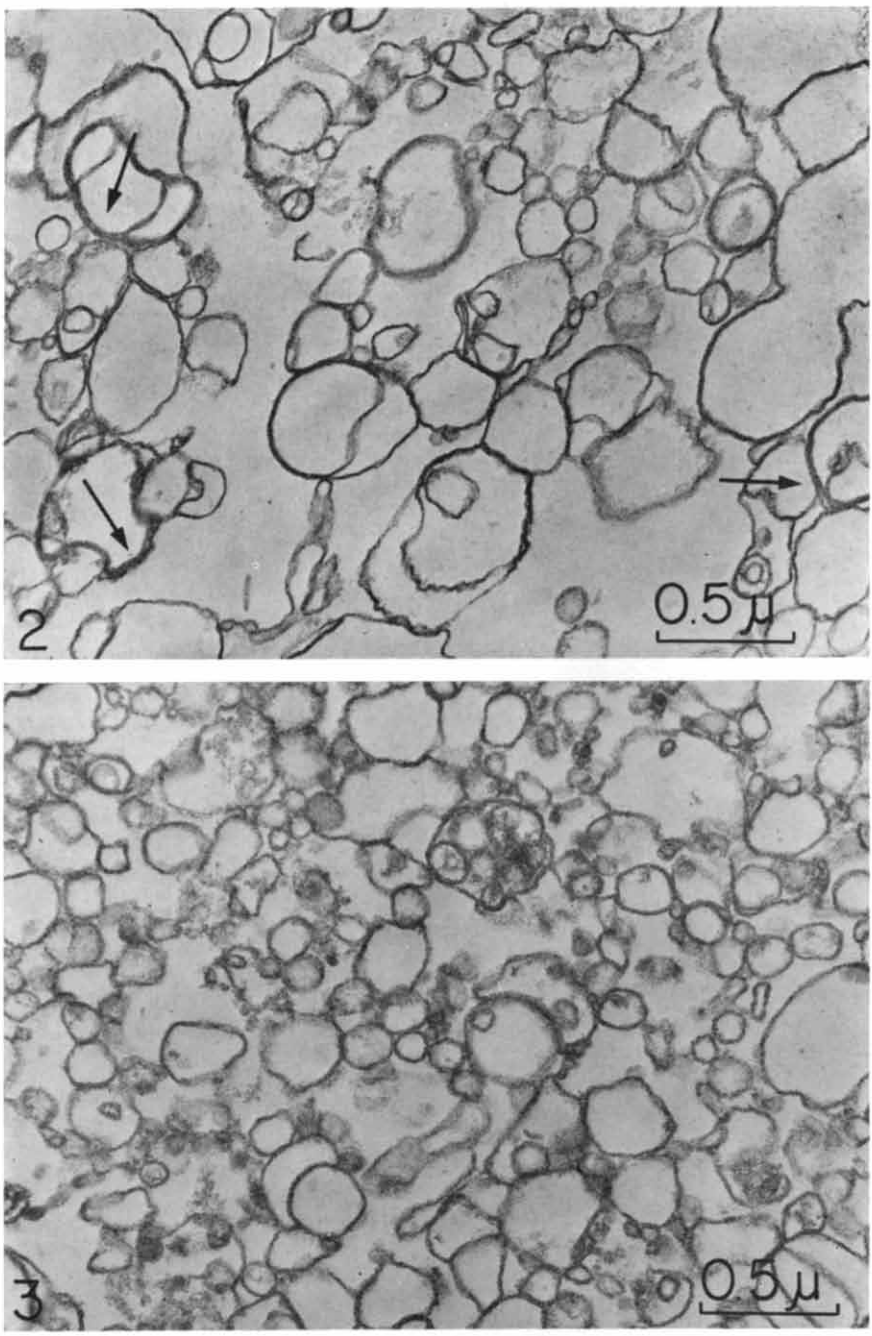

Fig. 2 and 3. Low magnification electron micrographs of Fractions I (Fig. 2) and II (Fig. 3) showing their homogeneous composition. Only membranous profiles of variable sizes are observed. Notice that the smaller-sized profiles predominate in Fraction II. In Fraction I, some profiles show a thicker wall formed by multilaminated myelin-like structures (arrows).

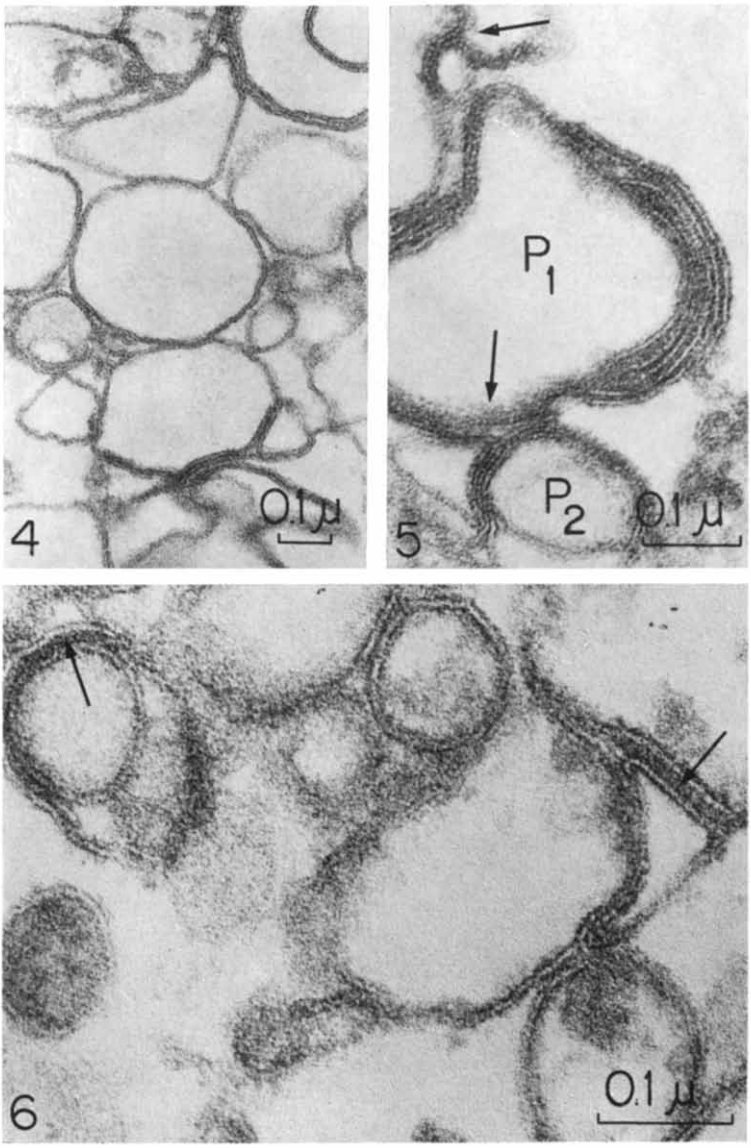

Fig. 4 and 5. High magnification electron micrographs of membranes forming Fraction I. In Fig. 4, several single-walled profiles showing the trilaminar substructure of the membranes are observed. In Fig. 5, multilaminar-walled profiles are shown. The membranes forming profile P1 are not fused and present an intervening dense material between the apposed layers. This material is also observed at the inner side of the membranous wall as a "fuzzy" layer. On the other hand, membranes forming profile P2 appear fused in a myelin-like fashion. Arrows indicate the regions where the globular substructure of the membrane is observed.

Fig. 6. High magnification electron micrograph of the membrane components of Fraction II, including single membranes showing the trilaminar pattern in several profiles. Notice the abundance of the dense material attached to both surfaces of the membranes. Arrows show the dense layer interposed between adjacent membranes.

Composition

Analysis of the overall relative composition revealed a larger amount of protein in Membrane II and a higher content of hydrocarbon in Membrane I (Table I). This hydrocarbon has been tentatively identified by means of gas–liquid chromatography as *n*-pentacosane (n-$C_{25}H_{52}$). The protein moiety present in both membranes contains 5–10 % carbohydrate. Fractionation of the polar lipids show that 80 % of them are phospholipids and 20 % is made up by a lipid which in two-dimensional thin-layer chromatography exhibits the mobility of monoglycerides. This lipid does not react with ninhydrin, the phosphatide reagent, or the glycolipid reagent used. Four phospholipids were identified (Table II): sphingomyelin, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. In Membranes I and II the phosphatidylcholine is the most abundant phospholipid. No significative difference in the relative proportions of polar lipids was found. Table III shows the percentage distribution of fatty acids identified in sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and free fatty acids of Membrane I and II. Since these lipids were separated in one-dimensional thin-layer chromatography the fatty acids isolated from phosphatidylcholine include those of phosphatidylserine (approx. 20 % contribution). The overall most abundant fatty acid is the odd-carbon member tricosanoic acid but there are differences in the fatty acid composition of the lipid fractions examined and between similar lipid fractions isolated from Membranes I and II. The most significant differences are pointed out (**) in Table III. A low content of RNA, less than 8 μg/mg of membrane protein (>0.8%), could be detected in the isolated membranes.

TABLE I

PERCENTAGE WEIGHT COMPOSITION OF MEMBRANE FRACTIONS ISOLATED FROM GIANT SQUID NERVE FIBERS

Data are expressed as mean ±S.E. Number of individual membrane preparations are given in parentheses.

Membrane fraction	*Protein*	*Total lipids*	*Polar lipids*	*Cholesterol*	*Fatty acids*	*Hydrocarbons*
I	29.5 ± 1.4 (11)	70.5 ± 1.5 (11)	58.5 ± 3.5 (5)	28.1 ± 2.3 (5)	6.2 ± 0.9 (5)	7.3 ± 1.2 (5)
II	48.3 ± 1.9 (6)	51.7 ± 1.9 (6)	66.4 ± 2.1 (5)	25.2 ± 1.7 (5)	6.2 ± 1.0 (5)	2.1 ± 0.3 (5)

TABLE II

PERCENTAGE COMPOSITION OF PHOSPHOLIPIDS PRESENT IN THE POLAR LIPID FRACTION OF MEMBRANES I AND II

Data are expressed as mean ± S.E. Number of individual membrane preparations are given in parentheses.

Membrane	*Sphingomyelin*	*Phosphatidyl-choline*	*Phosphatidyl-ethanolamine*	*Phosphatidyl-serine*
I	10.0 ± 1.6 (5)	45.9 ± 2.9 (5)	34.4 ± 1.7 (5)	10.4 ± 2.3 (5)
II	9.0 ± 1.4 (5)	44.2 ± 2.4 (5)	32.6 ± 2.4 (5)	14.5 ± 2.8 (5)

Enzyme determinations

A high "diaphorase" activity was detected in the total homogenate and its crude mitochondrial fraction. This enzyme(s) was therefore selected as a parameter to evaluate mitochondrial contamination in Membranes I and II. ATPases appear as the only marker available for the plasma membranes of the axon and the Schwann cell[4,19]. Measurements of this enzyme activity in total homogenates were made difficult by interfering substances present in the sea water or interstitial fluid present. Therefore, we decided to compare the activity of ATPases in the membrane fractions with that of the first pellet obtained in the isolation procedure (P-1, see METHODS). This pellet contains nuclei, mitochondria, microsomes, connective tissue fibers and plasma membranes. In Table IV representative results on the determination of ATPases and "diaphorases" are reported. No "diaphorase" activity was detected in

TABLE III

FATTY ACID COMPOSITION OF POLAR LIPIDS ISOLATED FROM MEMBRANE FRACTIONS I AND II OBTAINED FROM NERVE FIBERS OF *D. gigas*

Fatty acids	*Lipid*							
	Sphingomyelin		*Phosphatidyl-choline**		*Phosphatidyl-ethanolamine*		*Free fatty acids*	
Membrane Fraction	*I*	*II*	*I*	*II*	*I*	*II*	*I*	*II*
$C_{13:1}$					0.5	0.6		
C_{14}	—	—	1.9	1.5	0.6	1.1	4.0	1.5
C_{15}	—	—	0.6	0.5	1.5	1.7	—	—
C_{16}	20.7**	31.8**	43.2	35.3	5.8	4.9	29.2	22.7
$C_{16:1}$	—	—	1.0	0.9	—	—	1.7	2.1
C_{17}	1.4	4.6	1.5	2.6	2.5	1.8	1.7	2.0
C_{18}	3.8**	17.6**	3.5	8.4	9.0	8.9	15.1	11.1
$C_{18:1}$	0.5	3.7	11.1	7.7	2.4	3.3	7.9	6.4
$C_{19:1}$	—	—	—	—	—	—	—	26.7**
C_{20}	1.2	1.8	—	—	—	—	—	—
$C_{20:1}$	2.0	7.0	7.8	11.8	11.2	15.8	5.9	7.2
C_{21}	—	1.9	5.7	4.6	5.2	4.8	4.0	4.6
C_{22}	0.6	2.5	4.6	4.0	10.0	9.3	24.3 **	5.6**
(?)	7.3	7.9	6.0	4.3	—	—	—	—
C_{23}	61.4**	21.0**	12.9	18.4	49.8	45.3	6.0	3.1

* Including the fatty acids of phosphatidylserine, approx. 20% contribution.

** Indicates substantial differences in the relative proportions of individual fatty acids in the same lipid fraction of Membranes I and II.

TABLE IV

"DIAPHORASE" AND ATPASE ACTIVITIES IN NERVE FIBER SUBCELLULAR FRACTIONS

Fraction	*"NADH diaphorase"*	*ATPase (μmoles P_i/mg per min)*		
	(μmoles/mg per min)	*(K^+, Na^+)*	*(K^+, Na^+, ouabain)*	*($-K^+$, $-Na^+$)*
P-1	0.20	3.1	2.8	1.7
Membrane I	< 0.001	36.2	22.3	11.0
Membrane II	0.02	12.0	9.6	3.1

Membrane I. In Membrane II, however, NADH-oxidizing activity up to 10% of that observed in Fraction P-1 was measured. The ouabain-sensitive (Na^+-K^+)-dependent ATPase activity was always higher in the membranes than in Fraction P-1 containing the particulate structures of the homogenates.

DISCUSSION

Previous studies have shown that the plasma membrane of the Schwann cell and axolemma of the giant nerve fibers of *D. gigas* form a complex and large system in which the contribution of plasma membranes from other cells (*i.e.*, connective tissues) is small[2,20]. Based on the assumption that the plasma membranes in the giant fibers are made of lipoprotein, the present isolation procedure has been developed which yields two fractions with the morphological appearance of the plasma membranes seen in the intact fiber (Fig. 7). Although we are not yet able to correlate unequivocally isolated Fractions I and II with the membrane types observed in the intact tissue, the following observations have led us to a tentative assignment. Electron microscopic studies of the membrane components of Fractions I and II reveal differences in thickness of both membranes, as well as differences in the size and number of the membrane profiles and in the behaviour of the membranes (appearance of myelin figures). The lack of myelin-like structures observed in Fraction II seems to be due to the large amount of dense material attached to the surface

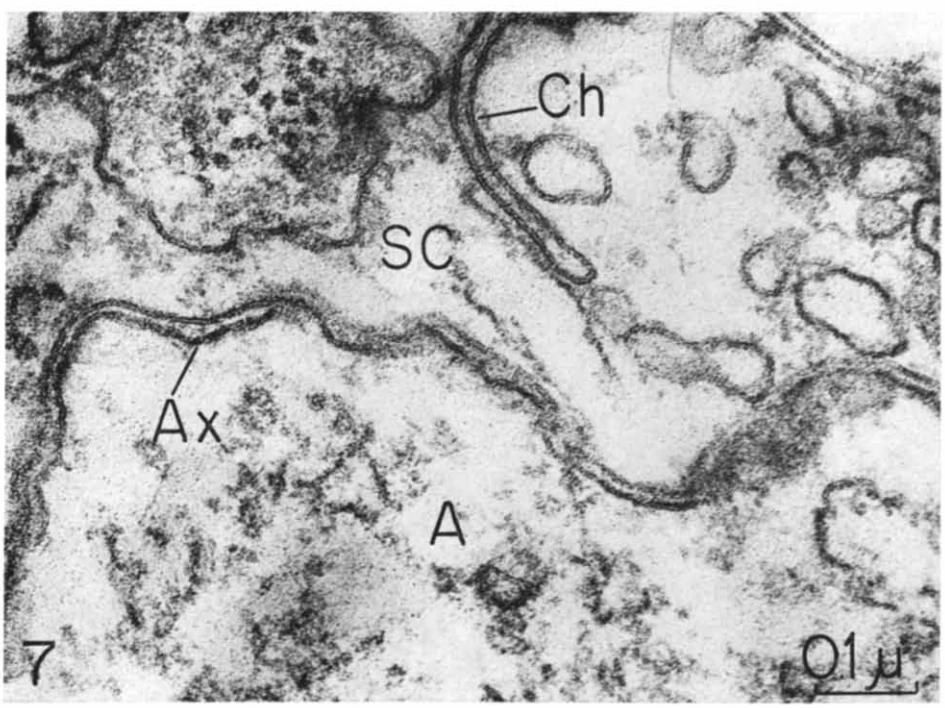

Fig. 7. Section from an intact giant nerve fiber showing part of the axon (A) bound by the axolemma (Ax), and the Schwann cell (Sc) crossed by a channel (Ch). At the right, channel segments are observed as round membranous profiles.

of the membranes of this fraction. As observed, no more than two apposed membranes fuse together, and more frequently a dense layer separates both membranes. It appears feasible that the coalescence is present at surfaces where the dense attached material is either scarce or can be easily squeezed out.

As compared with the studies in the intact squid giant nerve fiber[2,20], it seems worth pointing out the following correlations. (1) In the giant fiber the Schwann cell membranes (plasma membrane and channel walls) are thinner and more abundant than the axolemma, especially in *D. gigas* where the channel pathways are more tortuous[20]. (2) Intercellular spaces (channel lumina and axon–Schwann cell space) are filled with mucopolysaccharides as demonstrated by histochemical techniques[2]. (3) The effect of a detergent (sodium lauryl sulphate) on the axolemma induces the formation of myelin-like structures, while the Schwann cell membranes appear unaltered after the same treatment. Fraction I shows a higher activity of (Na^+-K^+)-dependent, ouabain-sensitive ATPase than Fraction II. SABATINI *et al.*[19] have demonstrated histochemically that the axolemma has more ATPase than the Schwann cell plasma membrane. These results, those obtained with the electron microscope, the yield of the fractions, their lipid composition[21,22] (specially its high cholesterol content) and the low content of "diaphorases" and RNA of the two membranes suggest, firstly, that we have isolated the plasma membranes present in the nerve fibers, and secondly, that Fraction I may be related to the axolemma and Fraction II to the Schwann cell plasma membrane. Although the lipids present in Fractions I and II are similar (Tables I and II), their fatty acid composition is different (Table III), another fact indicating a different origin of the two membranes. However, for a final assignment of the fractions obtained by the described procedure, the use of more direct techniques for identification and cellular localization of isolated subcellular fractions is required.

Using a different fractionation scheme, FISCHER *et al.*[4] have isolated two membrane fractions with the characteristics of plasma membranes from the optic nerve of *D. gigas*. The apparent density and the composition of these fractions are different from those of the fractions that we have isolated from the stellar nerve of the same animal. It would be important to establish whether the differences observed are caused by the dissimilar isolation procedure or whether the plasma membranes of these two nerves have a different composition.

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REFERENCES

1 I. SINGER AND I. TASAKI, in D. CHAPMAN, *Biological Membranes*, Academic Press, London, 1968, p. 347.
2 G. M. VILLEGAS AND R. VILLEGAS, *J. Gen. Physiol.*, 51 (1968) 44.
3 E. CONDREA AND P. ROSENBERG, *Biochim. Biophys. Acta*, 150 (1968) 271.
4 S. FISCHER, M. CELLINO, X. GOMES AND M. CANESSA-FISCHER, *Biophys. J.*, 9 (1969) A-176.
5 R. VILLEGAS AND G. CAMEJO, *Biochim. Biophys. Acta*, 163 (1968) 421.

6 G. CAMEJO AND R. VILLEGAS, *Biophys. J.*, 9 (1969) A-162.
7 M. WATSON, *J. Biophys. Biochem. Cytol.*, 4 (1958) 475.
8 E. S. REYNOLDS, *J. Cell Biol.*, 17 (1963) 208.
9 J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 32.
10 E. CONDREA, P. ROSENBERG AND D. DETTBARN, *Biochim. Biophys. Acta*, 135 (1967) 669.
11 D. ABRAMSON AND M. BLECHER, *J. Lipid Res.*, 5 (1964) 628.
12 J. M. BEVERIDGE AND S. E. JOHNSON, *Can. J. Res.*, 27 (1949) 1959.
13 J. C. DITMER AND R. L. LESTER, *J. Lipid Res.*, 5 (1964) 126.
14 R. E. BOWMAN AND R. C. WOLF, *Clin. Chem.*, 8 (1962) 302.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
16 E. J. KING, *Biochem. J.*, 26 (1932) 292.
17 D. F. H. WALLACH AND V. B. KARNAT, *Methods Enzymol.*, 8 (1966) 1964.
18 M. DUBOIS, K. A. GILLER, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
19 M. SABATINI, R. DIPOLO AND R. VILLEGAS, *J. Cell Biol.*, 38 (1968) 176.
20 G. M. VILLEGAS, *J. Ultrastruct. Res.*, 26 (1969) 501.
21 S. FLEISCHER AND G. RAUSER, *J. Am. Oil Chemists' Soc.*, 42 (1966) 588.
22 J. B. FINEAN, in J. A. V. BUTLER AND H. E. HUXLEY, *Progress in Biophysics and Molecular Biology*, Pergamon Press, Oxford, 1966, p. 143.