Lipid and Fatty Acid Composition of Frog Photoreceptor Outer Segments†

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ABSTRACT: Frog rod outer segments purified by density gradient centrifugation were studied to determine the phospholipid and neutral lipid contents as well as the respective fatty acid profiles of these classes. The outer segment photoreceptor membranes contained large quantities of palmitic (16:0), stearic (18:0), linolenic (18:3), and docosahexanoic (22:6) acid. Glycolipid was found to be in relative abundance (25% of the total lipid) and cholesterol in smaller quantities (2%). The photoreceptor membrane consists of 61% protein and 39% lipid; rhodopsin was 27% of the total lipoprotein of the membrane on a dry weight basis. Phosphatidylcholine (45%), phosphatidylethanolamine (26%), phosphatidylserine (15.1%), and sphingomyelin (6.4%) were the predominant phospholipid components. These results confirm the general fluidity characteristics associated with frog disk photoreceptor membranes on the basis of more physicochemical studies.

The primary photopic receptor of the vertebrate retina is the rod outer segment. These vertebrate rods consist of highly organized laminar stacks of flattened saccules surrounded by a plasma membrane. The outer segments are in turn connected to the remainder of the cell body containing the energetic and synaptic machinery of the cell. The flattened disk-like membranes of the rod contain the photosensitive visual pigment rhodopsin, a hydrophobic protein of mol wt 40,000 (Robinson *et al.*, 1972).

Due to the strongly hydrophobic nature of rhodopsin and the apparent bilayer character of the disk membranes (Blasie and Worthington, 1969; Worthington, 1971; Blasie, 1972) it seems likely that membrane lipid plays an important role in the visual excitation process. Indeed, on the basis of freeze–cleave–etch electron microscopy experiments (Mason *et al.*, 1972, 1973b) and X-ray diffraction data (Blasie, 1972), it has been suggested that rhodopsin may become more deeply immersed in the lipid phase of the bilayer upon light exposure. Because a strong protein–lipid interaction does appear to be present in disk membranes, it is of importance to characterize as fully as possible the lipid of rod outer segments.

Aside from the work of Eichberg and Hess (1967), neither the phospholipid, glycolipid, and neutral lipid nor the fatty acid moieties of these fractions have been studied in the frog photoreceptor membrane. This disparity is even more notable because the frog retina has been the single most important tissue in the biochemical and physiological investigations of the visual process. We therefore have investigated the lipid nature of these photoreceptors and have found some basic similarities to other well-studied systems such as the cattle photoreceptors (Borggreven *et al.*, 1970; Nielsen *et al.*, 1970; Anderson *et al.*, 1970; Poincelot and Abrahamson, 1970; Poincelot and Zull, 1969) as well as dissimilarities to the photopic system of the invertebrate squid (Mason *et al.*, 1973a). The data obtained here lend strong support to the view of the frog photoreceptor membrane as a highly fluid milieu, one in which chromophore rotation and a gross movement of the light-sensitive protein may readily occur.

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Experimental Procedure

Methods. Rod outer segments were prepared under red light from the retinas of dark adapted *Rana catesbiana.* Retinas were gently dissected and shaken in 50% sucrose in 0.1 M sodium phosphate buffer, pH 7.2. Buffer was then layered over the sucrose and the preparation centrifuged for 30 min at 25,000*g*. Material at the buffer–sucrose interface was primarily rod outer segments and mitochondria; this material was placed on a 25–40% sucrose continuous density gradient and centrifuged at 25,000*g* for 1 hr. The purified rods were removed from the gradient by means of a hole punctured in the tube bottom. Rod outer segment material was then washed once with phosphate buffer and three times with distilled water, and finally lyophilized to dryness. The final material was also extracted with 3% emulphogene in 0.15 M hydroxylamine, and this extract examined for spectral absorbance on a Cary 14 spectrophotometer.

Rods prepared in this way were examined for purity by negative staining with 3% phosphotungstate in 1% bovine serum albumin (pH 7.0) on carbon-coated copper grids. Grids were examined on a Hitachi 11-UA electron microscope. Samples were also examined by light microscopy on a Reichert optical microscope. All results given are for extracts purified from four separate frog preparations in an identical manner.

Pure lyophilized frog rods were doubly extracted under N_2 with $CHCl_3$–CH_3OH (2:1) by shaking in the light for 4 hr per extraction. The extracted rod material was removed by filtration and the resulting lipid extract reduced in volume by evaporation under N_2. Aliquots of this total lipid extract were taken for determination of lipid phosphorus, percentage of glycolipid (as hexose), and total cholesterol; additional samples were used for fractionation of the lipid classes by thin layer chromatography as well as for methylation and gas–liquid chromatographic analysis. A value of the total dry weight percentage lipid was also obtained from quantitative extraction of the pure frog rods.

Separation of phospholipid components for analytical purposes was accomplished on plates prepared by the method of Peifer (1962), and additional samples were chromatographed two dimensionally by a modified technique of Rouser *et al.* (1963). When phospholipid was studied, samples of the extract were spotted at 5–7 mm from the base of the plate and

TABLE I: Chemical Composition of *Rana catesbiana* Rod Outer Segments.

Class	% of Total Membrane Content
Protein	60.4[a]
Lipid	39.6[a]
(a) phospholipid	74.6[b] (29.5)
(b) glycolipid (as hexose × 4.6)	25.4[b] (10.1)
Cholesterol	2.18[c]
Rhodopsin as lipoprotein	27.0[d]

[a] Expressed as percentage of dry weight rod outer segments. [b] Expressed as the percentage of total lipid; values in parentheses are the percentage of total dry weight. [c] Value is the average of six separate determinations by chemical and gravimetric analyses. [d] Determined using ϵ 42,000 cm^{-1} and a mol wt of 40,000 for frog rhodopsin; value is calculated as a percentage of the dry weight of the purified membranes and reflects the rhodopsin content relative to the total membrane constituents.

chromatographed in petroleum ether to carry neutral lipid to the top of the plate. The plates were dried and redeveloped in $CHCl_3$–CH_3OH–acetic acid–H_2O (75:25:5:4); samples were identified by means of a chromic acid spray and subsequent charring of the sample and a standard plate containing phospholipids. Uncharred plates were then scraped to remove the isolated phospholipid class; the silica gel scraped from the plate was then eluted into $CHCl_3$–CH_3OH (2:1) and centrifuged. The organic solvent containing phospholipid was evaporated under N_2 and used for further analysis of phosphorus and fatty acid determination as the methyl ester. Samples were additionally rechromatographed to achieve higher purity or better separation of the desired component.

Neutral lipids were studied in a similar fashion. The solvent system used for separation of cholesterol, triglyceride, and other components was hexane–ethyl ether–acetic acid (85:20:1). Samples were obtained for further analysis by elution into hexane. With the above techniques, excellent chromatographic separation was achieved and samples were readily identified by means of comparison of R_F values with those obtained from similarly chromatographed standards.

Cholesterol was determined by the method of Abell *et al.* (1952) using a Lieberman–Burchard reagent. Phosphate hydrolyzed from phospholipid was determined by the technique reported by Mason *et al.* (1973a). Hexose was assayed using the anthrone reaction, *i.e.*, 10 ml of 0.2% anthrone in concentrated H_2SO_4 was added to an aliquot of the total lipid sample and incubated at 90° for 20 min, and the optical density was determined at 625 nm.

Methyl esters from phospholipids, neutral lipid, and the total lipid fraction were prepared by a modified technique of Metcalfe and Schmitz (1961). Samples were subjected to saponification with methanolic KOH followed by methyl esterification with methanolic boron trifluoride. The esters were extracted into hexane and 2–3-μl aliquots of this solution containing 15–30 μg of fatty acid esters were injected into a 5 ft × $^1/_8$ in. stainless steel column (temp, 198°; N_2 carrier, 11 psi) containing 20% diethylene glycol succinate on a Chromosorb W (80–100 mesh) support. A Varian A-600 gas chromatograph with flame ionization detector was utilized (temp, 189°; hydrogen, 15 psi). Fatty acid components were identified on the basis of: (1) comparison with methyl ester standards (Supelco); (2) a semilog plot of carbon number *vs.* retention time; and (3) hydrogenation of the fatty acid sample and rechromatography.

Fatty acid methyl esters were catalytically hydrogenated by the addition of 2–2.5 mg of palladium oxide to 2 ml of a hexane–ethanol (2:1, v/v) solution containing the methyl esters. Hydrogen gas was then bubbled through this solution; when the catalyst darkened and settled out, the reaction was presumed to have reached completion.

Quantitative measurement of fatty acid components was achieved by estimation of peak areas, this value being directly proportional to the retention time and peak height. An additional correction factor derived from chromatographic analysis of pure methyl esters was utilized in these studies. This value was determined for the particular column and packing in use according to the method of Tandy *et al.* (1961).

Materials. Fatty acid methyl ester standards and column packings for gas–liquid chromatography were obtained from Supelco. Silca gel G was purchased from Merck, and the detergent emulphogene was obtained from GAF, Inc. Phospholipid standards were purchased from Sigma.

All organic solvents were of spectrally pure, reagent grade quality. Methanolic BF_3 was prepared by bubbling gaseous BF_3 through 1 l. of methanol until 125 g had been dissolved.

Results

The lipid composition reported here is the result of determinations on rod outer segment membranes which appear to be pure by several criteria. Upon detergent extraction of these membranes, a spectral ratio (A_{278}/A_{500}) of 2.2 was obtained. Additionally, examination of isolated membranes by light and electron microscopy revealed few if any noticeable mitochondria or other organelles. On this basis, it seems likely that the gradient-purified membranes were probably contaminated with less than 5% of other non-outer segment material.

Chemical Composition. The chemical composition of rod outer segments from the frog is given in Table I. The fraction of the membrane which is protein is 61.4%, a value similar to that found for bovine rod outer segments by other workers, *i.e.*, approximately 62% (Poincelot and Zull, 1969; Borggreven *et al.*, 1970; Adams, 1967; Sjostrand, 1959; Hubbard, 1953; Collins *et al.*, 1952). Furthermore, the values obtained for total protein and lipid percentages are close to those obtained for *Rana pipiens* by Eichberg and Hess (1967). The outer segments of the frog retina also contain approximately 2% cholesterol. This value is similar to the value of 1–3% cholesterol obtained for the vertebrate cattle retina by Borggreven *et al.* (1970). The frog rod outer segments also contain 27% rhodopsin as lipoprotein, as determined from the dry weight of purified lyophilized material, and using an extinction coefficient of 42,000 cm^{-1} and mol wt of 40,000 for rhodopsin. The value obtained here may be a slight underestimation due to the hygroscopic properties of lyophilized biological material, but does reflect the amount of rhodopsin present in the membrane relative to lipid, protein, polysaccharide, and other components. The membranes as prepared here contain rhodopsin as the major protein component (≫80%).

It is also of interest to note that not a small fraction of the total lipid is glycolipid (25.4%); this value indicates that ap-

TABLE II: Phospholipid Composition of *Rana catesbiana* Rod Outer Segments.

Phosphatidylcholine	44.6
Phosphatidylethanolamine	26.1
Phosphatidylserine	15.1
Phosphatidylinositol	2.1
Sphingomyelin	6.4
Phosphatidic acid	5.3
Other minor components	1.3

proximately 10% of the total membrane is glycolipid, a value in good agreement with that found by Eichberg and Hess (1967).

Phospholipid Composition. The phospholipid composition of rod outer segments of the frog is given in Table II. Rod outer segments of the frog retina contain 45% phosphatidylcholine, 26% phosphatidylethanolamine, 15% phosphatidylserine, and 5.6% sphingomyelin. Phosphatidic acid also migrates near the front of the phospholipid solvent system used here and this component is estimated to comprise approximately 5% of the total phospholipid.

Additionally, several minor components (1–2%) were detected upon two-dimensional chromatography. These components strongly resemble diphosphatidylglycerol and possibly some small amounts of lysophosphatidylethanolamine or lysophosphatidylcholine components.

Fatty Acid Composition. The major fatty acid components of frog rod outer segments are palmitic acid (16:0), stearic acid (18:0), linolenic acid (18:3), and docosahexanoic acid (22:6). In Table III is given the complete fatty acid composition of the total lipid extract as well as of each phospholipid class. From the fatty acid profile given in this table, it is apparent that the major fatty acids predominate in only two or three of the major phospholipid classes, and are systematically excluded from other phospholipids. Docosanoic acid (22:0) appears to be a major constituent of several of the phospholipids, although it is only present as 4% of the total lipid.

The photoreceptor membranes studied here are also observed to contain predominantly long chain fatty acids (18 carbons and above) as well as a high degree of unsaturation (61%).

TABLE III: Fatty Acid Composition of Phospholipid Components of Frog Rod Outer Segments.

		Component[a]			
Fatty Acid	Total Lipid	PC	PE	PS	Sph
10:0	0.44	0.12	0.73	0.34	
11:0		Trace	0.42		2.03
12:0	3.02	0.21	4.96	3.12	2.36
13:0	2.93	2.07	5.29	2.46	3.81
14:0	3.01	0.55	3.93	4.15	6.33
15:0	4.48	2.23	7.24	4.20	4.23
16:0	13.02	3.96	11.26	13.65	14.14
16:1	4.51	2.45	6.64	2.78	1.38
17:0	0.52	0.25	1.67	1.16	4.40
18:0	8.65	13.8	5.90	7.61	0.31
18:1	4.48	6.10	3.28	4.28	2.66
18:2	3.40	4.03	2.26	2.17	13.57
18:3	6.93	17.36	13.88	5.61	12.19
19:0	0.53	Trace	0.89		0.23
20:0	1.02		1.04	0.41	1.43
20:3	1.63	Trace	1.72	1.14	1.26
20:4	1.21	1.36	1.60	1.37	4.30
20:5	3.11	8.23	1.10	2.04	0.71
21:0	Trace		Trace	0.56	
22:0	4.15	11.0	9.18	3.12	0.87
22:4	4.50	4.91	4.24	5.43	1.06
22:5	3.29	3.93	0.94	4.12	3.14
22:6	15.85	6.84	4.16	24.16	3.90
24:0	1.37	6.55	1.32	3.20	6.69
24:1	2.54	1.41	1.10	1.07	0.41
% below 18:0	31.93	11.84	42.14	31.87	38.69
% above 18:0	68.07	88.16	57.86	68.13	61.31
% saturated	39.01	40.74	44.73	40.86	45.96
% unsaturated	60.99	59.26	55.37	59.14	59.05

[a] Abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin.

Discussion

Frog rod outer segments are composed almost exclusively of disk membranes containing rhodopsin, the plasma membrane envelope being almost negligible in terms of total membrane. It is therefore possible to assume that the lipid composition reported here reflects that of the saccule membrane as well as the probable lipid environment of rhodopsin.

Recent experiments suggest rhodopsin may prove laterally and horizontally in the disk membrane. These studies indicate that rhodopsin in the frog disk membrane appears to undergo a change in the amount and type of induced birefringence (Brown, 1972) as well as free rotation in the plane of the disk membrane (Cone, 1972) upon light exposure. Mason *et al.* (1972, 1973b) have shown by freeze–etch electron microscopy that rhodopsin appears upon light exposure to translocate from the intradisk hydrophilic membrane surface into the median hydrophobic lipid region. Blasie (1972) has also demonstrated by X-ray diffraction that rhodopsin "sinks" into the lipid phase upon light exposure. Blasie's further experiments showing that X-ray diffraction maxima arising from frog disk membranes are markedly broadened as a function of temperature convincingly demonstrated the fluidity of this membrane.

The above experiments suggest that rhodopsin is in some way intimately associated with the lipid milieu of the disk membrane. The studies reported here demonstrate a high degree of unsaturation in the fatty acid moiety as well as a large percentage of long-chain hydrocarbons. The low cholesterol level present in the frog rod outer segment further suggests that the disk membranes containing the photopigment do indeed possess the characteristics of a highly fluid membrane. This conclusion is supported by the work of Hubbell and McConnell (1971) on model membrane systems. It was found by Hubbell that spin-labeled membranes similar in composition to the frog disk membranes show a low "packing" efficiency and a high degree of molecular motion in the lipid region. In light of the lipid composition reported here for frog disk membranes, Hubbell's experiments lend some credence to the observation that rhodopsin is capable of dynamic molecular movement in the frog disk membrane.

An interesting comparison is now possible between the three most studied visual pigment systems, namely, the cattle, frog, and squid membrane systems. It was established by Mason *et al.* (1973a) that the squid visual pigment membrane system was unlike the bovine system in terms of the cholesterol content as well as the phospholipid and fatty acid composition. It is apparent from these studies that similar to other vertebrate membranes, frog photoreceptor membranes are distinct from invertebrate visual pigment membranes. While certain similarities do exist between bovine and frog rod outer segment membranes, the bovine rod outer segment membranes contain decidedly more docosahexanoic acid (22:6), the fatty acid observed to play a major structural role in the fatty acid moiety of many membranes. Frog disk membranes contain 15% of the total fatty acid as 22:6, while bovine disk membranes have been reported to contain from 22 to 35%, and squid only 9% of the total fatty acid as 22:6.

The phospholipid profile of frog rod outer segments is similar to that reported by Eichberg and Hess (1967) with the exception that we find nearly 15% of the phospholipid to be phosphatidylserine whereas they find only 9%. Other minor components such as phosphatidic acid and possibly two diphosphatides were detected, although by reason of the relatively small amounts of these substances detected they probably do not play an important structural role in the membrane. The presence of glycolipid in large quantities was also confirmed. Although the presence of this latter component is now well documented, its function in the membrane is relatively obscure.

One interesting aspect of fatty acid metabolism was noted in the frog membranes studied here. Five major fatty acid components were detected, namely palmitic, stearic, linolenic, docosanoic, and docosahexanoic acid. These components are related metabolically by the palmitic → stearic → docasanoic and the linolenic → docosahexanoic pathways first proposed by Noller and Bannerot (1934). It is of interest that these components in the frog disk membranes show an accumulation of shorter chain fatty acids in the pathway rather than the preferential completion of the pathway to docosahexanoic acid noted in the bovine rod outer segment membranes. This observation may cast some light on differences in metabolic machinery within vertebrate visual systems. The frog disk membranes are also unlike invertebrate squid rhabdomes in this metabolic preference for fatty acid buildup. The consideration of these metabolic factors with other evidence on similar membrane systems may in some way be correlated with the position of these animals on the evolutionary pathway.

In summary, the composition of frog rod outer segment disk membranes is here reported to be qualitatively and quantitatively similar to other well-defined visual membrane systems, such as the cow, with respect to phospholipid composition and is in turn unique with respect to fatty acid composition. Furthermore, large differences between the frog and squid visual membrane systems were also noted. The characteristics of the frog visual pigment membrane system make it appear likely that indeed the fluidity of the lipid region facilitates molecular motion of the chromoprotein.

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