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The Molecular Organization of Nerve Membranes

VII. Solubilization and Characterization of Proteins from Squid Retinal Axon Membranes

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Summary. Soluble proteins have been obtained from squid retinal axon membrane preparations. By the use of a high concentration of iodide (I^-) or thiocyanate (SCN^-), proteins were solubilized containing less than 5% of the original membrane lipids. These proteins according to their behavior in vacuum filtration, can be divided into high and low molecular weight proteins. They have been partially characterized by exclusion column chromatography, polyacrylamide gel electrophoresis, gradient centrifugation, and analytical ultracentrifugation. They show some tendency toward reaggregation in the absence of sodium dodecyl sulfate (SDS).

The soluble proteins do not show any activity of membrane enzymes and this activity was not recovered upon the incubation with membrane liposomes. Only with Cl^- solubilized preparations was some enzymatic activity observed.

The amino acid composition of both families of proteins was obtained. The high molecular weight proteins are similar to other plasma membranes. The amount of basic amino acids in the low molecular weight proteins was significantly greater than in the high molecular weight fraction.

The axolemma-rich membranes ("light membranes") were isolated from the Schwann cell membranes and the protein solubilized by SDS. By sucrose gradient centrifugation a unique protein band was found in the "axolemma" proteins.

The relationship of the presence of unusual proteins and phospholipids with polyenoic chains is discussed with regard to the molecular organization of an excitable membrane.

In 1967 our group developed a procedure for the isolation and purification of plasma membranes from retinal axon in amounts sufficient for biochemical studies [13, 14]. Because of the particular ultrastructural arrangement of the retinal axons of the squid, the axolemma prevails over the "Schwannlemma" assuring adequate material to obtain nerve membranes. Further investigations made it possible to characterize rather extensively the enzymes, ultrastructure, and lipidic organization of the axolemma preparation [6, 32].

A study of the behavior in density gradient sedimentation for membranes isolated from giant and retinal fibers, which contain opposite ratios of axolemma/Schwannlemma was carried out in our laboratory [22]; it was established that 60 to 75% of the membranes isolated from the retinal axon homogenates came from the axolemma.

The increasing interest in the role of membrane proteins led us to intensify our effort in the fractionation and characterization of the proteins of nerve membranes isolated from retinal axons. This paper reports our studies on the characterization of proteins from this axonal membrane preparation.

Although it is not yet clear whether this membrane retains all the functional attributes of the excitable membrane, evidence obtained in our laboratory indicates that tetrodotoxin binding sites are recovered in the isolated membrane[1]. The characterization of the membrane proteins from the axonal membrane is of special relevance for the understanding of the molecular organization of excitable membranes.

The axonal proteins have been solubilized by various agents and characterized by polyacrylamide gel electrophoresis, exclusion chromatography, sedimentation behavior, and amino acid composition. The soluble proteins have been resolved into two discrete classes with distinct physico-chemical characteristics.

Materials and Methods

Retinal axons were obtained from squid (*D. gigas*) as described by Fischer *et al.* [13]. Axons were kept in 0.75 M sucrose at −30 °C for no more than 60 days. The lyophilized membrane preparation was obtained as described elsewhere [14]; this membrane material was always resuspended in sucrose 0.25 M − Tris-HCl 0.03 M (pH 9.0) (ST) at an approximate concentration of 5 mg of protein/ml. The suspension was homogenized gently with a Teflon pestle and centrifuged for 90 min at 105,000 × *g*. The pellets were resuspended and centrifuged once more. This "washed membrane fractions" was utilized as our starting material.

The solubilized membrane was centrifuged 90 min at 105,000 × *g* and filtered under vacuum against 5 mM Tris-HCl, pH 8.5 in collodion filters to eliminate the solubilizing agent (Schleicher and Schuell Type B-6). By this procedure the membrane proteins were separated into two types of proteins: (a) The high molecular weight (HMW)[2] soluble proteins remained within the filtration device, and (b) the low molecular weight (LMW) soluble proteins which were recovered from the ultrafiltrate.

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1 E. Jaimovich, S. Fischer and C. Urbina. Titration of TTX binding sites in intact axons and isolated plasma membranes from nerve fibers. (*In preparation.*)

2 Abbreviations used: HMW, high molecular weight; LMW, low molecular weight; BuOH, butanol; SDS, sodium dodecyl sulfate; OD, optical density; EM, electron micrograph; AA, amino acid; F-100, membrane fraction.

Solubilization Procedures

Solubilization by Butanol (at 2 and 20 °C). The procedure used was as described [14] with the following modifications: the washed membrane was resuspended in BuOH–H_2O (9:1) at a concentration of 1.0 mg of protein/ml. The aqueous phase was centrifuged at 30,000 × *g* for 30 min and the supernatant concentrated through the collodion filter.

Solubilization by Solvent and Detergent. The washed membrane was suspended at a concentration of 1.0 mg/ml of protein in BuOH, 0.5 M urea, 1 % SDS and 0.01 M mercaptoethanol (MeOH). After incubation at 30 or 37 °C for 60 min, the suspension was centrifuged at 30,000 × *g* for 30 min. The supernatant was then filtered.

Solubilization by SDS. The washed membrane fraction was suspended at a concentration of 1.5 mg of protein/ml in Tris-HCl, 10 mM (pH 8.5). SDS was added to bring the concentration to 1 % and heated at 90 °C for 1 min. After quick cooling the protein concentration was adjusted to 0.5 mg/ml and submitted to filtration. Centrifugation at 45,000 × *g* for 60 min yielded no precipitate; however, one did appear after a partial removal of SDS by exhaustive dialysis (16 hr or more).

Solubilization by the Chaotropic Agents. Washed membrane was resuspended in 1 mg of protein/ml in 50 mM Tris-HCl (pH 8.5) and 1 mM mercaptoethanol. Different anions (Cl^-, I^-, SCN^-) were added to a final concentration of 2, 3 or 4.0 M and stirred for 2 hr at 2 °C centrifuged at 105,000 × *g* for 90 min; the solution was filtrated until negative reaction of the anion occurred.

In some experiments, the incubation time of the membrane suspension with the chaotropic agents was increased without significant change in the yield of soluble proteins.

Polyacrylamide Gel Electrophoresis of Soluble Proteins

Polyacrylamide gels of 6.5 % were prepared according to the method of Maizel *et al.* [21] containing 0.1 % SDS and used without a spacer gel. The electrophoresis tubes had an internal diameter of 0.8 cm and a length of 10 cm. The gels were stained with Amido Schwarz or Coomassie blue and destained in 7 % acetic acid. The distribution of protein bands was obtained with a Canalco densitometer. Essentially similar resolution was obtained by the procedure of Takeyama *et al.* [30] with 7.5 % acrylamide in 5.0 M urea.

Determination of Enzymatic Activities

The (Na + K)-ATPase and NADH oxidoreductase activity in the membrane fraction and the soluble protein were assayed as described previously [5, 33].

The procedure of Lowry *et al.* [19] was used for protein determination. Crystalline bovine serun albumin was used as a standard. Because of color interference, the micro-Kjeldahl technique was used for protein analysis in samples solubilized by NaI.

Lipid Analysis

The lipid content of the washed membrane fraction and the solubilized proteins was analyzed. Lipids were extracted according to the method of Rouser *et al.* [27] with the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (BTH). The lipids were oxydized with acid K dichromate incubated at 100 °C for 30 min and the concentration determined spectrophotometrically (580 nm) with recrystallized cholesterol as standard [4]. Lipid

phosphorus was analyzed as inorganic phosphate [1]. A factor of 22 based on a mean molecular weight of 716 was used to compute the phospholipid content from the phosphate values [32].

Amino Acid Analysis

The solubilized proteins were hydrolyzed with 6.0 M HCl in sealed ampoules evacuated under N_2 for 24 or 48 hr at 112 °C. The acid was eliminated by successive evaporations and the residue suspended finally in 0.2 M citrate buffer (pH 2.2). The AA's were determined in an automatic amino acid analyzer (Beckman model 120-C). The analysis of each sample was repeated at least 3 times and the difference between runs never exceeded 5%.

Sedimentation Coefficient

The soluble proteins were sedimented at 40,000 rpm in a Spinco model E ultracentrifuge and equipped with ultraviolet optics. S^0_{20} was extrapolated graphically for infinite dilution of the sample, using three different protein concentrations.

Column Chromatography

The soluble proteins were separated by exclusion column chromatography (Sephadex G-100 medium) and eluted with 30 mM Tris-HCl (pH 8.5) at 20 °C unless otherwise stated. The flow rate was adjusted to 0.5 ml/min, and the eluate monitored at 280 nm using a Gilson monochromator. The void and the inclusion volumes were determined with blue dextran and ferricyanide, respectively.

Electron-Microscopy

The membrane fraction was examined as described previously [14] using a Phillips electron-microscope (Model EM 300) centered at 60 kV with a built-in anticontaminant device. The soluble protein was suspended in 10 mM Tris-HCl (pH 8.5) and stained with 1% Na phosphotungstate (pH 4.2).

Liposome Preparation

The washed membranes were homogenized in 20 volumes of chloroform-methanol (2:1) saturated with N_2 and centrifuged at 12,000 $\times g$ for 10 min. The pellet was extracted once more and both supernatants dried under a N_2 atmosphere for the precipitation of proteolipids [15]. Observation in the electron-microscope of negatively stained preparations showed the formation of typical liposome vesicles.

Sucrose Gradient Centrifugation

The membrane fraction was resolved by sucrose gradient centrifugation into two protein bands, as previously described [14]. Each band was collected separately, and concentrated by high-speed centrifugation. The proteins of both bands, were solubilized by the SDS procedure. Between 1.4 to 2.0 mg of the solubilized proteins were analyzed by sucrose gradient centrifugation (5 to 20%) in an SW-39 rotor at 35,000 rpm for 12 hr.

Fractions of 6 drops were collected, diluted to 0.3 ml and their optical densities at 280 nm were determined. The linearity of each gradient was verified by measurements of the refractive index.

Results

The lyophilized retinal axon membrane can be stored under vacuum in a dessicator without loss of its enzymatic activity or alteration in its lipid composition and ultrastructure [14, 31]. Evidence presented elsewhere [6] indicates that the extent of contamination with endoplasmic reticulum is negligible in this membrane preparation. However, when the lyophilized membrane was resuspended in ST some loosely bound proteins were recovered in the $105{,}000 \times g$ supernatant [6]. The washed membrane fraction maintained a constant protein/lipid ratio, a constant activity of ouabain-sensitive ATPase and bound glycolitic enzymes. Cytoplasmic contamination was negligible. Therefore, all solubilization procedures were initiated by two washings of the membrane in ST at a concentration of 5 mg of protein/ml. In the last supernatant only trace amounts of proteins were found.

Fig. 1 shows a flow diagram of the procedure used to solubilize and separate membrane proteins by a variety of chemical agents. This was utilized in all experiments with the modifications indicated in each case.

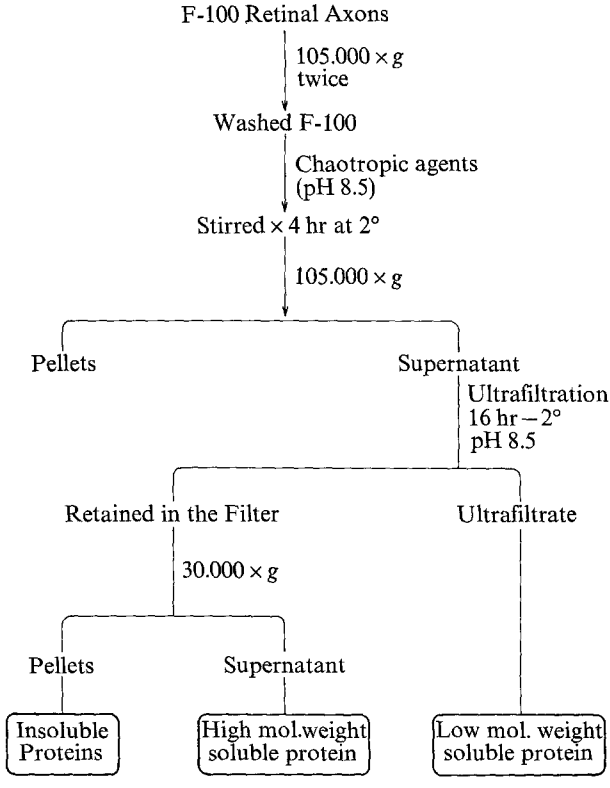

Fig. 1. Flow diagram of the solubilization of axonal membranes

Table 1. Solubilization of proteins from retinal axon membranes

Solubilization procedure	No. of exps.	Soluble proteins		Recovery of soluble proteins[d] (%)
		High molecular weight[a] (%)[c]	Low molecular weight[b] (%)	
Butanol (2–20 °C)	6	12.5 (8.6–17.1)[e]	31 (19.0–43.2)	43 (32.0–51.6)
Butanol-SDS-urea mercaptoethanol	5	64.8 (52.0–69.0)	9.3 (4.1–16.3)	72.4 (64.5–79.8)
SDS −90 °C	3	92.1 (90.0–96.0)	4.3	96

[a] The HMW proteins were those retained by a membrane filtration but not sedimented at 105,000 × *g*.

[b] The LMW proteins were those collected from the filtrate (*see* Materials and Methods).

[c] The percentage of soluble proteins was calculated as $\frac{\mu g \text{ of solubilized proteins}}{\mu g \text{ of membrane proteins}} \times 100$.

[d] The soluble proteins were those floating at 105,000 × *g* for 90 min after the chemical treatment of the retinal axon membranes. The procedures were started with 11 to 65 mg of membrane proteins, equivalent to 30 to 75 mg of membrane dry weight [14].

[e] Range of the experiments.

As an operational definition, the proteins unable to dialyze during filtration through the acetate filters were defined as high molecular weight (HMW) proteins. The exclusion limit of this membrane filter varied between 18,000 and 22,000 molecular weight. The low molecular weight (LMW) soluble proteins where those filtrated and further concentrated in a flash evaporator; they are probably formed by proteins under 22,000 molecular weight. The proteins retained in the filter (HMW) gave a precipitate after a high-speed centrifugation, which varied in size according to the procedure.

Table 1 shows the results obtained by several procedures used to solubilize and characterize the squid axon membrane proteins. It can be seen that the solubilization of the membrane using BuOH at 2 or 20 °C resulted in a low yield of soluble proteins as only 43% were solubilized. The low recovery of HMW proteins is noteworthy.

After the addition of 0.5 M urea and 1% SDS to the butanol at 20 °C, solubility increased significantly reaching 72%, mainly by an increase in the HMW proteins; in contrast, the proteins recovered by butanol alone were mainly LMW proteins. Very small amounts of proteins (under 10%) were solubilized by the use of 6 M urea alone.

Table 2. Phospholipid content of soluble proteins from axonal plasma membrane

Solubilization procedure	No. of exps.	Axonal membrane	Soluble material	
		Phospholipids / Protein	Phospholipids / Protein	Phospholipids[c] (%)
Butanol (2–20 °C)	6	0.83[a]	0.65	23.5
Butanol-SDS-urea mercaptoethanol	5	0.82[a]	1.12	69.4
SDS (90 °C)	3	0.82[a]	1.21	95.5
NaI 3 M (4 °C)	2	1.33[b]	0.10	1.6
NaSCN 4 M (4 °C)	2	1.13[b]	0.17	5.5

[a] Chemical determinations performed on nonwashed membrane fraction.
[b] Chemical determinations performed on membrane fraction washed two times.
[c] $\frac{\text{Phospholipids of the soluble material}}{\text{Phospholipids of the washed membrane fraction}} \times 100$. The phospholipids content of the axonal membrane was $\pm 29.4\%$ of dry weight.

If the membrane suspension was made to 1% SDS and heated for 1 min at 90 °C all membrane proteins were solubilized. After 16 hr of filtration only 4% of the HMW proteins precipitated as insoluble material upon ultracentrifugation; however, when the filtration was extended (48 hr) a significant fraction of the proteins were precipitated. It is clearly shown that the soluble proteins are mainly formed by HMW, in contrast with the recovery obtained with BuOH. Solubilization by SDS at room temperature, or at 37 °C, yielded a lower recovery of soluble proteins.

The proteins solubilized by the last two procedures have a great tendency to reaggregate as judged by their behavior in column chromatography and gel electrophoresis. The attempts to fractionate the proteins by Sephadex-G-100 without the use of detergent in the elution buffer always produced a single peak running with the exclusion volume, containing 55% of the proteins. Similarly, polyacrylamide gel electrophoresis without SDS did not resolve these proteins; they remained close to the origin without further resolution.

Phospholipids were determined in the solubilized materials obtained by different chemical treatment. Table 2 shows the phospholipid/protein ratio of axonal membrane and of a solubilized material. It can be seen that the axonal membrane contains a ratio close to one by weight of phospholipids and proteins. If the lyophilized membrane fractions are resuspended and centrifuged twice some loosely bound proteins are released from the membrane pellets. This increases the ratio to 1.33 and 1.13 which is only slightly

modified by the BuOH-SDS-urea or the SDS treatment. Seventy and 90% of the initial phospholipids were present, respectively, in the solubilized proteins. Therefore, these findings suggest that the detergent acts on the membrane by disrupting lipid-lipid interactions thus leading to the formation of lipoprotein units that do not precipitate at $105{,}000 \times g$. We note that the proteins solubilized by butanol contain 23% of the original membrane phospholipids despite the lower yield of soluble material (40%).

Another procedure used for membrane fragmentation was the application of anions of the "chaotropic" series. These anions cause the solubilization of biological membranes [16, 18]. The action of chaotropic agents is related to their ability to decrease the "ordered" structure of water and increase its lipophilicity. The change in the lipophilicity and in the structure of water weakens the hydrophobic bonds of membranes suspended in aqueous media and increases the solubility of their component proteins and lipids.

To perform these experiments, the membrane suspension was stirred with these anions for 1 to 16 hr at 4 °C. It was established that after 2 hr the maximal solubilization of membrane proteins was achieved and later this period of treatment was used throughout. The yield of soluble proteins was strongly pH dependent and the best recovery was obtained for membrane suspensions at around pH 8.5. Below or above this value the yield of soluble proteins was usually less. After centrifugation of the "chaotropic" membrane suspension a transparent layer was observed floating on the top of the tube; this was easily separated by suction and identified as lipid by chemical analysis.

Table 3 shows the yield of soluble proteins obtained by treatment of axonal membranes with several chaotropic salts. With 2 or 4.0 M NaCl a low recovery of soluble proteins was obtained. A lower concentration of Cl^- (between 0.1 and 0.6 M) yielded negligible amount of soluble proteins. In contrast, a high recovery of soluble proteins was obtained by treatment with I^- or SCN^- (76 and 90%, respectively). The use of SCN^- increased the overall yield of soluble proteins as compared with I^--treated samples. When the chaotropic anions were removed by extensive dialysis (against 5 mM Tris-HCl, pH 8.5), the majority of these proteins still remained in solution. Increasing the anion concentration above 4.0 M had no further effect on solubility.

Another important feature of this procedure is that the phospholipids were almost completely separated from the soluble proteins. Table 2 shows that less than 5% of the initial phospholipids are recovered within the soluble proteins. The lipid/protein ratio of 1.2 for the squid axon membrane de-

Table 3. Solubilization of nerve membrane proteins by chaotropic salts

Solubilization procedure	No. of exps.	Insoluble protein[a] (%)	Recovery of soluble protein[b] (%)	Soluble protein	
				High molecular weight[c] (%)	Low molecular weight[d] (%)
NaCl 4 M	2	71.8 (59.6–84.0)[e]	18.4 (8.4–28.4)	13.5	4.9
NaI 4 M	3	14.0 (9.2–16.3)	76.0 (68.3–84.7)	29.8	46.2
$NaSCN^-$ 4 M + mercaptoethanol	2	12.3 (9.8–14.8)	91.0 (87.1–94.9)	42.9	48.1

The percentage refers to original membrane proteins.

[a] Insoluble proteins are those sedimenting at 105,000 × *g* after the chaotropic treatment of the membranes.

[b] Soluble proteins are those not sedimentable at high speed after the anion treatment.

[c] High molecular weight soluble proteins are those retained by a collodion membrane, but not sedimentable at 30,000 × *g*.

[d] Low molecular weight soluble proteins are those filtered through the collodion membrane, and not sedimentable at high speed.

[e] Range of the experiments.

creased to 0.1. One of the most important achievements of this procedure is that the membrane proteins can now be studied almost free of lipids.

We have shown previously that (Na + K)-ATPase and NADH oxidoreductase are two membrane-bound enzymes present in the axolemma preparations [14]. It was important to determine if the soluble proteins retained this enzymatic activity. In the intact membranes the mean specific activity of both enzymes were 74.3 and 835, respectively, and 49% of the ATPase was inhibited by ouabaine. In the proteins solubilized with 2 or 4 M Cl^-, close to 40% of the ATPase originally present in the membrane fraction was recovered. About 55% of the ouabain-insensitive ATPase and only 11% of the original NADH oxidoreductase activity was found in this fraction. However, using I^-, SCN^-, butanol or detergent, the activity of both enzymes was lost after solubilization (Table 4).

It is possible that the removal of the majority of the membrane lipids by the I^- or SCN^- solubilization procedure could be responsible for the loss of enzyme activity. To test this hypothesis, liposomes were prepared with total lipid extracted from the nerve membrane preparation. No con-

Table 4. (Na + K)-ATPase and NADH oxidoreductase activities of soluble proteins from axo plasma membranes

Solubilization procedure	No. of exps.	Intact membranes			Soluble proteins		
		(Na + K)-ATPase		NADH Oxido-reductase[b]	(Na + K)-ATPase		NADH Oxido-reductas
		Total	+ Ouabaine[a]		Total	+ Ouabaine[a]	
BuOH (2–20 °C)	4	83	–	940	none	–	–
BuOH–SDS-urea mercaptoethanol	3	79	–	820	none	–	none
SDS (90 °C)	2	81	–	919	none	–	none
NaCl 2 M (4 °C)	1	45	26	–	19.2	15.7	none
NaCl 4 M (4 °C)	2	78.5	43	665	28.7	22.5	77.2
NaI 2 M (4 °C)	1	69	43	–	none	none	–
NaSCN 4 M (4 °C)	2	80	40	790	none	none	30.7

[a] Specific activity = μmoles phosphate/mg protein per hr.
[b] Specific activity = μmoles $K_3Fe(CN)_6^{3-}$/mg protein per min.
The incubation media contained in a final volume of 1.0 ml (mM): 160 Tris-Cl (pH 8.: 2 Tris-ATP; 4 Mg; 80 Na; 120 K; and approximately 20 μg of membrane proteins. The ouabai concentration was 5×10^{-4} M. The specific activity of total ATPase varied between 45 and in the 15 experiments; and for NADH oxidoreductase it varied between 586 and 1039.

vincing demonstration of this point could be obtained from incubating liposomes with several concentrations of soluble proteins. No reactivation of enzyme activity was obtained by varying the temperature or incubation time. Therefore, it seems that the native configuration of the soluble proteins cannot be recovered by recombination with liposomes.

To gain information concerning the number of proteins in the soluble material, polyacrylamide gel electrophoresis was performed. Fig. 2 shows the densitometric tracings of membranes and soluble proteins. Several protein bands were obtained from axonal membrane solubilized by SDS; their number is similar to the number of bands reported for other plasma membranes (Fig. 2*a*). The HMW proteins obtained with SCN^- or I^- presented only a few protein bands (Fig. 2*b* and *c*). As stated before, after the removal of the anions by dialysis, only a small amount of insoluble proteins was recovered after centrifugation at $30{,}000 \times g$ for 60 min. From several experiments the insoluble material was pooled and solubilized with 1% SDS and resolved in several protein bands, as shown in Fig. 2*d* and *e*. When the HMW proteins were resolved by gel electrophoresis in the absence of SDS almost all of the proteins remained close to the origin. The LMW proteins obtained by SCN^- treatment were also analyzed by gel electro-

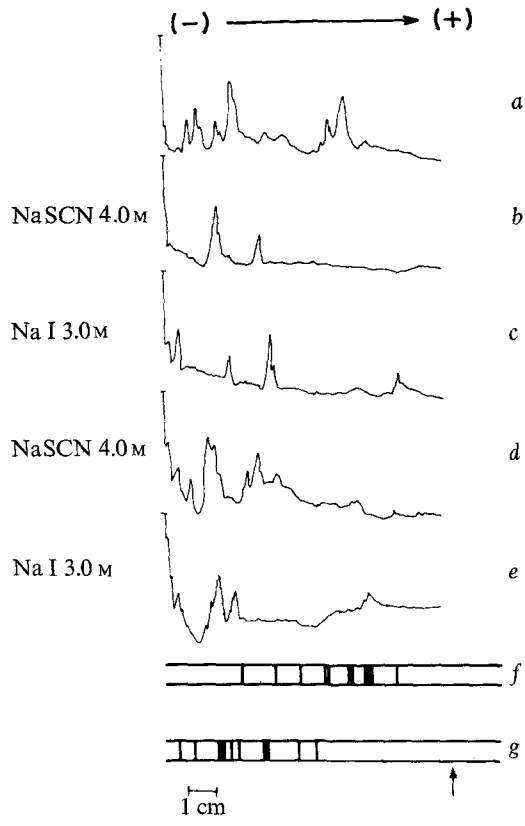

Fig. 2. Polyacrylamide gel electrophoresis of soluble and nonsoluble proteins from plasma membranes isolated from squid retinal axons. (*a*) Densitometric tracing of membrane fraction. SDS was utilized as solubilizing agent. (*b*), (*c*) HMW soluble proteins obtained by the use of chaotropic agents. (*d*), (*e*) Insoluble proteins obtained after removal of the anions. The pellet was collected by high-speed centrifugation from HMW proteins after extensive membrane dialysis and solubilized with 1% SDS. (*See* Materials and Methods for further details on the procedures.) 50 to 100 μg of proteins were applied per gel prepared with SDS. They were run at room temperature for approximately 6 hr at 6 mamps/tube. The gels were stained with Coomassie blue or Amido Schwarz and the absorbancy obtained with a Canalco densitometer. (*f*), (*g*) LMW and HMW proteins solubilized by 4.0 M SCN^-. The conditions for electrophoresis were the same as above. Drawing protein bands were obtained after staining. The arrow represents the migration of the tracking dye

phoresis. Three clearly stained bands and some minor ones were obtained. For ease of comparison the HMW proteins obtained from the same preparation are also shown. It is clear that the bulk of the LMW proteins migrated more than the HMW proteins (Fig. 2 *f* and *g*).

The soluble proteins were analyzed by Sephadex G-100 column chromatography. HMW proteins obtained from membrane treated with 2.0 M

SCN^- were recovered almost completely in the void volume of the column when SDS was not present in the Tris-HCl buffer (Fig. 3*a*). Only 40% of the HMW proteins obtained from membranes treated with 4.0 M SCN^- were retained by the gel and they were separated into three distinct bands when eluted with this buffer (Fig. 3*b*). On the contrary, LMW proteins resolved clearly into four protein bands and none of these proteins were recovered within the void volume of the column (Fig. 3*c*). This may indicate that the LMW proteins were unable to reagreggate when they were chromatographed in the absence of SDS.

To demonstrate if the HMW and LMW proteins resolved by Sephadex were formed by different proteins they were analyzed by gel electrophoresis. The proteins recovered in the void volume and the retained ones from Fig. 3*b* were concentrated and run. Three to four main bands were obtained in both samples (Fig. 3*b'* and *b''*). The proteins recovered from the main optical density peak of the LMW protein (Fig. 3*c*) were equally treated. The three protein bands obtained migrated more than the HMW ones (Fig. 3*c'*). These patterns obtained under total dissociating conditions suggest strongly that HMW and LMW fractions are formed by different peptide species.

The axonal membranes were solubilized with SDS and resolved by Sephadex chromatography in the presence of the detergent; under these experimental conditions the protein profile changed significantly (Fig. 3*d*). Only a small percentage of the proteins were found in the void volume and the remaining material resolved into six bands; this finding indicates that the presence of SDS during solubilization and Sephadex chromatography decreases the tendency of the membrane proteins to reaggregate.

HMW proteins obtained by SCN^- and I^- were also analyzed by linear sucrose gradient centrifugation and resolved into two main components. As shown in Fig. 4, the major peak from SCN^--treated membranes bands at a higher sucrose concentration (around 15%) whereas proteins obtained by I^- banded in two peaks at lower sucrose density.

Attempts were made to obtain an approximate S^0_{20} value for the proteins solubilized by these anions. In the absence of detergent the proteins always banded in a single ultraviolet peak when analyzed in the analytical ultracentrifuge. Three different concentrations of the proteins were analyzed and by graphical extrapolation mean values of S^0_{20} of 4.0 and 1.4×10^{-12} were obtained for proteins solubilized with SCN^- and I^-, respectively.

Protein solubilized by I^- or SCN^- were hydrolyzed and their AA content determined. Table 5 shows that the overall AA content of the HMW proteins solubilized by these anions is quite similar to the values reported

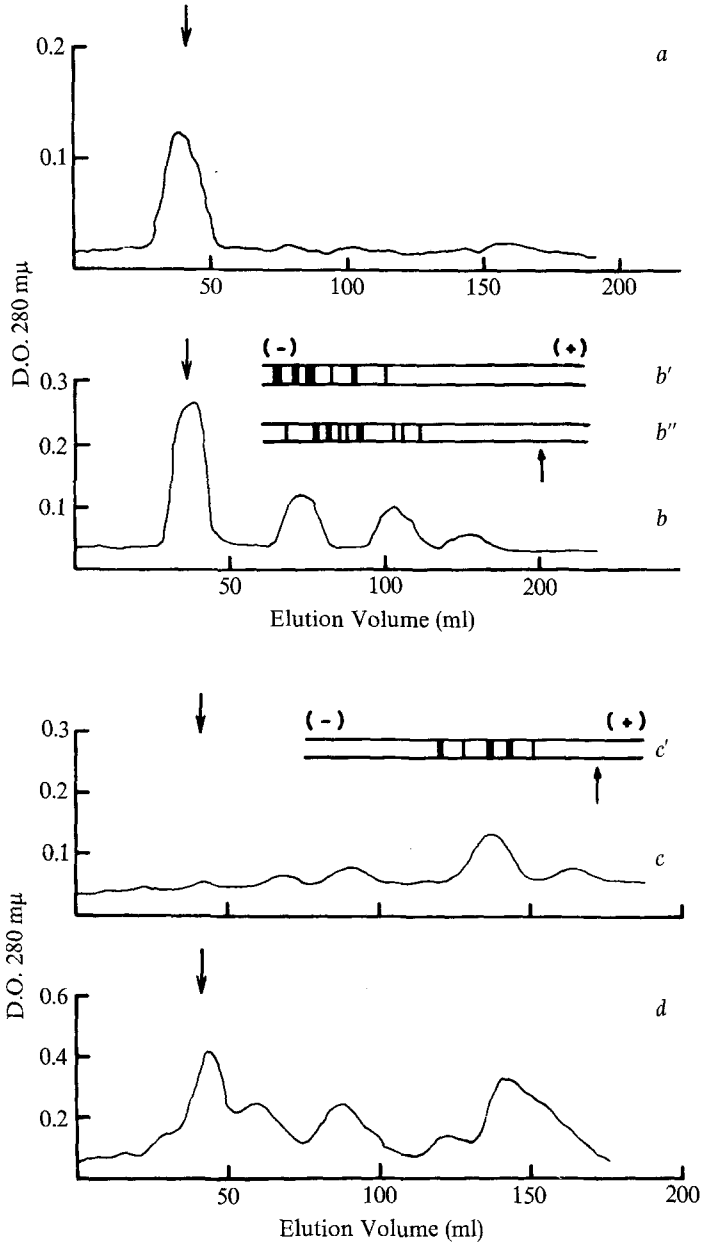

Fig. 3. Sephadex G-100 column chromatography of solubilized proteins from plasma membranes. (*a*) Soluble proteins obtained with 2.0 M SCN^-. (*b*) Soluble proteins obtained with 4.0 M SCN^-. (*b'*), (*b''*) Gel electrophoresis of proteins obtained from the void volume and proteins retained by Sephadex. The tubes were pooled and concentrated separately. The rest of the conditions were as described in Fig. 2. (*c*) Low molecular weight soluble proteins obtained with 4.0 M SCN^-. (*c'*) Gel electrophoresis of the proteins concentrated from the main optical density peak. Conditions as described above. (*d*) Membrane solubilized by SDS and eluted in the presence of 0.8% SDS. In all conditions the eluant was 30 mM Tris-Cl (pH 8.5). The arrow in each chromatogram represents the void volume (determined with blue dextran). Columns of 1.8 × 24.0 cm were used and the runs performed at 4 °C. In (*d*) the column was eluted at room temperature

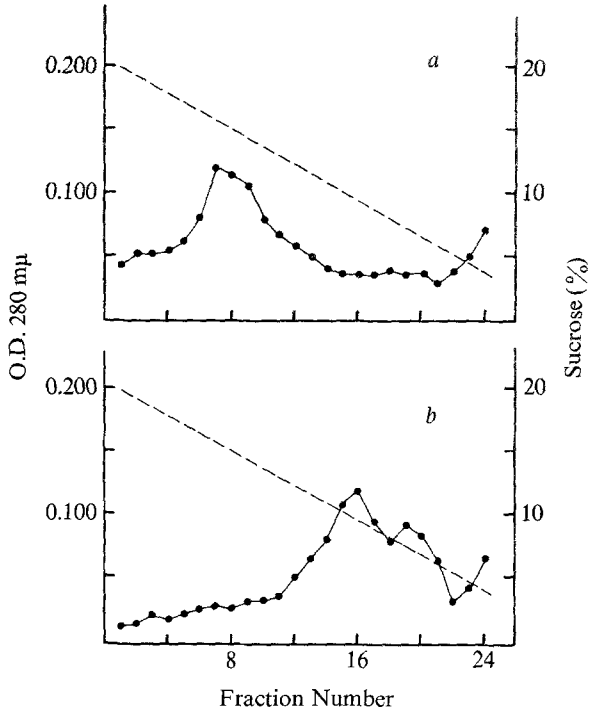

Fig. 4. Sucrose gradient centrifugation of protein solubilized by I^- and SCN^-. HMW proteins were applied to linear sucrose gradient, pre-equilibrated for 4 hr. Approximately 0.8 mg of protein per tube. (*a*) Protein solubilized in 4.0 M SCN^- (*b*) Protein solubilized in 3.0 M I^-

for other cell membrane proteins [17]. However, the LMW proteins had an unexpectedly different AA content. This can be observed easily from Table 6 where the AA's have been arranged in three groups. The amount of basic AA's is almost two times greater in the LMW proteins than in the HMW proteins. This is partially balanced by a relative reduction of the nonpolar AA's. Consequently, the ratio of hydrophobic/polar AA's drops quite significantly in the LMW proteins as compared with HMW material.

One important question is to establish whether the soluble proteins obtained with the chaotropic agent contains vesicles of the intact membrane. Negatively stained preparations of the membrane and of soluble proteins were observed in the electron-microscope. Fig. 5*a* shows a typical appearance of plasma membranes purified from squid retinal axons. They are formed mainly by vesicles of different diameters enclosing multiple vesicles of smaller sizes as described before [14]. Contamination by other subcellular structures is almost absent. This point was also confirmed by an EM obtained from fixed and stained preparations.

Table 5. The amino acid content of membrane proteins solubilized by various chaotropic agents

Amino acids	Proteins[a]			
	High molecular weight			Low molecular weight
	NaI (3 M)	NaSCN (2 M)	NaSCN (4 M)	NaSCN (4 M)
Lysine	5.9[b]	8.4	6.6	9.1
Histidine	2.8	2.3	1.7	6.3
Arginine	4.3	4.8	4.3	7.8
Aspartate	11.4	11.9	13.2	22.6
Threonine	4.0	6.0	6.6	7.4
Serine	6.4	7.6	8.0	5.8
Glutamate	14.6	11.7	12.4	7.6
Proline	4.0	5.3	5.3	3.9
Glycine	7.1	7.0	6.7	4.4
Alanine	8.3	8.3	8.2	6.0
Valine	6.6	6.2	6.2	4.7
Methionine	1.1	2.0	2.9	1.5
Isoleucine	5.0	4.5	4.4	3.0
Leucine	9.7	8.6	8.4	6.0
Tyrosine	1.9	1.5	1.2	0.7
Phenylalanine	3.9	3.9	4.0	3.0

[a] *See* Materials and Methods for the definition of high and low molecular weight proteins.

[b] The results are expressed as: $\frac{\text{moles of AA}}{\text{100 moles of AA}}$. The mean value of at least three determinations are given. The variations were not higher than 5%.

Table 6. The amino acid content of soluble protein obtained from axonal membrane

Amino acids	Proteins			
	High molecular weight			Low molecular weight
	NaI (3 M)	NaSCN (2 M)	NaSCN (4 M)	NaSCN (4 M)
Basic[a]	13.6	15.5	12.6	23.2
Acidic[b]	26	23.6	25.6	30.2
Hydrophobic[c]	28.2	26.7	27.1	18.1
$\frac{\text{Hydrophobic}}{\text{Polar}}$	0.71	0.68	0.70	0.34

[a] Lysine; histidine; arginine.
[b] Aspartic; glutamic.
[c] Valine; methionine; isoleucine; leucine; tyrosine; phenylalanine.
The results were calculated from Table 5.

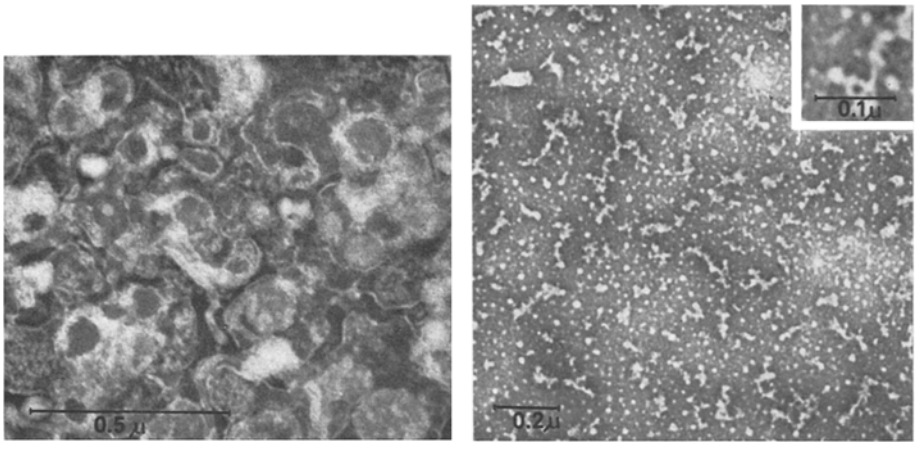

Fig. 5. Electron-micrograph of retinal nerve plasma membrane and soluble proteins. (*a*) Plasma membrane purified, as described [2]. The membranes were suspended in 0.25 M sucrose (pH 8.7). Negative staining was performed with 1 % PTA. Magnification × 40,000; scale: 0.5 μ. (*b*) Negative staining soluble proteins obtained by I^- treatment. The proteins were extensively dialyzed to remove the anion. Magnification × 40,000; scale: 0.2 μ. *Insert*: the same preparation at higher resolution. Magnification × 230,000; scale: 0.1 μ

The soluble proteins obtained by I^- treatment are shown in Fig. 5*b*; an amorphous material is seen in the majority of the fields while in some regions they show a tendency to form rather geometrical arrangement. Membrane vesicles were completely absent. The EM of SCN^--solubilized proteins displayed similar features. However, the proteins obtained by Cl^- treatment show membrane vesicles in a few EM fields although they are of much smaller diameter than the original membranes. The presence of these vesicles may be responsible for the enzyme activity recovered in this preparation.

We have demonstrated that the axonal membrane fractions resolve into two protein bands in sucrose gradient centrifugation [22]. The membrane floating at low sucrose concentration ("light membranes") was tentatively assigned to the axolemma while the membrane recovered at high sucrose concentration ("heavy membranes") was tentatively ascribed as the Schwannlemma and basement membranes.

To study whether or not both types of membranes were formed by different proteins the following experiments were performed. From several sucrose gradient runs the two OD bands were pooled and the membrane pellets recovered by high-speed centrifugation. Both membrane fractions were solubilized by SDS and the soluble proteins resolved by a second

Table 7. Recovery of SDS solubilized protein by sucrose gradient centrifugation from heavy and light axonal membranes[a]

Recovery of soluble proteins	Sucrose density		
	1.073 to 1.061	1.033 to 1.025	1.020 to 1.014
Light membranes	6.0[b]	19.0	44.0
Heavy membranes	27.0	none	47.0

[a] Membrane pellets were purified by lineal sucrose gradient centrifugation and resolved into two protein bands; the first one was located around 38% sucrose (heavy membranes) and the second protein band at 27% sucrose (light membranes). From six runs the heavy and light membranes were separated. Each membrane fraction was centrifuged at $145{,}000 \times g$ for 120 min and from the pellets the proteins were solubilized by SDS. The soluble proteins were then applied to a new sucrose gradient (5 to 20%) made 0.5% with respect to SDS and run at 36,000 rpm in the SW-39 rotor for 16 hr. Nineteen fractions were collected, the optical density (280 nm) obtained, and the soluble proteins recovered from each membrane calculated.

[b] The % recovery was obtained from $\frac{\text{protein in the OD peak}}{\text{total protein in the tube}} \times 100$.

sucrose gradient (*see* Table 7). The results are presented as the percentage of the soluble proteins recovered in the two or three protein bands observed. It can be seen that the "heavy" membrane had more proteins in the high density sucrose area than the "light" membranes, while the amount of proteins found at low density sucrose is similar for both membranes. The light membrane showed a protein band floating at intermediate density ($d = 1.343$ to 1.346) which was not present in the solubilized proteins from the "heavy membranes". This protein fraction accounts for 31% of the light membrane proteins.

Discussion

A number of reports have been published on the solubilization of membranes from erythrocytes [26, 28], rat liver [24], mycoplasma laidlawii [25], synaptic vesicles [7], mitochondria [10] and from other sources. A similar solubilization of the proteins from axonal membranes may allow some structure-function comparison. Squid retinal axons offer the advantage of obtaining large amounts of membrane required for protein studies. However, the membranes prepared from retinal nerves are heterogeneous since Schwann cell membranes account for 30% of the axonal preparation [22]. Accordingly, the heterogeneity of the proteins solubilized from this membrane fraction has to be considered for the interpretation of the results.

Our experiments showed quite clearly that some procedures developed for the solubilization of erythrocyte (RBC) membrane proteins, give poor results on axonal membranes. A good example is the butanol treatment where the protein yield was adequate (around 40%) but the lipid content was similar to that of the original membrane. Several reports have shown that butanol almost completely solubilizes the RBC ghosts' protein while 90 to 95% of the lipids are removed to the butanol phase, achieving complete solubilization and separation of the main molecular components of RBC membrane [20, 34]. This finding contrasts sharply with the effect of this solvent on the axonal membrane where the extent of protein solubilization is lower and a high percentage of the lipids remains associated with the protein. This result suggests that the type and extent of lipid-protein interaction within the axonal membrane is of a different nature than in RBC membranes.

The treatment of erythrocyte membrane with concentrated salt solutions (0.6 M NaCl) solubilized close to 20% of the membrane proteins [23]. If the ghosts were preincubated in the presence of EDTA the yield of soluble proteins increased to 40% [26]. Treatment with even higher concentrations of NaCl (2 to 4 M) solubilized only 15% of the axonal membrane proteins. The presence of EDTA did not modify the recovery in our preparation. This type of experiment shows that charge attraction between membrane lipids and proteins cannot be neutralized by raising the ionic strength. Thus, by means of the NaCl solubilization, large amounts of protein remain membrane-associated or aggregated.

However, when hydrophobic protein-protein, lipid-lipid and/or protein-lipid interactions were affected by detergent or anions most of the membrane protein was solubilized. Almost all the protein was removed by the anionic detergent SDS, but the lipid-protein ratio of the axonal membranes remained unchanged. This result suggests that probably the membrane is being dissolved into small lipoprotein subunits by initially altering lipid-lipid interaction. However, it cannot be excluded that lipid may be in miscellar solution separated from proteins or that lipoproteins are re-formed after SDS removal.

Several anions of the chaotropic series were found suitable for solubilizing as much as 90% of membrane proteins, which contained low amounts of phospholipids, and that over one-half of the material obtained were LMW proteins. It should be noted that HMW proteins, after the removal of the anions, yielded only 20% of the material sedimenting at $30{,}000 \times g$. This insoluble material treated with 2% SDS was resolved into numerous different protein bands by gel electrophoresis. This data may

imply that the insoluble material separated from the HMW proteins is extensively heterogeneous and it can be explained tentatively, as a protein aggregate.

The HMW soluble proteins on the contrary are formed by a rather small number of different polypeptide chains as judged by polyacrylamide gel electrophoresis.

Evidence was obtained demonstrating that HMW proteins obtained by high concentration of anions were able to reaggregate despite their low lipid content. When these proteins were analyzed without SDS, either by density gradient centrifugation, column chromatography, analytical centrifugation or gel electrophoresis, a single protein band was obtained. These results may be explained tentatively as interactions between the polypeptide chains of hydrophobic or electrostatic nature. The use of SDS during these procedures resolved them into several fractions.

In the axonal membrane proteins the overall content of hydrophobic amino acids is rather low (hydrophobic/polar = 0.70) in contrast with a ratio close to unity for RBC ghosts [2, 26] and synaptic membrane proteins [8]. On the other hand, the amino acid content of several membrane proteins gives no indication of a high proportion of nonpolar amino acids. Thus, the hydrophobic interactions in the membrane must have their origin in certain specialized amino acid sequence. The overall amino acid content of the HMW proteins is similar to RBC ghost proteins obtained by NaCl–EDTA [26] and to pyridine-solubilized RBC protein [3].

The LMW proteins have a higher content of basic AA than the HMW proteins with a corresponding decrease of nonpolar amino acids. The LMW proteins isolated from erythrocyte membranes [26] or mitochondrial structural proteins [9] have a much lower content of basic AA, but they resemble more the basic proteins of nerve myelin [12]. We are aware of the difficulties in calculating the net charge of proteins by amino acid analysis since glutamine and asparagine are converted to free acids by the hydrolysis procedure.

Some speculation seems valid at this point. The overall analysis of membrane proteins indicates the presence of an excess of polar residues. However, the axonal membrane has close to 47% acidic lipids, such as phosphatidyl serine and phosphatidyl ethanolamine [32]; the rest of the lipids have no net charge at neutral pH. Without an excess of basic AA in the LMW soluble proteins a marked instability of the overall structure would occur in the absence of counter-ions caused by charge repulsion between the negatively charged phospholipids and proteins. This may be the explanation for the remarkable stability of the axonal membranes at low ionic strength and in the presence of a chelating agent (EDTA) which destroys any structure

stabilized by divalent ion bridges. This contrasts with the remarkable fragmentation of the RBC ghost membrane obtained under those conditions [11].

Previous work has shown that nerve membrane fractionated by sucrose gradient centrifugation from giant and squid retinal axons reflected their cellular origin. The "light membrane" is more abundant when isolated from retinal axons, an axolemma-rich structure [22]. A smaller amount of "light membrane" was obtained from giant axons, the Schwannlemma-rich fibers. It was considered important to analyze the protein solubilized from both types of membrane: the axonal and Schwann membranes ("light" and "heavy" membranes, respectively). Our results suggest the presence of different proteins within both membranes (Table 7). The proteins solubilized from "light membrane" are different from those of "heavy membrane" at least in their behavior during density gradient centrifugation. The axonal membrane is characterized by (a) a low amount of HMW proteins, (b) a higher percentage of LMW proteins, and (c) a protein fraction floating at intermediate density. Further work is required to ascertain these findings.

Recent evidence indicates that the specificity of the lipid-protein interaction seems to be given by the polar groups and the fatty acid chain of the phospholipids as well as the polar and nonpolar regions of the proteins [29]. The axonal membrane, as well as other excitable membranes, were characterized by an unusually high content of polyenoic fatty acid chains in the phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine species [32]. Chains containing four or six double bonds may lead to molecular patches within the membrane greatly different from those containing saturated chains of just one double bond. It is proposed that the particular lipid matrix formed by the high content of polyenoic fatty acid chains and the LMW proteins of the axonal membrane may have functional meaning for an excitable membrane.

Although not one of these questions is actually answered in this paper, the isolation and partial characterization of axonal proteins and lipids would represent an important accomplishment in the study of the molecular organization of nerve membranes. Many of the questions raised in this discussion are now open to experimental approach.

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