The Molecular Organization of Nerve Membranes

IV. The Lipid Composition of Plasma Membranes from Squid Retinal Axons*

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Summary. The lipid content and composition from an axolemma-rich preparation isolated from squid retinal axons was analyzed.

The lipids, which accounted for 45.5% of the dry weight of this membrane, were composed of 22% cholesterol, 66.7% phospholipids and 5.2% free fatty acids. The negatively charged species phosphatidyl ethanolamine (37%), phosphatidyl serine (10%) and lysophosphatidyl ethanolamine (4%) made up 51% of the phospholipids. The amphoteric phosphatidyl choline and sphingomyelin accounted for 39% and 4%, respectively.

The relative distribution of fatty acids in each of the isolated phospholipids was studied. The most remarkable feature of these phospholipids was the large proportion of long-chain polyunsaturated fatty acids. The 22:6 acyl chain accounted for 37% in phosphatidyl ethanolamine, 21.7% in phosphatidyl choline, 17.5% on phosphatidyl serine and 20.3% in sphingomyelin (all expressed as area%).

The molar fraction of unsaturated fatty acids reached 65% in phosphatidyl ethanolamine and 42.0 and 44.8% in phosphatidyl choline and phosphatidyl serine, respectively. The double bond index in these species varied between 1.0 and 2.6.

The lipid composition of the axolemma-rich preparation isolated from squid retinal axons appears to be similar to other excitable plasma membranes in two important features: (a) a low cholesterol/phospholipid molar ratio of 0.61; and (b) the polyunsaturated nature of the fatty acid of their phospholipids.

This particular chemical composition may contribute a great deal to the molecular unstability of excitable membranes.

The development of certain concepts about biological membranes has been prompted by studies of membrane constituents.

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It is well known that lipids may play an essential role both in the molecular organization as well as in the function of membranes. Cholesterol has an important role in increasing the degree of molecular packing in monolayers and enhancing the stability of bilayers (Van Deenen, 1965). The effect of removal and readdition of lipid on enzymic activities of mitochondrial membranes also suggests a high degree of specificity in regard to the function of these membrane constituents (Fleischer, Brierly, Klouwen & Slautterback, 1962; Racker & Bruni, 1968). However, little is known about the components of axonal membranes that may aid in understanding their specialized function.

Several molecular models of nerve excitation have been developed. Goldman (1967) has postulated that the dipoles of certain phospholipids can change their orientation in such a way that the phosphate groups may act as ion exchange gates under the influence of an electric field. A thorough characterization of the lipid components of excitable membranes may strengthen the theoretical approach to the molecular basis of excitation.

Fischer, Cellino, Zambrano, Zampighi, Téllez-Nagel, Marcus and Canessa-Fischer (1970) have recently reported a procedure for isolating an axolemma-rich preparation from the retinal axon of the squid (RAS). By different experimental criteria such as electron microscopy, membrane enzymic markers and chemical analysis, the fraction which sedimented at $100,000 \times g$, was enriched in plasma membranes. This membrane preparation also contained glycolytic enzymes coupled to the synthesis of ATP and GTP (Cecchi, Canessa-Fischer, Maturana & Fischer, 1971).

Plasma membranes isolated from squid nerve fibers exhibiting different axolemma/Schwannlemma morphological ratios were studied by means of differential and density gradient centrifugation (Marcus, Canessa-Fischer, Zampighi & Fischer, 1971). The term axolemma is used here for the external cell membrane of the axon; the term "Schwannlemma" is used for the external membrane of the Schwann cells surrounding the axon.

Retinal axons yielded a greater amount (4:1) of plasma membranes floating at low sucrose concentrations than giant axons (Schwannlemma-rich). On the basis of these experiments it was estimated that about 75% of the membranes isolated from retinal axons were derived from the axolemma.

In the present paper we are reporting studies carried out to determine the lipid composition of this axolemma-rich preparation. The various lipid classes present in the fraction as well as the fatty acid residues of each phospholipid species were identified and quantified.

The data suggest that the excitable membranes might have regions molecularly unstable due to the high percent of polyenic fatty acid species attached to their phospholipids, and to the low cholesterol/phospholipid molar ratio.

Materials and Methods

The plasma membrane fraction (F-100) was obtained from squid retinal axon homogenates as described by Fischer, Cellino, Gariglio and Téllez-Nagel (1968). Each preparation was subsequently analyzed for protein content and membrane enzyme markers [(Na + K)-ATPase and NADH-ferrycyanide oxidoreductase activities]. The F-100 was liophylized in 0.25 M sucrose and stored in sealed tubes at −10 °C prior to its use.

Chemical Determinations of Isolated Plasma Membranes

The membrane dry weight was determined as described by Fischer et al. (1970) and the protein content was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Lipids were extracted from 100 mg of F-100 under dry conditions as described by Rouser, Kritchevsky, Heller and Lieber (1963), except that 50 mg of 2,6-di-tert-butyl-4-methylphenol (BTH)/100 ml of solvent were used. This antioxidant was employed because it allowed more reproducible results than nitrogen flushing. The total lipids were determined in triplicates using Bragdon's procedure (1951), with recrystallized cholesterol as standard.

Lipid phosphorus was analyzed as inorganic phosphate by the Ames's method (1966). The absorbancy was measured either at 820 nm or 700 nm according to the amount of the phosphate in the sample. All glassware used for this analysis was acid cleaned. A factor of 22.37 was used to calculate the amounts of phospholipids based on a mean molecular weight of 716. This mean molecular weight was calculated from the percent of every phospholipid and its average chain length as determined by the fatty acid analysis.

Cholesterol was first separated from the phospholipids in order to avoid interference in the chemical determination of the steroid. (Parson & Yano, 1967). The separation of non-polar lipids from the total extract was accomplished by chromatography on thin-layer (TLC) plates of Silica gel G 20×20 cm and 250 μ in thickness heated for 30 min at 120 °C. The plates were developed with chloroform/methanol/water/acetic acid (25:15:2:4 v/v) up to 13 cm (Skipsks, Peterson & Barclay, 1964), dried for 10 min and redeveloped in the same direction with ethyl ether/petroleum ether/acetic acid (85:15:2 v/v) (Helson, 1967). The spots were visualized by iodine vapor and the cholesterol identified by comparison to the standard R_F.

The two fractions that were oserved (cholesterol and another unidentified neutral lipid) were scraped from the plates. The silica gel was suspended in chloroform/methanol (2:1), and then shaken and centrifuged at $5,900 \times g$ for 10 min. The procedure was repeated twice in duplicate samples. Cholesterol was subsequently determined in pooled supernatants by Lieberman's method (Stadtman, 1957), and the total non-polar fraction by the Bragdon procedure (1951).

Identification and Determination of Individual Phospholipids

We extracted 30 to 40 mg of F-100 by the method of Rouser et al. (1963), and triplicates of aliquots were subjected to two-dimensional TLC. The solvent system I was chloroform/methanol/water (65:25:4 v/v) and solvent system II was butanol/acetic acid/water (60:20:20 v/v), as described by Rouser, Galli and Lieber (1964).

The phospholipids visualized after the exposure to iodine were identified by comparison of their R_F's with standards. Phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE) were prepared from the cephaline fraction of beef extract (Merck) by diethylaminoethanol (DEAE) cellulose column chromatography. Phosphatidyl choline (PC) and sphingomyelin (SM) were prepared from the lecithin fraction of egg yolk extract Type II-E (Sigma) using silicic acid columns according to Rouser *et al.* (1963). Lysophosphatidyl choline (LPC) and lysophosphatidyl ethanolamine (LPE) standards were obtained from Applied Science Laboratories, Inc.

Subsequent characterization was accomplished by the determination of the nitrogen-containing bases. A sufficient amount of every phospholipid for base identification was obtained from 10 plates developed as described above.

Each phospholipid was scraped from the plate and extracted twice with chloroform/methanol/water (65:25:4 v/v) and once with butanol/acetic acid/water (60:20:20 v/v). After evaporation of the solvent, acid hydrolysis was carried out in sealed ampoules with 1.0 ml of methanol/chlorhydric acid (10% w/w) and hydrolyzed at 110 °C for 72 hr. Afterwards, the solvent was evaporated and the HCl was eliminated by adding water. To remove the fatty acids and other materials 1.0 ml of water was added to the samples and extracted twice with 1.0 ml of petroleum ether. We then extracted 2 ml of the organic phase with 1.0 ml of water and evaporated the pooled aqueous phase. The residue was suspended in 0.1 ml of water for high voltage electrophoresis on Whatman 3 MM paper (Katz, Dreyer & Anfinsen, 1959), using formic acid 8% (v/v) pH 1.9 at 36 volt/cm for 90 min.

The nitrogen bases were identified by comparing the R_F with known standards. Ethanolamine, serine and sphingosine were detected with 0.2% ninhydrin in ethanol, after heating for 5 min at 100 °C. Choline was detected with the modified Dragendorff's reagent (Bregoff, Roberts & Delwiche, 1953). Quantitative analysis for every individual phospholipid was carried out by determination of the inorganic phosphate in the presence of silica gel after scraping the plates.

Identification and Determination of Fatty Acid Chains

Preparative TLC. The lipids obtained from 1.0 gr of F-100 (Rouser *et al.*, 1963) were applied as bands on preparative TLC of 500 to 750 μ in thickness. The polar lipids were separated with chloroform/methanol/water (65:25:4 v/v) according to Rouser *et al.* (1964) and the spots revealed by the iodine vapor. This step allowed the separation of PE, LPE, LPC and SM from the PC and PS mixture. The separation of the last two was accomplished by extraction of the silica with chloroform/methanol/water (65:25:4 v/v) and subsequent chromatography in preparative TLC developed by butanol/acetic acid/water (60:20:20 v/v).

The six purified fractions were extracted twice from the silica with 20 ml of chloroform/methanol/water mixture, once with 25 ml of butanol/acetic acid/water mixture and once with 20 ml of methanol. The extract was evaporated and dissolved in ethanol.

Preparation of Fatty Acid Methyl Esters and Gas-Liquid Chromatography (GLC). The purified phospholipid was saponified by adding 1.0 ml of 33% aqueous KOH and 9.0 ml of ethanol and heating it for 20 min at 55 °C. After acidification (HCl), the fatty acids were extracted with petroleum ether and the solvent evaporated. The resulting fatty acids were methylated with methanol using borium trifluoride as the catalytic agent. Two water extractions were used for the removal of the catalyst.

Chromatography of the methylated fatty acids was carried out in a Hewlett-Packard GLC (Model 5750) equipped with the flame iozination detector. Cu-silvered columns of 1/8 inch × 280 cm packed with 18% diethyl glycol succinate on Diatoport (S 80–100

mesh) were used. The temperature of injection was 230 °C for detection. The temperature of the column was first programmed between 130 to 200 °C at a rate of 4 °C/min up to 18:1, and later set in isothermic conditions (200 °C for the longer fatty acid chains). The carrier gas flow was 40 ml/min for nitrogen, and 250 ml/min for hydrogen and air.

Separation of saturated and unsaturated ester up to 22:6 was complete in 60 min. Peaks were identified by comparing their retention in volumes relative to methyl estearate with those obtained for standard methylated fatty acids. The area percent of each component was determined by triangulation.

Conversion factors for quantitative estimation of the mole percentage of the esters were obtained by the use of standard esters; fatty acid methyl ester mixtures corresponding to K 107 and mixture F, or mixture No. 3, or both from Applied Science Laboratories, Inc. This factor was calculated by dividing the known weight percentage in an appropiate synthetic mixture of purified esters by the observed area. The mass of each ester was calculated using the conversion factor. The percentage of the total mass contributed by each component was finally computed. These data were then converted to mole percent of fatty acid.

Identification of the Non-Polar Lipids. We used 1 gr of lyophilized membranes to prepare a Rouser's extract of lipids. Preparative TLC was carried out using ethyl ether/petroleum ether/acetic acid (85:15:2 v/v) to separate the phospholipids (which remain in the origin) from cholesterol and an unknown non-polar fraction. The unknown component was scraped from the plates, extracted with chloroform/methanol (2:1 v/v), methylated and analyzed by gas liquid chromatography as described above.

Standards of triolein and tripalmitin allowed to exclude the presence of glycerides in this fraction.

Results

The axonal membrane preparations used in this investigation have been previously characterized by a high specific activity of (Na + K)-ATPase and NADH-ferricyanide oxidoreductase, by an extremely low succinate dehydrogenase, and by the absence of cytochrome oxidase (Fischer *et al.*, 1970). Furthermore, electron microscopic and chemical analysis indicated that contamination due to mitochondria and endoplasmic reticulum was less than 5%.

Table 1 shows the lipid classes found in the membrane preparation obtained from retinal squid axons. A portion of 45.6% of the plasma mem-

Table 1. *Lipid composition of plasma membranes isolated from the retinal axons of the squid*

Class	% of membrane dry wt.	% of total lipids
Total lipids [a]	45.4 ± 7.5	100
Phospholipids	29.4 ± 3.6	66.7 ± 3.2
Cholesterol	9.8 ± 1.7	22.0 ± 1.6
Free fatty acids [b]	2.3 ± 0.4	5.2 ± 0.5

[a] Mean ± SD of nine different F-100 preparations.
[b] 16:0, 18:0 18:1, (22:1 or 20:4).

Table 2. *Phospholipid composition of plasma membranes from retinal axons of the squid*

Phospholipid	% of total phosphorus
Phosphatidyl ethanolamine	37.3 ± 1.6
Phosphatidyl choline	39.7 ± 2.7
Phosphatidyl serine	9.7 ± 0.9
Sphingomyelin	3.9 ± 0.7
Lysophosphatidyl ethanolamine	3.7 ± 0.5
Lysophosphatidyl choline	2.9 ± 0.7

Mean $\pm$ SD of six different preparations. Phosphorus recovery from the plates was $93.7 \pm 5.4\%$.

brane dry weight is lipid. The extraction of total lipids was carried out by the procedure of Rouser *et al.* (1963) to avoid the loss of cardiolipin and cerebrosides. However, extraction carried out according to Folch-Pi (1955) yielded similar results. The ratio of protein to lipid in this axolemma-rich preparation was calculated to be 0.81. However, since the procedure of Lowry *et al.* (1961) gives rather arbitrary values, it is quite possible that the protein/lipid ratio is really closer to one.

Phospholipids accounted for 66% of the total lipids of our preparation. In the neutral fraction, cholesterol (81%) was identified by comparison with a synthetic standard from its TLC behavior. Free fatty acids, which constitued the remaining 19% of this fraction, were determined by the GLC analyses of their methyl esters.

It should be noted that the sum of lipid phosphorus plus cholesterol and fatty acids gave values of 93.9% of the total lipids. The disagreement with the independent determination of total lipids in the extract by the Bragdon procedure (1951) can be explained by the different sensitivity of the dichromate oxidation technique.

Six phospholipid classes were identified by two-dimensional TLC in the polar fraction of RAS: PE, PC, PS, SM, LPE and LPC. They were characterized by comparison with the R_F of known standards of phospholipids. The purified phospholipids were also submitted to acid hydrolysis to identify the bases by specific sprays after high voltage electrophoresis. This pattern of phospholipids in TLC was confirmed by using different solvent systems, as well as by column chromatography on DEAE-cellulose.

The percent of each phospholipid in the polar fraction was determined by the phosphate analysis of the spots scraped from the plates. Table 2 shows the percent of the six species detectable in this fraction. It is quite possible that the 6% (dry wt.) of lysophosphatides may be derived from autolysis of PC and PE during their lengthly extraction procedure. This

value is of the same order of magnitude as the free fatty acids (5%) determined by a different procedure. It is quite interesting to notice that only four phospholipid species were found in the axolemma-rich preparation. It should be pointed out that the cardiolipin, a typical mitochondrial component, was absent in these membranes. This gives a good indication that contamination by mitochondrial membrane is negligible as established previously by morphological and enzymic criteria (Fischer *et al.*, 1970).

Cerebrosides, typical components of myelin, were not detected in the squid membrane preparation. This is in accordance with the absence or extremely low concentration of myelin in the nervous system of mollusks and some arthropods. PC and PE are the major phospholipids of membrane from RAS since they accounted for 77% of the lipid phosphorus. These values are quite similar to those reported for plasma membrane of squid giant axons (Camejo, Villegas, Barnola & Villegas, 1969), synaptic plasma membrane (Cotman, Blank, Moehl & Snyder, 1969) and retinal outer segment (Nielsen, Fleischer & MacConnell, 1970; Anderson, Feldman & Feldman, 1970). The content of PS is similar to the values reported for these membranes.

The low percent of SM is another important chemical difference of the axolemma-rich preparation in comparison with another neural membrane such as myelin. It should be noted that sphingolipids in nervous tissue seem to have a relative recent evolutionary development, appearing in some but not in all ganglia of cephalopods.

As reported previously (Fischer *et al.*, 1970), RAS membranes can be kept for various months when lyophylized in sucrose solutions.

In the lyophylized membranes, sucrose constituted 75% of its dry weight. It was found to be very important to avoid water uptake during storage; otherwise the TLC pattern appeared with slurred spots. This slurring of spots usually happened after 4 to 6 months in storage and it was correlated with a decrease in the activities of the enzymic markers. Thus, the criteria for the selection of the membrane preparation were three: neat spots in the TLC, maintenance of (Na + K)-ATPase activity and a recovery of 90% of the phosphorus applied. The data presented here were obtained from the squids collected during 1969 and 1970 and no seasonal variation in the lipid composition of the membrane was found.

The nature and chain length of the fatty acid are of vital importance in determining the structural or functional roles which a given lipid may play. Table 3 contains the fatty acid composition of PC, PE and PS which accounted for 87% of the lipid phosphorus. Table 4 contains fatty acid composition of SM, LPE, and LPC. It should be noted that for protection of

Table 3. *Fatty acid composition of the three major phospholipids of plasma membranes from retinal axon of the squid*

Fatty[a] acid	Phosphatidyl choline		Phosphatidyl ethanolamine		Phosphatidyl serine	
	(area %)	(molar %)	(area %)	(molar %)	(area %)	(molar %)
14:0	0.8 ± 0.3	1.0 ± 0.4	0.1 ± 0.04	0.2 ± 0.1	1.5 ± 0.5	1.9 ± 0.7
15:0	—	—	—	—	0.7 ± 0.3	0.8 ± 0.4
16:0	36.4 ± 0.1	44.7 ± 0.2	2.1 ± 0.6	3.0 ± 0.9	9.6 ± 1.5	11.8 ± 1.9
16:1	—	—	—	—	2.1 ± 0.1	2.6 ± 0.1
17:0	1.5 ± 0.5	1.8 ± 0.7	1.6 ± 0.5	2.3 ± 0.8	2.3 ± 0.4	2.8 ± 0.5
18:0	7.6 ± 0.1	9.4 ± 0.2	20.5 ± 2.5	29.5 ± 3.6	32.3 ± 6.2	39.8 ± 7.7
18:1	8.0 ± 0.8	9.9 ± 1.1	6.0 ± 0.7	8.6 ± 1.1	9.4 ± 3.4	11.6 ± 4.2
18:2	4.5 ± 0.6	5.5 ± 0.8	0.4 ± 0.1	0.6 ± 0.2	1.2 ± 0.6	1.5 ± 0.8
18:3 } 20:1	2.7 ± 0.1	3.4 ± 0.1	3.3 ± 0.8	4.8 ± 1.3	1.6 ± 0.5	2.0 ± 0.7
20:2	1.5 ± 0.3	1.6 ± 0.4	4.1 ± 1.0	5.2 ± 1.3	2.6 ± 0.6	2.7 ± 0.7
20:4 } 22:1	10.7 ± 2.3	9.3 ± 2.0	18.3 ± 1.3	18.6 ± 1.4	9.5 ± 1.6	8.3 ± 1.4
20:5	3.0 ± 0.1	2.3 ± 0.1	7.3 ± 1.4	6.6 ± 1.3	7.6 ± 3.1	5.9 ± 2.4
22:4	—	—	—	—	—	—
22:6	21.7 ± 2.3	10.2 ± 1.1	37.3 ± 5.2	20.5 ± 2.9	17.5 ± 2.9	8.2 ± 1.4
Different Membranes	(4)		(6)		(3)	

[a] Number carbons: number double bonds.

unsaturated fatty acids from autoxidation during TLC, BTH was added to the solvents at a concentration of 50 µg/mg of lipid.

The most striking characteristic of the fatty acid present in the axolemma lipids is the unusually high concentration of long-chain polyunsaturated fatty acids. The most abundant unsaturated fatty acid in phospholipids was 22:6. This type prevailed over all others, representing 48% of the total of these unsaturated chains.

It is also notable that the two lysophosphatides possessed almost half of the percent of unsaturated fatty acids than their respective phosphatides. None of the lipid classes contained chains with 24 carbons.

Among the saturated fatty acids, the predominant form in PC was 16:0, while in PE, SM, LPC and LPE, the 18:0 chains represented one-third of the total measured. We have not performed hydrogenation of the fatty acids to elucidate between 20:4 and 22:1 and between 18:3 and 20:1; but it is most probable from data obtained in brain lipids that these fatty acids are 20:4 and 18:3.

Table 4. *Fatty acid composition of sphingomyelin, lysophosphatidyl ethanolamine and lysophosphatidyl choline*

Fatty[a] acid	Sphingomyelin		Lysophosphatidyl ethanolamine		Lysophosphatidyl choline	
	(area %)	(molar %)	(area %)	(molar %)	(area %)	(molar %)
14:0	0.9 ± 0.1	1.1 ± 0.1	2.4 ± 0.8	2.5 ± 0.8	1.4 ± 1.0	2.8 ± 1.5
15:0	—	—	1.6 ± 0.9	1.8 ± 0.9	—	—
16:0	13.6 ± 0.6	16.3 ± 0.7	26.1 ± 10.8	27.7 ± 11.5	28.6 ± 8.1	35.1 ± 9.7
16:1	1.4 ± 0.8	1.7 ± 0.2	—	—	1.0 ± 0.7	1.6 ± 0.7
17:0	3.8 ± 1.7	4.5 ± 2.0	4.1 ± 1.1	4.4 ± 1.1	2.7 ± 0.5	2.6 ± 0.4
18:0	35.5 ± 4.9	42.4 ± 6.0	26.6 ± 6.6	28.3 ± 7.6	38.9 ± 10.4	38.2 ± 10.4
18:1	6.0 ± 1.2	7.2 ± 1.5	12.2 ± 5.6	12.9 ± 5.9	6.3 ± 2.7	8.5 ± 3.7
18:2	0.5 ± 0.1	0.6 ± 0.1	3.7 ± 1.1	3.9 ± 1.2	0.5 ± 0.1	0.5 ± 0.1
18:3 20:1	5.5 ± 0.9	6.5 ± 1.1	4.3 ± 0.8	4.5 ± 0.9	3.9 ± 0.3	4.0 ± 0.3
20:2	1.0 ± 0.6	1.0 ± 0.6	1.7 ± 0.3	1.6 ± 0.3	0.8 ± 0.6	0.7 ± 0.4
20:4 22:1	6.5 ± 2.4	5.5 ± 2.0	5.7 ± 0.7	4.3 ± 0.6	3.7 ± 0.1	2.6 ± 0.1
20:5	4.4 ± 1.7	3.3 ± 1.3	3.1 ± 1.6	2.0 ± 1.0	2.1 ± 0.1	1.2 ± 0.1
22:4	0.8 ± 0.3	0.5 ± 0.2	2.8 ± 1.6	1.6 ± 0.9	—	—
22:6	20.3 ± 2.4	9.3 ± 1.1	11.1 ± 1.8	4.5 ± 0.7	10.4 ± 1.2	3.6 ± 0.4
Number of different membranes	(4)		(3)		(3)	

[a] Number carbons: number double bonds.

Table 5. *Fatty acid unsaturation of phospholipids isolated from retinal axon membranes*

Phospholipid	Molar unsaturation (%)	Double bond index[a]
Phosphatidyl choline	42.1 ± 2.1	1.1–1.4
Phosphatidyl ethanolamine	65.0 ± 2.9	2.0–2.6
Phosphatidyl serine	44.8 ± 4.3	1.1–1.4
Sphingomyelin	35.7 ± 3.5	1.0–1.3
Lysophosphatidyl ethanolamine	35.4 ± 1.7	0.8–1.0
Lysophosphatidyl choline	25.5 ± 1.4	0.5–0.6

[a] See text for additional details.

Another interesting molecular species is SM, which contains as much as 35.6% of unsaturated fatty acids.

Table 5 summarizes the molar percent of fatty acids and the double bond index in the six phospholipid species found in the axolemma-rich preparation.

The average degree of unsaturation based on double-bond index, according to Richardson, Tapell, Smith, and Houle (1962), is the sum of products of the molar fraction of each fatty acid and its number of double bonds. In order to calculate this index for RAS membrane the extreme values were used since we could not differentiate between fatty acids 18:3 and 20:1 or 22:1 and 20:4.

Discussion

The chemical composition of several plasma membranes seems to indicate some correlation between the function of these structures and their lipid composition as proposed by Fleischer and Rouser (1965). Myelin that represents the metabolically more-stable membrane, has a high content of cholesterol. Excitable membranes such as rat brain synaptosome, bovine retinal outer segments and our axolemma preparation, show instead a low cholesterol content, a remarkably higher protein/lipid ratio and a lower cholesterol/phospholipid ratio.

The cholesterol/phospholipid molar ratio found for retinal axon plasma membranes was 0.61, significantly lower than 0.9 of myelin (Table 6). Cholesterol has been normally visualized in association with membrane phospholipids, and its interactions with different lecithins has been studied extensively in monolayers (Demel, Van Deenen & Pethica, 1967; Shah &

Table 6. *Protein and lipid composition of several isolated plasma membranes*

Membrane	Protein/lipid (w/w)	Cholesterol (% weight of total lipids)	Molar ratio (cholesterol/ phospholipid)	Double bond index	
				(PC)	(PE)
Myelin (1)[a]	0.25	25.00	0.90	0.69	0.96
Mitochondria from guinea-pig liver (2, 3)[a]	6.36	1.40	0.03	1.01	1.16
Bovine retinal outer segment fragments (4)[a]	0.85	5.93	0.24	0.98	1.93
Synaptic membrane from rat brain (5)[a]	1.12	8.74	0.50	0.64	2.95
Membrane from giant I.	0.42	19.80	0.82	0.22	0.14
squid axons (6)[a] II.	0.93	13.00	0.70	0.22	0.20
Membrane from retinal squid axons (7)[a]	0.81	8.00	0.61	1.10 to 1.44	1.99 to 2.65

[a] References: (1) O'Brien and Sampson (1965a, b); (2) Parsons *et al.* (1967); (3) Parkes *et al.* (1970); (4) Nielsen *et al.* (1970); (5) Cotman *et al.* (1969); (6) Camejo *et al.* (1969); (7) from Tables 1, 2 and 3 of this paper.

Schulman, 1967). It seems that cholesterol increases the apolar interaction of the hydrocarbon area of phospholipid (Chapman, Kamat, De Gier & Penkett, 1968) and for this reason, membranes whose cholesterol content is low are more loosely organized and possess a less stable structure. Also, in studies using liposomes, the role of cholesterol can be interpreted as reducing the chain mobility and consequently, the permeability of this model system (De Gier, Mandershoot & Van Deenen, 1968). Accordingly, the low cholesterol content found in retinal plasma membranes may be an important requirement for a very active membrane.

Only four phospholipids were detectable in the axolemma-rich preparation plus two lysophosphatides probably formed by autolysis of PE and PC. The lipids of these membranes were devoid of diphosphatidyl glycerol, phosphatidyl inositol and N-acetyl neuraminic acid gangliosides. However, the presence of glycolyl groups in the sialic acid as described by Warren (1963) was not investigated.

Our phospholipid composition agrees more closely with those reported by Camejo et al. (1969) for membranes isolated from the entire trunk which contains the giant axons. However, it should be noted that the free fatty acid content of giant axon membrane is similar to retinal axon membranes, despite the fact that lysophosphatides were not detected by these authors in the TLC separation.

The major axolemma phospholipids were PC, PE and PS, the sum of negatively charged molecules being 50% of the total lipid phosphorus.

The results are also in accordance with the phospholipid composition of Loligo axons (Condrea & Rosenberg, 1968).

The most noticeable feature of RAS membranes was that PE, one of the major phospholipids, has 65% of the fatty acid molecules unsaturated; other phospholipids contained approximately 50 molar percent of highly unsaturated long-chain fatty acids. Most of the polyunsaturated fatty acid is 22:6, which accounted for 37% of the area in PE and 21.7% in PC.

The data here reported for the fatty acid composition of squid retinal axon membranes differ from those obtained by Camejo et al. (1969) from giant axons. The low percentage of monoenoic fatty acids and the high cholesterol content described by these authors might be caused either by the prevalence of Schwannlemma in this preparation or by their different method of isolation.

The distribution of fatty acid residues is not fixed for any given tissue or specie, but depends upon enviromental conditions, such as temperature and diet. Thus, it is quite possible that this particular fatty acid composition of RAS only represents adaptation of these squids to their environment. It

should be mentioned that the brain fatty acids of goldfish acclimatized to lower temperatures showed a greater degree of unsaturation (Johnston & Roots, 1964). However, the 22:4 and 22:6 content increased approximately only 5% when the temperature decreased from 30 to 5 °C. The fatty acid composition of mitochondria, a well known unsaturated membrane, can also be altered with changes in the diet. Studies done by Witting, Harvey, Century and Zlowith (1961) have showed that in rats maintained on a cod liver oil rich diet for five months, the 22:6 fatty acid content of liver mitochondria increased up to 14% (w/w). Since the increase of polyunsaturated fatty acid observed after drastic changes in temperature or diet never reaches the values reported here for retinal axon plasma membranes it seems rather unlikely that they are caused by the environmental conditions of the squid from our coast.

Only two membranes with similar high content of longer polyunsaturated fatty acids have been reported: (1) synaptic plasma membrane isolated from rat brain (Cotman et al., 1969); and (2) retinal outer segment (Anderson et al., 1970; Nielsen et al., 1970). Table 6 shows that the double bond index of the PE in these two excitable membranes is quite similar to RAS and approximately duplicates the values reported for guinea pig liver mitochondria, another metabolically active membrane-structure (Parkes & Thompson, 1970).

The cohesion between lipid molecules is significantly disturbed by the presence of unsaturated bonds, and there is general agreement that such a factor would greatly influence the packing of the lipid molecule in the membrane (Van Deenen, 1965). The introduction of unsaturated fatty acids into a bimolecular leaflet would lead to a more loosely packed and less stable structure (O'Brien, 1963). Studies carried out in liposomes (De Gier et al., 1968) and in erythrocyte membranes (Walker & Kummerow, 1964) showed an important enhancement of permeability when the number of double-bonds increased in the phospholipids.

The presence of a low cholesterol content in a long chain of polyunsaturated fatty acids in RAS may have important consequences for its physico-chemical properties. Thus, the lipid arrangement in these membranes gives rise to a more fluid and dynamic structure.

Monolayers formed with the phospholipids of retinal axon membranes carried out by Wolff, Canessa-Fischer, Vargas and Diaz (1971) have demonstrated an expanded structure with a mean molecular area of 80 Å/molecule.

Studies with synthetic and natural phospholipids have suggested that the lipids in biological membranes are normally in a liquid expanded state (Van Deenen, 1966). We would like to suggest that the high content of long-

chain polyunsaturated fatty acids may provide the appropiate solid/liquid ratio, degree of expansibility and hydrophilic properties necessary for the maintanance of a liquid-crystalline system (Johnston *et al.*, 1964). Excitable membranes containing these molecules can undergo suitable phase transition when influenced by an electrical or chemical event.

These fatty acid residues would not only influence the molecular arrangement of the lipid molecules, but also, the types of lipid-protein interaction holding the structure of the axolemma.

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