J. Membrane Biol. 9, 209–228 (1972)

The Molecular Organization of Nerve Membranes

VI. The Separation of Axolemma from Schwann Cell Membranes of Giant and Retinal Squid Axons by Density Gradient Centrifugation

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Received 13 December 1971

Summary. Plasma membranes were isolated from two types of squid nerves which have morphologically, different ratios of axolemma/Schwannlemma (A/S). These membranes were studied by means of differential and density gradient centrifugation.

Thoroughly dissected giant axons were used as membrane source having low A/S ratio. Retinal fibers were used as membrane source with high A/S ratio. A similar procedure for the isolation of the plasma membranes was used for both types of squid axons.

Differential centrifugation showed that at $1{,}500 \times g$, the yield of membrane enzymes (Na, K-ATPase and NADH-ferricyanide oxidoreductase) from giant axon homogenates was 2 to 5 times greater than from retinal nerve homogenates, but at $105{,}000 \times g$ the opposite was the case, the yield from retinal axons being about two times greater. Thus, the major part of the membrane material from the retinal nerve seems to be less dense than the membrane material from giant axons.

The behavior of the $105{,}000 \times g$ fraction from both types of fibers was studied by determining protein, Na, K-ATPase, and NADH-oxidoreductase along a lineal sucrose gradient (10 to 40%; centrifuged at $40{,}600 \times g$ for 90 min). By any of the three measurements, retinal axons yielded a greater amount (2:1) of plasma membranes sedimenting at low sucrose concentration (16 to 25%) as compared to that observed at high sucrose concentration (35 to 38%). Giant axons, on the contrary, yielded a higher proportion of membranes (2.5:1) sedimenting at high sucrose concentrations (over 40%).

The experimental data indicate that a different cellular origin can account for the behavior of nerve membranes along lineal gradient centrifugation. The membranes floating at low sucrose concentration ("light membranes") can be tentatively ascribed to the axolemma; the membranes found at high sucrose concentration ("heavy membranes") to the Schwannlemma and basement membranes.

In accord with their high A/S morphological ratio, squid retinal axons yielded 5 times more light membranes (axolemma) than dissected giant axons.

We have recently reported that squid retinal nerves can be used to isolate an axolemma-rich preparation owing to their high ratio of axonal to Schwann cell surface membranes [3, 4]. Since it is well known that unmyelinated fibers are enveloped by a Schwann cell and fibrous tissue, a plasma membrane fraction is likely to contain a mixture of "axolemma" and "Schwannlemma" and internal membranes. The term "axolemma" (A) is used here for the surface cell membrane of an axon; the term "Schwannlemma" (S) is used for the surface membrane of the Schwann cell.

Fischer, Cellino, Zambrano, Zampighi, Téllez-Nagel, Marcus, and Canessa-Fischer [4] reported that sucrose gradient contrifugation of the plasma membrane fraction permitted the separation of two chemically different types of plasma membranes. To explain these findings it was postulated that their cellular origin were different; by several chemical, enzymic and ultrastructural criteria it was concluded that endoplasmic reticulum could not contribute significantly to the heterogeneity of the plasma membrane fraction.

It was considered necessary to study the fate of both types of surface membranes when nerve homogenate are submitted to differential and density gradient centrifugation. The problem was approached by comparing the profile of membrane obtained from nerve fibers which exhibited different "axolemma/Schwannlemma" (A/S) morphological ratio.

A great deal of electron-microscopic studies have been made on the giant nerve fibers from several species of squid [1, 5, 10]. Investigation of the ultrastructure of axons from *Dosidicus gigas* carried out by Villegas [8] stated that the Schwann layer is formed by a single row of Schwann cells crossed by channels. The tortuosity of these channels greatly increase the surface of the Schwann cell plasma membrane, as shown in that report. Therefore, the morphological features of giant axons are very unfavorable for axolemma and the A/S ratio should be below one.

In the retinal nerves of the squid, the relation between the Schwann cells and the axons is different. Each bundle contains approximately 10 unmyelinated axons enclosed by one layer of Schwann cell membrane. The amount of excitable membrane turns out to be 5 times higher than membranes from the enveloping cells. Hence, based only on morphological grounds, it can be stated that the A/S ratio in retinal nerves must be inverted in the giant axons.

The present communication reports experiments designed to compare the distribution of cell membranes from giant and retinal *Dosidicus* axons on density gradient centrifugation.

The data support the starting hypothesis that from these different types of nerves, two chemically different types of membranes could be separated in inverse proportions.

The experimental data indicate that the membranes isolated from retinal axons floating at low sucrose densities are formed mainly by the axolemma.

Materials and Methods

The squid, *Dosidicus gigas*, were caught and killed a few miles offshore from Montemar, Chile. Fishing and laboratory facilities of the Laboratorio de Fisiologia Celular were used. Within 2 hr of capture of the animal the eyes and nerve trunks containing the first stellar nerves were removed and placed in ice-cold artificial seawater. The retinal axons were dissected out in 0.75 M sucrose as described elsewhere [4] and stored in this solution at -20 °C up to 10 days. Under appropriate illumination, the giant axons were carefully dissected from the nerve trunk in cold artificial seawater and thoroughly cleaned of connective tissue and small nerve fibers. They were tied at both ends and the excitability was measured by recording action potential with external electrodes. This precaution was important to detect and to discard biological material collected in bad conditions. Such material usually yielded very low specific activities for membrane-bound enzymes. Afterwards, the axons were washed in 0.75 M sucrose and used immediately or kept at -20 °C not more than 10 days.

Isolation and Purification of Plasma Membranes

Plasma membranes were prepared from 10-g wet weight of retinal nerve as described by Fischer *et al.* [4].

A similar procedure was used to isolate membranes from giant axons. Cleaned axons were rinsed in 0.25 M sucrose, 10 mM Tris-EDTA, pH 7.5 (SE) and finely cut with scissors. Between 50 and 100 giant axons were homogenized in 10 ml of 0.25 M sucrose, 5 mM Tris-EDTA, and 30 mM histidine, pH 7.4 (SEH) using a motor driven glass-glass homogenizer in the presence of acid-washed glass beads (150- to 200-μ diameter). Sonication was performed at 20-kc output for 20 sec (Bronwill Biosonic B sonicator). The suspension was spun for 10 min at $1{,}500 \times g$ in a Sorvall refrigerated centrifuge, and the supernatant was spun twice at $10{,}000 \times g$ for 20 min. To obtain the final membrane fraction, the last supernatant was centrifuged for 90 min at $105{,}000 \times g$ in a rotor 40 in a Spinco L ultracentrifuge. The pellet was resuspended twice by gentle homogenization with a Teflon homogenizer in 0.25 M sucrose, 30 mM Tris-chloride, 1 mM EDTA, pH 7.4 (SET) and centrifuged for 60 min at $105{,}000 \times g$. The final pellets were resuspended in 1.5 ml of 30 mM Tris-chloride, 1 mM EDTA, pH 7.4 (F-100). The whole procedure was performed at 2 °C.

Linear gradients were established between 10 and 40% sucrose containing 30 mM Tris-chloride, 1 mM EDTA, pH 7.4. A fraction of 0.5 ml of 50% sucrose was added as a cushion; 0.6 ml of F-100 from giant axons containing about 0.5 to 0.9 mg of protein was placed on the pre-equilibrated gradient and centrifuged for 90 min at 22,500 rpm in an SW-39 rotor at a temperature setting of 22 °C in the Spinco L ultracentrifuge. Fractions of 0.3 to 0.5 ml were collected in pre-cooled tubes by a Sigma motor pump, and the optical density was measured at 280 nm in a Unicam spectrophotometer. The linearity of the gradient was checked routinely by measurements of the refractive index.

Samples of 0.6 ml of F-100 from retinal axons containing about 2 to 5 mg of protein were placed in identical gradients in the SW-25 rotor and centrifuged for 90 min at 20,000 rpm.

Enzymic Assays

The Na, K-ATPase was assayed at 37 °C in duplicates and in a final volume of 1 ml containing: (mM) Tris-chloride 160 (pH 8.5), Tris-ATP 2; $MgCl_2$ 4; NaCl 160; KCl 40; ouabain 0.2. The membrane fractions were pre-incubated 10 min with the ion mixture at 37 °C before addition of ATP. The reaction was stopped after 10 to 30 min of incubation with acid ammonium molybdate used for the analysis of inorganic phosphate.

The amount of protein used was such that no more than 25% of the substrate was hydrolyzed during the incubation period. 60 to 100 μg of F-100 protein from giant axons and 20 μg from retinal axons were used, suspended in 0.05 ml of 30 mM Tris-chloride, 1 mM EDTA (pH 7.4). The nonenzymic hydrolysis of ATP was measured in each experiment. Care was taken not to denature the membrane enzymes by dilution.

When F-100 from giant axons was resolved by gradient centrifugation, aliquots of 0.05 ml were used to measure the phosphate released in the presence of (Mg + Na + K) and (Mg + Na + K + ouabain), as described above. The reaction was stopped by 1 ml of cold 5% ammonium molybdate, 5 N H_2SO_4. Later, 5 ml of isobutanol/benzene (1:1 v/v) was used to extract the phosphomolybdic complex in glass-stoppered tubes; 2 ml of the organic phase were analyzed by Ernster's method [2] with a final volume of 5 ml.

NADH- (acceptor) oxidoreductase (NADHDase) was assayed at 37 °C in a Unicam SP 800 recording spectrophotometer in 0.2 ml aliquots of the F-100 suspension as follows: (mM) NADH 0.2; K ferricyanide 0.66; Tris-chloride 86 (pH 8.5). The reaction was started with the addition of NADH and absorbances at 420 nm were recorded differentially as a function of time against the nonenzymic reaction. NADHDase activity was analyzed in the sucrose gradient fraction in 0.2 ml aliquots from each tube and incubating them in a similar media; the absorbance was recorded at 340 nm.

The millimolar extinction coefficients used were: NADH, 622 (340 nm) and K ferricyanide, 0.96 (420 nm).

Proteins were determined in F-100 fractions by the Lowry method [7].

Reagents

NADH, ATP, histidine, TRIS-121 and ouabain were from the Sigma Chemical Co. Inorganic salts, sucrose and organic solvent (p.a.) were from Merck, Darmstadt, W. Germany.

Ultrastructural Studies

To carry out electron-microscopic studies on the retinal nerves, squids were perfused through the aorta upon being caught while still in the boat. The perfusion solution contained 6.25% glutaraldehyde 0.2 M collidine buffer, pH 7.45 in artificial seawater. Later, the eyes were removed and the nerve bundles dissected out carefully and immersed for 2 hr in the same fixing solution.

They were rinsed afterwards in collidine buffer 0.2 M, pH 7.45. Post-fixation was carried out in 2% osmium tetroxide in collidine buffer followed by dehydration in a series of 1:1 acetone and araldite mixtures for 6 hr. Thin sections were obtained in a Porter Blum MT 2 ultramicrotome equipped with a diamond knife and placed on

collodium carbon-coated grids of 100 mesh. After double staining with uranyl acetate and lead citrate they were observed in a Siemens Elmiskop 1A and a Phillips EM 300 electron-microscope.

To study the ultrastructure of plasma membranes, F-100 from giant axons was suspended by homogenization in 0.25 M sucrose buffered with 30 mM Tris-chloride. Drops of this preparation and the pellet separated by density gradient centrifugation were deposited on the grid, stained with 1 % Na phosphotungstate (pH 4.2) and observed with a Phillips EM 300 electron-microscope centered at 60 kV and provided with an anticontamination device.

Results

Ultrastructural Studies

Fig. 1 shows a comparative diagram of the structure of giant and retinal axons of the squid. As mentioned before, *Dosidicus* giant axons have been studied by Villegas [8]. Retinal fibers were studied by one of us (G.Z.) to establish the relationship between the various surface and internal membranes.

Fig. 2 shows an electron-micrograph of a cross-section of retinal nerve fibers. The axons are organized in packages surrounded by the Schwann cell and communicated to the exterior by several mesaxons. Each Schwann

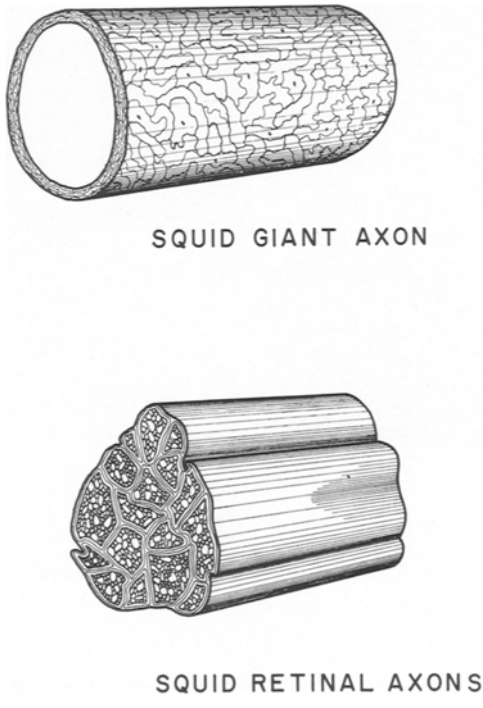


Fig. 1. Comparative diagram of the structure of giant and retinal nerves

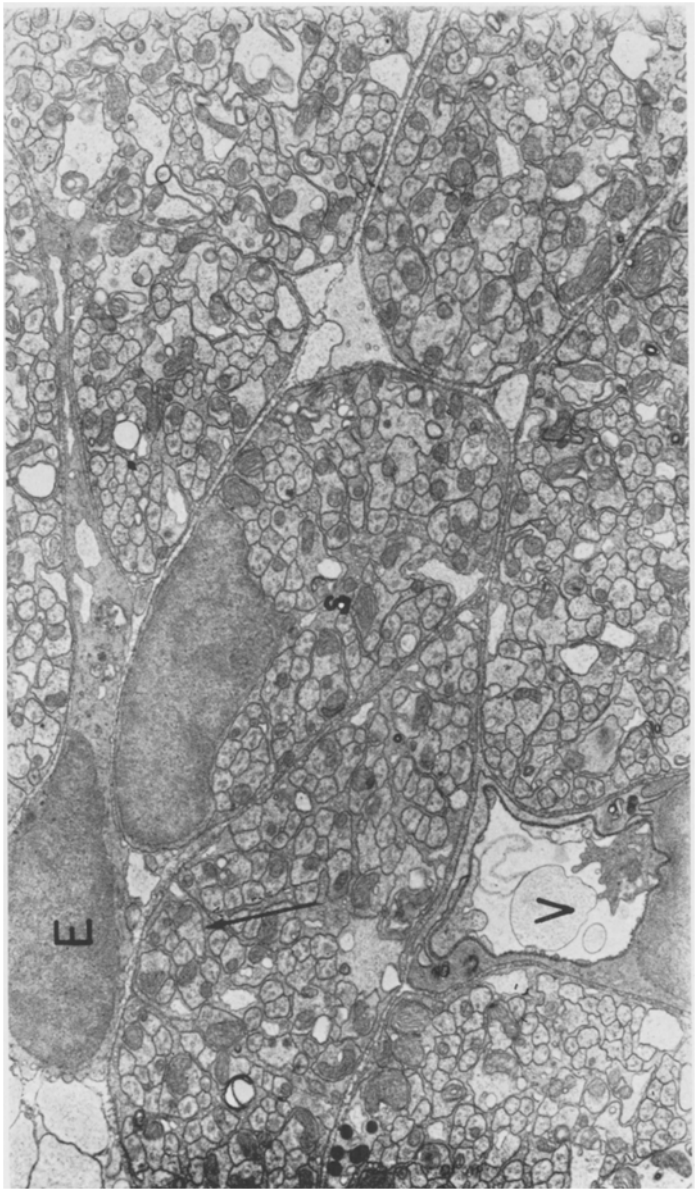


Fig. 2. Electron-micrograph of squid retinal fibers. Several axons can be observed surrounded by one Schwann cell whose cytoplasm rarely penetrated inside the bundle (arrow). *S*: Schwann cell nuclei; *E*: endoneurium cell surrounding various nerve bundles; *V*: blood vessel between the bundles of axons

cell contains numerous axons of 10-μ mean diameter. The assembly of several of these units is made by one connective cell leading to the formation of the fasciculus. The blood vessels are arranged in the endoneurium space and their structure is similar to that described by Gray [6]. It should be noted that in the retinal fibers the basement membrane is present only in

the blood vessels. This is another marked difference as compared with giant axons which have a thick basement membrane [8].

The A/S ratio was measured to be in the order of 5:1. However, this value might be altered by the presence of membranes from other cells such as connective cells and blood vessels. The electron-micrograph also shows many mitochondria contained in the axon cytoplasm. Rough endoplasmic reticulum is present in a small proportion in the connective cell cytoplasma close to the nuclei; it was not found in the elongation which separates the Schwann cells.

The A/S ratio was measured also in an electron-micrograph published by Villegas [8] which gave values in the order of 1:5.

It should be pointed out that both types of fibers contain scarce endoplasmic reticulum.

Differential Centrifugation of Squid Axon Homogenates

Fischer, Cellino, Gariglio, and Téllez-Nagel [3] selected the $100{,}000 \times g$ pellet from retinal axon homogenate as a plasma membrane-enriched fraction. The experimental criteria were: (*a*) The distribution of two membrane enzymes as the Na, K-ATPase and NADHDase. (*b*) The chemical composition which showed protein/lipid ratio, cholesterol, phospholipid, and RNA content typical of plasma membranes. (*c*) The ultrastructural studies which determined the purity of plasma membrane by negative staining and fixation techniques.

Table 1. The distribution of proteins and enzyme units upon fractionation by differential centrifugation of homogenates from giant and retinal axons of the squid

	NADH-Ferricyanide oxidoreductase (% of total units)		Na, K-ATPase (% of total units)		Proteins (%)	
	Giant	Retinal	Giant	Retinal	Giant [a]	Retinal
$1{,}500 \times g$ pellet	13	7.5	24	4	20	4
$1{,}500 \times g$ supernatant	75	69.0	53	83	31	90
$100{,}000 \times g$ pellet	19.4	36.0	18	38.4	9.6	8.6
Ratio $\frac{100{,}000}{1{,}500} \times g$	1.4	4.8	0.75	9.6	0.48	2.15

[a] The proteins were determined by their absorption at 260 and 280 nm. 10 g wet weight of retinal axons containing 234 mg of protein were processed. 0.8 to 1 g of giant axons containing 48 mg of proteins were processed. The units measured in homogenates were taken as 100%.

Table 1 shows a comparison of the enzyme units and proteins obtained from giant and retinal axons. Since the yields of giant axons were low in a single preparation, the axons were collected for several days in different batches. For this reason, the data are presented as per cent of the starting material to allow comparison with retinal fibers, as well as between different batches.

It can be seen that both types of nerves yielded similar per cent of protein in the $100{,}000 \times g$ pellet. However, retinal axons contained twice the amount of ATPase and NADHDase activity as compared to giant axons.

It should also be noted that only 4% of the proteins from retinal axons sedimented at $1{,}500 \times g$ whereas 20% sedimented from giant axons. The latter fibers also have 6 times more ATPase activity in the low-speed centrifugation pellet. Moreover, when giant axon homogenate was not sonicated for 15 sec, 50% of the ATPase units remained in the $1{,}500 \times g$ pellet. These data suggest that a great deal of giant axon membranes was probably trapped by the thick endoneurial connective tissue and basement membrane present in these fibers.

It would be important to clarify the sedimentation behavior of internal membranes from the axons which might be present in a higher proportion in giant than in retinal axons.

From dissected giant axons, the axoplasm can be extruded from the remaining sheath. The axoplasm will contain the axonal internal membranes; the sheath will contain the "axolemma", the basement membrane and the surface and internal membranes of the Schwann cell.

Table 2 shows the Na, K-ATPase activity determined in homogenates of both structures. It can be seen that even though axoplasm accounted for 60% of the nerve proteins, its enzyme specific activity is 8.5 times lower than the sheath. This would indicate that the ATPase activity of internal membranes belonging to the axon can not contribute significantly to the enzyme units collected on the $100{,}000 \times g$ pellet from intact giant axons or retinal axons. This explains also why the sheath and intact giant axons yielded a similar per cent of ATPase activity in the high-speed pellet. Since the per cent of proteins recovered was also similar using either intact nerves or sheaths, it can be concluded that internal membranes from the axon cytoplasm are a minor component of the $100{,}000 \times g$ pellet.

Table 3 shows that F-100 isolated from giant axons of the squid (GAS) and retinal axons (RAS) oxidized only NADH with ferricyanide, but not succinate, NADPH or lactate as electron acceptors. The properties of this enzyme are similar to erythrocyte membranes [11–13]; this datum excludes

Table 2. Na, K-ATPase and proteins of axoplasm, sheath and intact giant axons

	Na, K-ATPase		Proteins	
	Na, K-ATPase specific activity (μm/mg prot per hr)	100,000 × *g* pellets (% units)	100,000 × *g* pellet (%)	% of axon wet weight
Axoplasm	2.8	—	—	13
Sheath	23.5	17	8.7	8.6
Intact axons	10.1	18	9.6	21.6
Periaxonal tissue	5.0	—	—	—

The units measured in the starting homogenates were taken as 100%. Axoplasm was obtained by rolling out into parafilm paper. The sheath comprises the axolemma, surface and internal membranes of the Schwann cell and the basement membranes. Periaxonal tissue was dissected out from the trunk containing the giant axons. Na, K-ATPase assay was carried out as described in Materials and Methods.

Table 3. Specificity of NADH oxidoreductase activity catalyzed by plasma membranes isolated from giant axons and retinal axons of the squid

Electron donors	Electron acceptors (nmoles/mg prot per min)		
	$K_3Fe(CN)_6$	Cytochrome *c*	Oxygen
Giant axons			
0.6 mM NADH	1,298	3.36	—
0.6 mM NADPH	37.42	—	—
6.7 mM succinate	—	—	—
1.7 mM lactate	—	—	—
Retinal axons			
0.6 mM NADH	1,360	73.7	16.7
0.6 mM NADPH	—	—	—
6.7 mM succinate	69	—	—
1.7 mM lactate	15	—	—

contamination by mitochondria and endoplasmic reticulum which oxidizes NADPH. It should be pointed out that the latter enzyme was present in the F-100 pellet, but it was removed by further hypotonic washings.

Fractionation by Density Gradient Centrifugation

Pellets isolated from both GAS and RAS were analyzed by sucrose gradient centrifugation, recording the optical density at 280 nm. Fig. 3*B* shows that membrane proteins of the retinal fibers were distributed in two

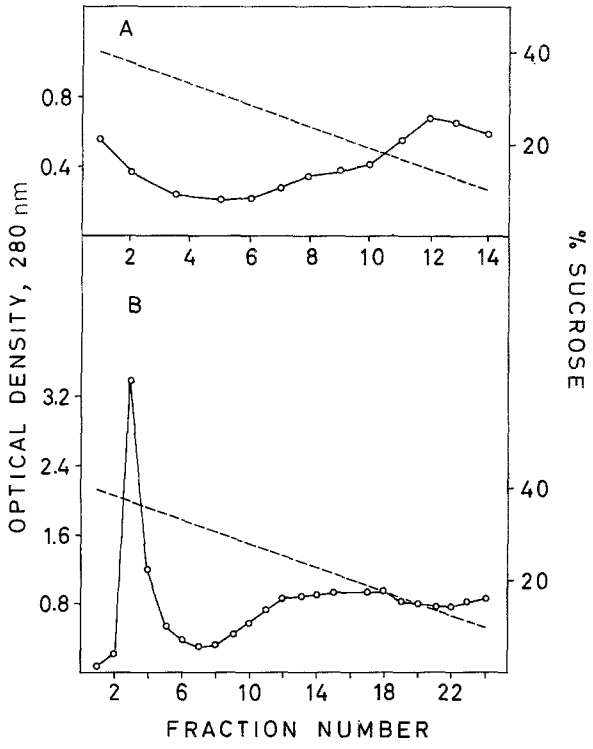


Fig. 3. The distribution of proteins along sucrose gradient centrifugation of membranes (F-100) isolated from giant (*A*) and retinal (*B*) axons of the squid. o-o-o, optical density at 280 nm; -----, % of sucrose. Linear gradients of sucrose were established between 10 to 40% made up in 1 mM EDTA, 30 mM Tris-Cl (pH 7.4) with 0.5 ml of 50% sucrose as cushion for the SW-39 rotor and 2.0 ml of 50% sucrose for the SW-25 rotor. (*A*) 636 µg of membrane proteins (F-100) from giant axons were centrifuged 90 min at 22,500 rpm in an SW-39 Spinco rotor. 365 µg of proteins collected in the sucrose cushion are not shown in the drawing (heavy membranes). (*B*) 3.6 mg of membrane proteins from retinal axons were centrifuged 90 min at 20,000 rpm in an SW-25 rotor

peaks along the gradient with an insignificant amount in the sucrose cushion. The first protein peak was located between 35 and 38% sucrose after centrifuging at 40,600 × *g* for 90 min (heavy membranes); a second band of proteins was distributed between 16 and 25% sucrose (light membranes).

In contrast, the membrane fraction isolated from GAS (Fig. 3*A*) and centrifuged under identical conditions, was distributed in one band between 10 and 20% sucrose (light membranes). But, most of the proteins sedimented as a pellet in the cushion of 50% sucrose (heavy membranes).

It was important to verify whether or not this fraction could be ascribed to aggregate membranes or other material. To enable a better resolution in the gradient, a number of modifications were made in the isolation of

Table 4. Distribution of proteins separated from membrane fraction isolated from giant and retinal axons of the squid by sucrose gradient centrifugation

	% Total membrane proteins			Ratio light/heavy
	Heavy membranes	Light membranes	Rest of the gradient	
Cleaned giant axons	58.0	16.8	25.2	0.29
	72.0	8.4	18.6	0.17
	48.0	25.0	27.0	0.52
	58.0	15.9	26.0	0.27
	59.7	20.5	19.8	0.35
	55.5	18.0	26.5	0.33
	55.4	17.8	26.8	0.32
	51.0	28.0	21.0	0.55
Mean values	57.4	18.5	23.8	0.35 ± 0.13*
Retinal axons	22.9	48.5	28.6	2.12
	29.2	32.7	38.1	1.12
	26.8	50.0	23.2	1.87
	31.3	36.5	32.2	1.62
	20.4	53.9	26.7	2.64
	27.7	52.7	19.6	1.92
	35.0	51.5	13.5	1.47
Mean values	27.7	46.5	25.9	1.82 ± 0.56*

* Mean value ± SD.

0.6 to 0.9 mg of membrane proteins (F-100) from giant axons were centrifuged 90 min at 22,500 rpm in an SW-39 rotor. 2 to 4.8 mg of membrane proteins from retinal axons were centrifuged 90 min at 20,000 rpm in an SW-25 rotor. Heavy membranes were collected from the gradient between 35 to 50% sucrose concentration. Light membranes were collected from the gradient between 12 to 25% sucrose concentration. To compare several membrane preparations, the amount of protein of the membrane fraction placed in the gradient was taken as 100%.

F-100 from GAS homogenates such as: (*a*) varying the sonication time from 0 on up to 30 sec; (*b*) centrifuging the 10,000 × *g* supernatant at 20,000 × *g* for 20 min; (*c*) increasing the EDTA concentration in the sucrose solutions for the gradient from 1 mM to 5 mM; (*d*) reducing the amount of membrane proteins placed on the gradient to eliminate the possibility of aggregation by overloading; (*e*) reducing the time allowed to equilibrate the density gradient centrifugation from 90 to 60 min. None of these modifications reduced the amount of membrane proteins collected in the sucrose cushion.

Table 4 shows the distribution of proteins separated by sucrose gradient centrifugation of the F-100 from retinal and giant axons. The membranes collected between 35 and 50% sucrose were described as heavy membranes.

The light membranes were those collected at low densities between 12 and 25% sucrose.

It was found that in retinal axons, 1.8 more membrane proteins floated at low densities than at high densities. Only 26% of the proteins was found to be outside of the optical density peaks.

The F-100 from giant axons, however, was found to have an inversely proportional distribution. The amount of membrane protein collected in the high density sucrose area (heavy membranes) was 2.8 times the amount of light membranes; 23.9% of the protein was found to be distributed in the rest of the gradient. These data indicate that retinal fiber F-100 contains 5 times more light membranes than do giant fibers. That is, the ratio light/heavy membranes is 1.8:1 in RAS and 1:2.8 in GAS.

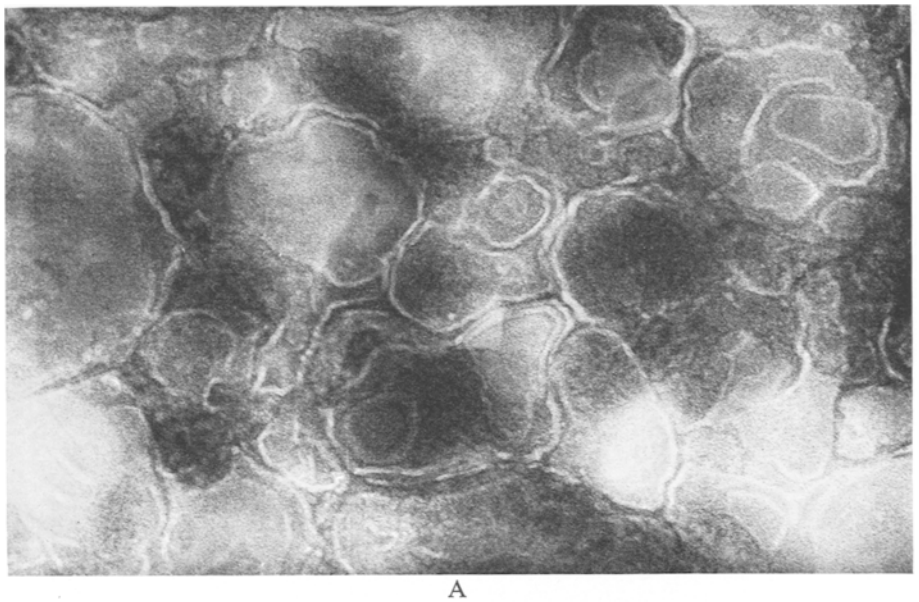
Both types of membranes were centrifuged at $100{,}000 \times g$ for 1 hr to obtain pellets adequate for ultrastructural studies.

Electron-micrographs of glutaraldehyde-fixed pellets from the two types of membranes isolated by density gradient were reported previously by Fischer *et al.* [3].

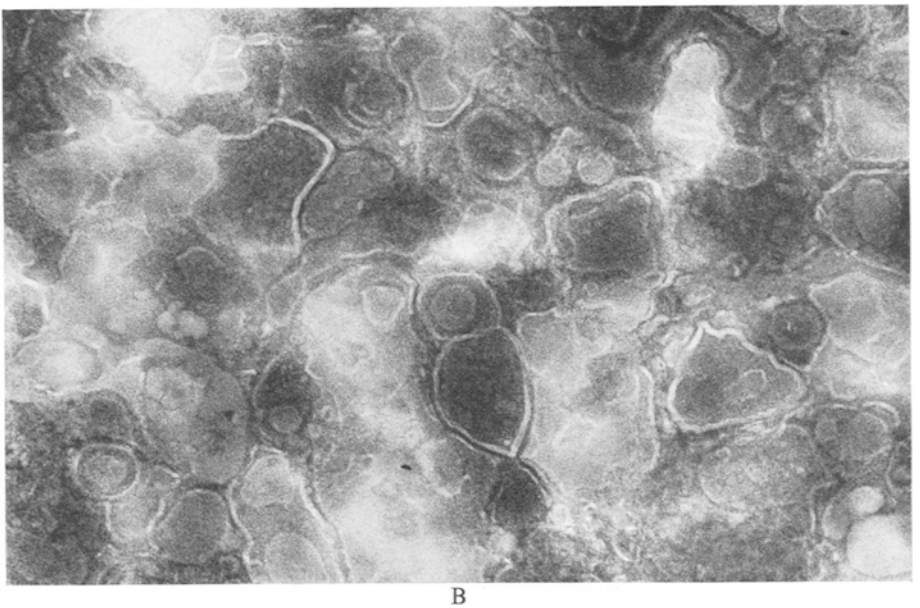
An alternative method was used to study the ultrastructure of both membranes isolated from giant and retinal axons. Fig. 4*A* shows an electron-micrograph of heavy membranes from RAS obtained by negative staining. The main structures observed are also vesicles formed by membranes with a diameter between 0.08 to 0.5 μ. The formation of vesicles inside of other vesicles was often seen. In Fig. 4*B* an electron-micrograph of light membranes material, negatively stained, can be seen. Again, the membranes are associated forming vesicles of slightly less diameter between 0.04 to 0.2 μ. Loose membranes, which did not associate in vesicles, were frequently seen. In both types of membranes, separated by density gradient centrifugation, mitochondrial or ribosomal contamination was not observed.

Negative staining of membranes separated from giant axons showed that heavy membranes were so packed that it was difficult to obtain a good photograph. Light membranes were not different from retinal axons.

Fig. 5 shows the profile of NADHDase specific activity along the sucrose gradient centrifugation of F-100 from RAS and GAS. A narrow band of high specific activity was found at high sucrose concentration in retinal nerves; at low sucrose concentration the membranes had a specific activity usually 3 to 5 times lower. F-100 from giant axons, on the other hand, sedimented most of the enzyme units in the sucrose cushion and they are not shown in the drawing. NADHDase displayed a rather homogenous distribution along the gradient. It is possible that a well-defined band could not be resolved because of its low specific activity.



A



B

Fig. 4. Electron-micrograph of heavy and light membranes from retinal axons of the squid. (*A*) Electron-micrograph of heavy membranes from RAS obtained by negative staining. Heavy membranes were obtained from F-100 resolved on sucrose gradient centrifugation, by concentrating the first optical density peak through centrifugation at 145,000 × *g* (35 to 37% sucrose). The pellet was suspended in 0.25 M sucrose 30 mM Tris-Cl (pH 7.4) and stained with 1% Na phosphotungstate (pH 4.2). Magnification: 120,800 ×. (*B*) Electron-micrograph of light membranes from RAS obtained by negative staining. Light membranes were obtained from the second peak of sucrose gradient centrifugation of F-100 (18 to 25% sucrose). Conditions similar to Fig. 4(*A*). Magnification: 120,800 ×

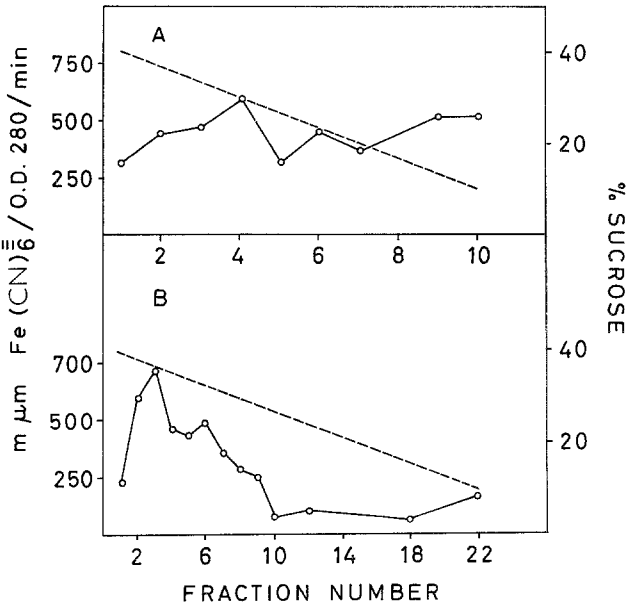


Fig. 5. The distribution of NADH dehydrogenase along sucrose gradient centrifugation of membranes (F-100) isolated from giant (*A*) and retinal (*B*) axons of the squid. -o-o-o-, NADH-ferricyanide oxidoreductase activity; -------, % of sucrose. (*A*) 0.6 ml of F-100 from giant axons containing 630 μg of membrane protein were placed on a pre-equilibrated linear gradient of sucrose established between 10 to 40% with 0.5 ml of 50% sucrose as cushion. 365 μg of membrane proteins were collected in the cushion (heavy membranes). The enzymatic specific activity of these membranes was 1,880 nmoles of reduced acceptor/mg protein per min. (*B*) 2.0 ml of F-100 from retinal axons containing 22.2 optical density units at 280 nm were placed on the gradient and 98% of them were recovered from the linear gradient. 1,620 NADHDase units (nmoles of reduced acceptor/min) were placed on the gradient. 690 enzyme units were collected between 25 to 15% sucrose (light membranes)

Table 5 shows the NADHDase specific activity of F-100, heavy and light membranes of giant and retinal axons. It can be seen that the RAS membranes collected at high sucrose concentration increased their specific activity 3 to 5 times. GAS heavy membranes increased their mean specific activity only 80%.

Table 6 shows the distribution of NADHDase units of F-100 from RAS and GAS, according to gradient centrifugation. It should be noted that 60% of the original units of GAS were recovered as heavy membranes while only 16% were recovered in the zone of low density. 50% of the enzyme units of RAS were recovered as heavy membranes and 38% as light membranes. Since the RAS heavy membranes had a specific activity at least 3 times higher than light membranes, it can be estimated that RAS F-100

Table 5. Na, K-ATPase and NADH oxidoreductase specific activities of plasma membranes separated by density gradient centrifugation

	Giant axons	Retinal axons
Sucrose concentration [a]		
Heavy membranes	40 – 50	35 – 38
Light membranes	12 – 18	18 – 25
NADH oxidoreductase [b]		
F-100	960 ± 381 (5) [d]	925 ± 560 (6)
Heavy membranes	1,279 ± 665	3,515
Light membranes	–	980
Na, K-ATPase [c]		
F-100	44.3 ± 8 (5)	110.3 ± 250 (11)
Heavy membranes	48.0 ± 12.5	74
Light membranes	–	40

[a] g %.
[b] nmoles of reduced acceptor/mg protein per min.
[c] μmoles/mg protein per hr.
[d] Number in parentheses indicates number of experiments.

Table 6. Distribution of enzyme units upon fractionation by sucrose gradient centrifugation of membrane fractions isolated from giant and retinal axons of the squid

Enzyme	Dissected giant axons (% of units)	Retinal axons (% of units)
NADHDase		
Heavy membranes	61.5 ± 15 (4)	49 ± 11 (5)
Light membranes	16.0 ± 9 (9)	38 ± 12 (5)
Rest in gradient	18.0 ± 6 (2)	13 ± 4 (5)
Light/heavy ratio	0.26	0.8
Na, K-ATPase		
Heavy membranes	58.0 ± 9 (4)	36 ± 6 (5)
Light membranes	23.0 ± 7 (3)	49 ± 8 (5)
Rest gradient	16.0 ± 8 (4)	15 ± 8 (5)
Light/heavy ratio	0.39	1.4

The units measured in the amount of F-100 placed on the gradient were taken as 100%. The units on heavy or light membranes were calculated from the profile of specific activity of the enzyme along the gradient.

Number in parentheses indicates number of experiments.

yields 3 to 4 times more light than heavy particles upon density gradient centrifugation. On the contrary, the NADHDase units from GAS F-100 favored the heavy membranes in a proportion of 1:4. Since NADHDase specific activity of heavy membranes was twice that of the light membranes,

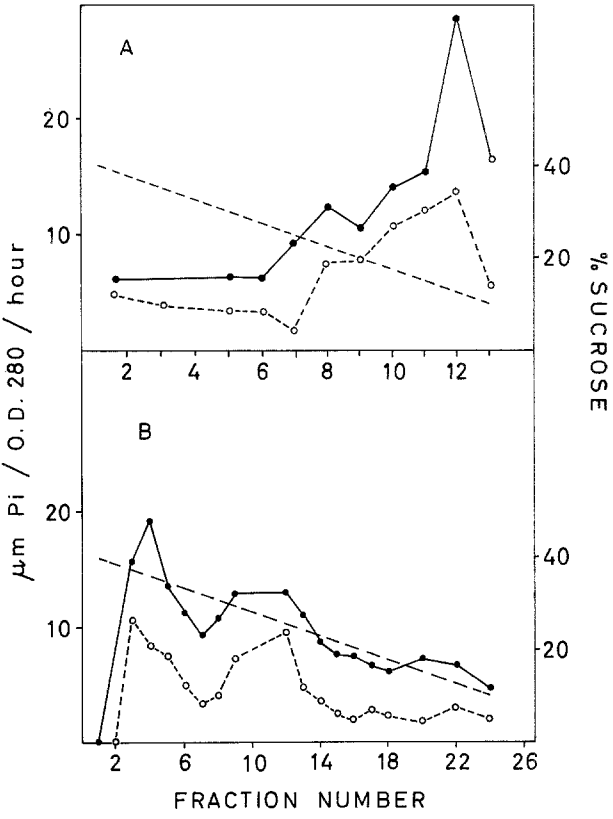


Fig. 6. The distribution of Na, K-ATPase along sucrose gradient centrifugation of membranes (F-100) isolated from giant (*A*) and retinal (*B*) axons of the squid. •-•-•-•, Na, K-ATPase activities; o-o-o, Na, K-ATPase + 10^{-5} M ouabain; -------, % sucrose. (*A*) 0.6 ml of F-100 from giant axons containing 529 μg of membrane proteins were placed on a pre-equilibrated linear gradient of sucrose established between 10 to 40% with 0.5 ml of 50% sucrose as cushion. 253 μg of protein were collected in the cushion (heavy membranes) and its specific activity was 24.6 μM P_i/mg protein per hr. 21.6 ATPase units (μM P_i/hr) were placed in the gradient and 12.4 units were recovered from the cushion (heavy membranes). From the 9.2 ATPase units distributed along the gradient, 6.4 units were measured at low density (light membranes). (*B*) 2.0 ml of F-100 from retinal axons containing 3.6 mg of protein were placed on a similar gradient built up on SW-25 rotor tubes. 3.24 mg of protein were found along the lineal gradient. 1,311 ATPase units were placed on the gradient. 541 units were collected between tubes 1 to 7 (heavy membranes) and 529 units between tubes 8 to 16 (light membranes)

the enzyme units distributed between light and heavy membranes in a ratio of 1:2.

Therefore, the number of membranes floating at low density is lower in GAS as compared with those from RAS.

Fig. 6 displays the distribution of Na, K-ATPase in the F-100 obtained from RAS and GAS by density gradient centrifugation. It can be seen that

retinal axons displayed two bands of specific activity, one in the heavy and one in the light region of the gradient.

The membranes floating at high sucrose densities had 1.4 times higher specific activity than membranes floating at lower density. The F-100 isolated from GAS displayed only one peak between 18 and 12% sucrose. As stated above, most of the enzyme units were recovered at the bottom of the tube.

Table 5 summarizes the specific activity of Na, K-ATPase of membranes isolated from RAS and GAS.

The ratio of ATPase units found between light and heavy membranes changed from 0.4:1 for GAS to 1.4:1 for RAS. To account for the RAS-ATPase activity measured in the area of low sucrose concentration despite its lower specific activity, there would have to be twice as many particles.

Discussion

Even though the morphological A/S ratio demonstrated the predominance of axolemma in the retinal fibers of the squid, it is impossible to estimate to what degree each type of cell membrane withstands the isolation procedure. Therefore, the observed morphological A/S ratio may not be accurately reflected by the ratio of membranes observed by density gradient centrifugation. The experiments described in this work were designed with the aim of comparing results obtained by morphological observation and such isolation procedures. To achieve this, two types of squid fibers with an inverse A/S ratio were exposed to identical isolation procedures.

By the use of two membrane enzyme markers, the particles sedimented at $100{,}000 \times g$ were selected as a plasma membrane-enriched fraction from homogenates of giant and retinal axons. Retinal axons yielded 10 times more ATPase activity in the high-speed than in the low-speed pellet. Giant axons however, had a higher ATPase and NADHDase content in the $1{,}500 \times g$ pellet. These data suggest that the more abundant Schwannlemma of the giant fibers have a greater tendency to pack or co-precipitate. Histochemical studies [9] have shown that the Schwann cell surface has a high concentration of mucopolysaccharides. It is quite possible that these molecules favor plasma membrane aggregation, forming large structures difficult to resolve by gradient centrifugation.

It should also be pointed out that even though retinal axons have a much smaller amount of internal axon membranes, they contained 10 times more ATPase units in the $100{,}000 \times g$ pellet than giant axons.

When the axoplasm was removed from giant axons, a similar amount of ATPase units was also recovered in the high-speed pellet from sheath homogenates. These results demonstrated that the axonal endoplasmic reticulum does not contribute significantly to the heterogeneity of the plasma membrane-enriched fraction.

Further analysis of F-100 by density gradient centrifugation indicated that both types of fibers render an inverse proportion of light to heavy membranes. The light/heavy membrane ratio determined by the distribution of proteins on the gradient, changed from 1.8:1 in RAS to 1:2.8 in GAS. Since only a small fraction of the heavy membranes from GAS entered the high sucrose zone, the exactness of the terms heavy and light membranes may be questioned. This is especially true if the fractionation by the sucrose gradient centrifugation involves size as well as density of the particles. However, we reported previously [4] that glutaraldehyde-fixed membranes did not show significant differences in size in the electron-micrograph.

Some probable explanation for the fact that the F-100 fraction is heterogeneous and contains two types of chemically different membranes are considered below.

(1) The heterogeneity of this fraction might be caused by a difference in the amount of axon internal membranes. Since, in giant fibers, axoplasm accounts for 60% of the proteins, axonal endoplasmic reticulum will prevail over surface membranes in the plasma membrane-enriched fraction of these fibers.

It was shown that the axoplasm extruded from giant axons has an ATPase specific activity 8 times lower than the remaining membranes. Since the per cent of proteins and enzyme units collected from intact axons and sheath were similar, it is difficult to ascribe the higher amount of heavy membranes sedimented from giant axons to their difference in axonal internal membranes. However, it can not be excluded that the membranes collected between 38 to 26% sucrose from F-100 RAS may belong to the scarce endoplasmic reticulum from these fibers.

(2) The heterogeneity of this fraction might be caused by contamination with the outer mitochondrial membranes. These large particulate structures could be broken by sonication and sedimented as small vesicles with endoplasmic reticulum. In our experiments, sonication was applied only in concentrated homogenates for 10 to 20 sec. This is a mild treatment compared with the hypotonic solutions used for homogenization which also disrupt mitochondria.

The ultrastructure of retinal fibers shows that they are richer in mitochondria per unit area than giant axons. It would be expected, therefore,

that the heavy membranes which oxidize NADH would be found in greater proportion in retinal axons. On the contrary, it was found that giant axons yielded a higher amount of these membranes.

On the other hand, the specificity of the electron transport reaction catalyzed by F-100 from both nerves demonstrated clearly that the external mitochondrial membrane is not likely to contribute to its heterogeneity.

(3) The heterogeneity might be caused by a different contribution of axonal and enveloping cell surface membranes.

It was shown by NADHDase and ATPase analysis throughout the gradient that in RAS, the quantity of light membranes prevailed 2:1 over the heavy membranes; in GAS, the ratio of light/heavy membranes decreased to 1:2. These ratios are more similar to those observed by morphological studies.

These findings support the conclusion that the membrane profile of each fiber as observed by density gradient centrifugation reflects their cellular origins.

It was clear from the results that light membranes were more abundant when isolated from retinal axons, the axolemma-rich structure. The amount of these light membranes decreased markedly in the Schwannlemma-rich fibers of giant axons. The experimental data support the hypothesis that the axolemma can be related to the light membrane fraction which was isolated in substantial amounts from the retinal axon fibers.

The technical assistance of Mr. R. Allan and Mr. J. Soto is gratefully acknowledged. Thanks are due to Drs. D. Tosteson, T. Shaw, and B. Fleischer for valuable discussion of these results.

This research was supported by the University of Chile, C.I.C.

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