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CHEMISTRY OF PHOTORECEPTOR MEMBRANE PREPARATIONS FROM SQUID RETINAS

ROBERT E. ANDERSON [a], ROBERT M. BENOLKEN [b], PAULA A. KELLEHER [a], MAUREEN B. MAUDE [a] and REX D. WIEGAND [a]

[a] *Cullen Eye Institute, Baylor College of Medicine, and* [b] *Graduate School of Biomedical Sciences, The University of Texas Health Science Center, Houston, Texas 77030 (U.S.A.)*

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Summary

Photoreceptor membrane preparations were made from retinas of the squid *Loligo (Doryteuthis) plei* for protein and lipid analysis. Lipid analysis was also completed on a single membrane preparation from *Loligo pealei.*

(1) The membranes contain 75 wt. % protein and 25 wt. % lipid. Neutral lipids make up 26 mol % of the total lipid, the remaining 74% being phospholipid. No glycolipids were observed.

(2) Free fatty acids and cholesterol comprise 8.6 and 17 mol %, respectively, of the total lipid. No other neutral lipids were found.

(3) Phosphatidylethanolamine and phosphatidylcholine are the major phospholipids. Lysophosphatidylcholine, lysophosphatidylethanolamine, sphingomyelin, and phosphatidylserine are present in small quantities. Phosphatidylinositol was not detected in the membranes.

(4) The levels of polyunsaturated fatty acids, principally 20:4ω6, 20:5ω3, and 22:6ω3 are higher in the squid membranes than in any other vertebrate or invertebrate retina that has been examined thus far. These acids account for 58 mol % of the fatty acids in phosphatidylcholine and phosphatidylserine, 75 mol % of the free fatty acids, and nearly 90% of the fatty acids of lyso- and phosphatidylethanolamine. The results from *L. plei* and *L. pealei* were indistinguishable.

(5) Rhodopsin is the major protein of the membrane preparations and has a molecular weight of 50 500 ± 850 determined by sodium dodecyl sulfate polyacrylamide gel disc electrophoresis.

Introduction

The chemistry of the photoreceptor membranes of vertebrate retinas has been studied in great detail (reviewed in ref. 1). Lipid and protein are the two

major components and on a mass basis they are present in roughly equal proportions. The majority of the lipids are phospholipids, and the hydrophobic domain of the bilayer is composed of large amounts of long chain polyunsaturated fatty acids, predominantly docosahexanoic acid (22:6ω3). The membrane-bound protein is almost all rhodopsin, a portion of which is imbedded in the lipid bilayer where it exhibits both rotational [2,3] and translational [4] mobility.

Invertebrate photoreceptor membrane chemistry has been studied less extensively. Hubbard and St. George [5] showed that 11-*cis*-retinaldehyde is the prosthetic group for the photopigment of squid. Hagins [6] found that squid rhodopsin has a molecular weight of 51 000, although Kimball et al. [7] and Fager et al. [8] reported a value of 70 200. Paulsen and Schwemer [9] found molecular weights of 43 000 and 35 000 for the visual pigments of the octopus *Eledone moschata* and the insect *Ascalaphus macaronius*, respectively.

The lipids of the moth eye were studied by Zinkler [10], who reported large amounts of 18:3ω3 and 20:5ω3, but no 22:6ω3. We analyzed the photoreceptor membranes of the lateral eye of the horseshoe crab *Limulus* and found 20:4ω6 and 20:5ω3 as the major polyunsaturated fatty acids [11]. Mason, Fager and Abrahamson [12] reported large percentages of short chain fatty acids, low levels of polyunsaturated fatty acids, and significant amounts of triglycerides and cholesterol esters in the squid *Loligo pealei*.

In this paper we report the lipid and protein chemistry of photoreceptor membrane preparations from the squid *Loligo (Doryteuthis) plei* and the lipid analysis of a single membrane preparation from *L. pealei*. The protein pattern by gel electrophoresis is similar to that given by Hagins [6], but the lipid chemistry is in sharp disagreement with that reported by Mason et al. [12].

Methods

L. plei (earlier classification, *Doryteuthis plei*, see ref. 13), were collected during the month of December, 1976, at the Bermuda Biological Station for Research, St. Georges, Bermuda. Squid were collected with a seine in water 2—3 feet deep and immediately transferred to a running seawater tank in a darkroom. After dark adapting overnight, healthy animals were decapitated under dim red light and whole eyes removed and frozen. All specimens were brought to Houston for analysis. *L. pealei* were captured in 20—40 feet of water in the Gulf of Mexico in August, 1976, at The University of Texas Marine Science Institute, Port Aransas, Texas. After dark adapting the animals in running sea water, the photoreceptor membranes were prepared, frozen and transferred to Houston for lipid analysis.

Three groups containing eight *L. plei* eyes each were thawed, the eyes were carefully dissected under dim red light, and the anterior portion of each eye including the lens removed and discarded. The remaining posterior half was homogenized in 15 ml 66 mM phosphate buffer (pH 7.2) and made up to a final volume of 40 ml, and centrifuged for 20 min at 27 000 $\times g$. The supernatant was discarded and the residue was homogenized in 20 ml of 42% sucrose (w/w) in buffer and made up to a final volume of 40 ml with sucrose buffer, and centrifuged at 60 000 $\times g$ for 60 min. The floating material was harvested

and the residue was resuspended in the sucrose buffer and centrifuged again at 60 000 × *g* for 60 min. The second float was combined with the first, made up to 40 ml with buffer, and the membranes pelleted at 27 000 × *g* for 20 min. The supernatant was discarded and the residue containing photoreceptor membranes was suspended in 40 ml buffered 42% sucrose. This suspension was spun at 60 000 × *g* for 60 min after which the float was removed, diluted with buffer, and pelleted at 27 000 × *g* for 20 min in 3 successive buffer washes. Three preparations of membranes yielded 11.5, 24, and 17 mg of protein.

For spectroscopy, aliquots of squid photoreceptor membranes were homogenized under dim red light in 1 ml of 2% digitonin in 66 mM phosphate buffer (pH 7.2). After 2 h, the debris was removed by centrifugation and the supernatant scanned from 650 to 250 nm in a Cary 118C recording spectrophotometer. Hydroxylamine was added to a final concentration of 50 mM and the sample rescanned, bleached, and scanned once again. The ratio of absorbance at 278 nm to 490 nm was determined without correction for light scatter.

Polyacrylamide gel disc electrophoresis was carried out according to a previously published procedure [14]. Plots of log molecular weights of standard proteins versus mobility relative to the dye front were used to determine the apparent molecular weights of the proteins from squid photoreceptor membranes. Total protein was determined by the procedure of Lowry et al. [15] using purified bovine serum albumin as the standard.

Wet photoreceptor membrane pellets were suspended in 0.4 ml water and extracted twice with 2 ml chloroform/methanol (2 : 1, v/v). The lipids from the combined chloroform extracts were partitioned into neutral and polar lipids by silicic acid column chromatography. Neutral lipids were eluted with 100 ml chloroform, and polar lipids with 75 ml chloroform/methanol/water (60 : 45 : 12, by vol.). Two-dimensional thin layer chromatography of phospholipids was carried out as previously described [16]. Thin layers of Silica Gel G were used to separate neutral lipids in one dimension using a solvent system of hexane/diethyl ether/glacial acetic acid (80 : 20 : 1, by vol.).

Methyl esters of neutral and phospholipids were prepared with boron trifluoride-methanol [17] and analyzed on a Varian 2400 gas chromatograph equipped with a 180 cm × 2 mm (interior diameter) column packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.). All runs were temperature programmed from 170 to 220°C at 1°C/min. Injector and hydrogen flame detector temperatures were maintained at 230 and 250°C, respectively. The area under each peak was determined by electronic integration and converted to relative mol %.

Neutral lipids were quantitated by gas-liquid chromatography using an internal standard. A known quantity of tridecanoin was added to an appropriate volume of the neutral lipid fraction and the mixture was hydrogenated over platinum oxide catalyst, separated from the catalyst, and dried by evaporation under N_2. An excess of *O*-bis-(trimethylsilyl)trifluoroacetamide was added and the mixture was heated in a sealed tube at 100°C for at least 30 min. The tube and contents were cooled, an equal volume of chloroform added, and a portion of the solution injected into a Varian Model 2100 gas chromatograph equipped with a flame ionization detector. The glass column (60 cm × 2 mm interior diameter) was filled with 1% OV-17 on 100—120 mesh Gas-Chrom-Q

(Supelco, Inc., Bellefonte, Pa.). The flow rate of the carrier gas (N_2) was 100 ml/min at 100°C and the air and hydrogen flows were adjusted to give maximum detector response. The detector and the on-column injector temperatures were 350°C and 300°C, respectively, and the temperature was linearly programmed at 4°C/min over the range of 100—340°C. A standard neutral lipid mixture consisting of known quantities of free fatty acid, monoglyceride, cholesterol, diglyceride, cholesterol ester and triglyceride was analyzed routinely to obtain detector response factors for each lipid class.

Protein and lipid data from *L. plei* are the average of a minimum of three independent determinations made in duplicate. A single determination was made of the lipid class and fatty acid composition of *L. pealei.* Although the lipid data shown are for *L. plei*, similar values were obtained for *L. pealei.*

Results

Purity of the squid photoreceptor membrane preparation. Since there are no published criteria for judging the purity of preparations of invertebrate photoreceptor membranes, the purity of the squid membranes was assessed by the methods generally used for vertebrate photoreceptors. The 278/490 nm absorbance ratios for several preparations averaged 3.6 ± 0.4. Polyacrylamide gel disc electrophoresis of the membrane preparations showed a major component with the molecular weight of squid rhodopsin [6] and a gel pattern similar to that published by Hagins [6] for preparations of squid photoreceptors. Electron microscopy of several preparations revealed a population of membranous vesicles of various sizes with occasional regions of intact microvillous processes. No organelles were identifiable in any of our preparations, although areas of amorphous, electron-dense material were present. Attempts to prepare membranes with better spectrophotometric ratios, gel patterns, and electron microscopic appearances using sucrose gradient centrifugation techniques did not result in improvement of the quality of the membranes. However, in view of the differences in morphology of invertebrate and vertebrate photoreceptors, it is not surprising that our preparations of squid photoreceptor membranes do not meet the standards of purity established for vertebrate membranes.

These membranes do not contain retinochrome, which would be indicative of contamination with other retinal membranes. The addition of 50 mM hydroxylamine to unbleached digitonin extract did not lower the absorbance at 490 nm more than 2—3%, conditions under which the chromophore of retinochrome is rapidly converted to the retinyl oxime [17].

Proteins of squid photoreceptor membrane preparations. Polyacrylamide gel disc electrophoresis (Fig. 1) of the photoreceptor membrane proteins showed two major peaks. One component (A) made up about 50% of the stainable protein and is rhodopsin. The other large component (B) has a molecular weight of 28 500 ± 3300 and amounts to 22% of the total protein. This latter protein was not a major component of Hagin's preparations.

Six independent preparations of squid photoreceptor membranes contained 75 ± 2.2 wt. % protein and 25 ± 2.2 wt. % lipid. This protein value is the highest yet reported for photoreceptor membranes. However, of all the analy-

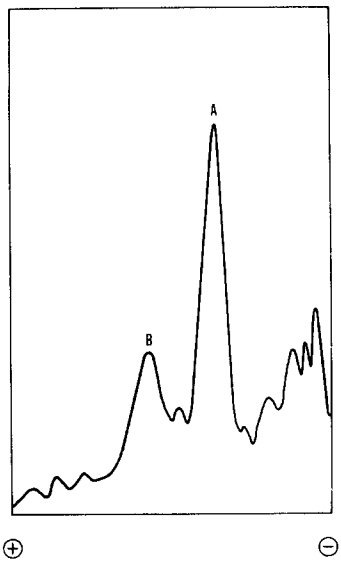


Fig. 1. Tracing of a polyacrylamide gel of squid photoreceptor membrane preparations. Components A and B have molecular weights of 50 500 ± 850 and 28 500 ± 3300, respectively. The length of the gel is 9.8 cm.

ses presented here, this estimate has the greatest uncertainty because of the possibility of protein contamination in our membrane preparations.

Lipid classes of squid photoreceptor membrane preparations. Fig. 2 is a two-dimensional, thin-layer chromatograph of the phospholipids of squid photoreceptor membranes. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were identified by their characteristic migration patterns [16]. The lipid identified as lysophosphatidylethanolamine migrated near the region of phosphatidylinositol and might have been confused with this phospholipid except that it gave a ninhydrin-positive reaction, whereas phosphatidylinositol does not. When authentic phosphatidylinositol was co-chromatographed with the membrane lipids, it was completely resolved from lysophosphatidylethanolamine and migrated to the position shown in Fig. 2. There was no measurable lipid phosphorus in the region of the thin layer chromatograph corresponding to phosphatidylinositol. Although no specific glycolipid stains were employed, charred spots corresponding to ceramide mono- and dihexosides were not observed on overloaded chromatoplates. Plasmalogens, if present, would have been identified by the characteristic gas chromatographic pattern of the dimethyl acetals produced by the acid methanolysis of the vinyl ether linkage. No aldehydes were observed in any of our preparations.

The neutral lipid class composition was determined by high temperature gas liquid chromatography and a chromatogram of standards is shown in Fig. 3A. Free fatty acids (FFA) are the trimethylsilyl esters of 16, 18, and 20 carbon acids; MG-16 is a monoglyceride of a 16 carbon fatty acid; Chol-27 is cholesterol; TG-30 is the triglyceride tridecanoin which is used as the internal standard; DG-32 and -36 are diglycerides containing a total of 32 and 36 carbons

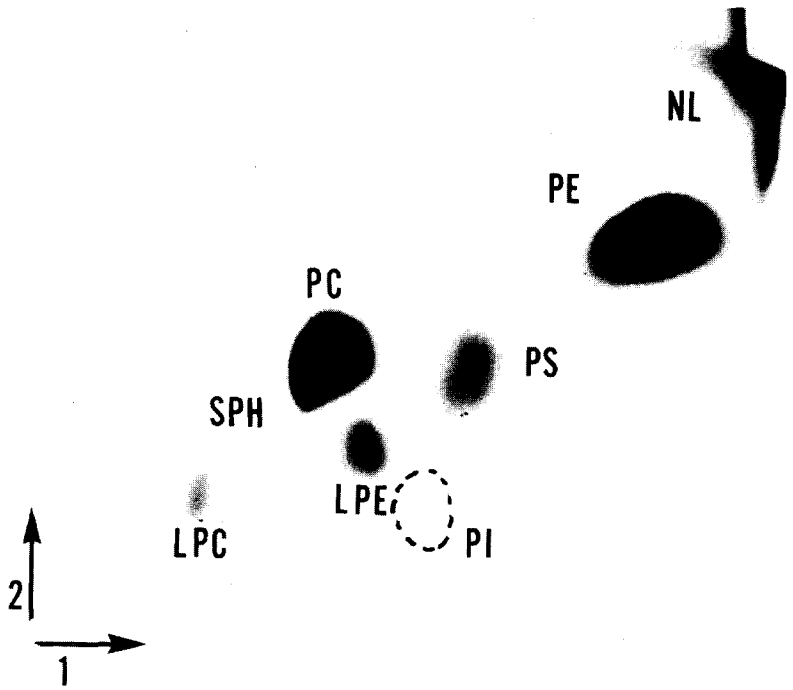


Fig. 2. Two-dimensional thin layer chromatograph of the phospholipids of squid photoreceptor membrane preparations. The lipids were visualized by spraying with 55% sulfuric acid containing 0.6% sodium dichromate and charring at 190° C for 30 min. LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; NL, neutral lipid.

in their fatty acids; CE-53 and -55 are cholesterol esters with fatty acids containing 16 and 18 carbons, respectively; and TG-48 and -54 are tripalmitin and tristearin, respectively. Note that each component is well separated from the others, permitting reliable identification. In the few instances where components overlap, such as a 22-carbon free fatty acid and a 16-carbon monoglyceride, separation of these classes by preparative thin-layer chromatography prior to gas chromatography facilitates identification and quantitation.

Fig. 3B is a chromatogram of the neutral lipids from squid photoreceptor membranes containing tridecanoin (TG-30) as the internal standard. Only free fatty acids and cholesterol are present. The 22-carbon fatty acids were identified after fractionation by thin-layer chromatography as just described. The absence of cholesterol esters and triglycerides in our membranes is in sharp contrast to the report of Mason et al. [12] on squid photoreceptor membranes.

The lipid class composition of squid photoreceptor membranes is given in Table I. On a molar basis, phospholipids make up 74.2% and neutral lipids 25.8% of the total lipid. Phosphatidylcholine and phosphatidylethanolamine are the major components in nearly equimolar amounts. Phosphatidylserine, sphingomyelin, lysophosphatidylethanolamine, and lysophosphatidylcholine are present as minor components. Cholesterol accounts for two-thirds of the neutral lipids (17 mol % of total lipid) and free fatty acids account for one-

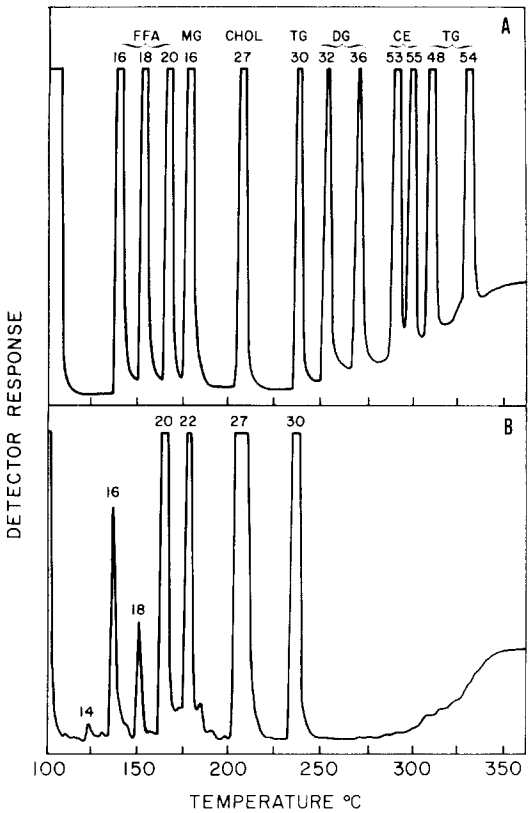


Fig. 3. (A) Gas-liquid chromatogram of a standard mixture of neutral lipids and (B) gas-liquid chromatogram of the neutral lipids of squid photoreceptor membrane preparations. (The abbreviations are given in the text.)

third of the neutral lipids (8.6 mol % of total lipid).

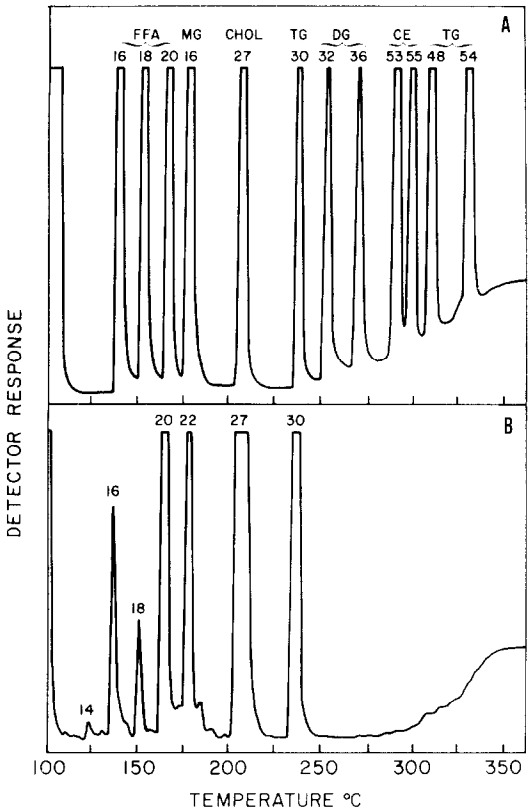
Fatty acid composition of the lipids of squid photoreceptor membrane preparations. The gas-liquid chromatographic pattern of the methyl esters of

TABLE I

LIPID COMPOSITION OF SQUID PHOTORECEPTOR MEMBRANES

The values are the average of duplicate determinations from three different samples of squid photoreceptor membranes and are expressed as relative mol% ± S.D.

Lipid class	mol%
Lysophosphatidylcholine	1.7 ± 0.2
Lysophosphatidylethanolamine	3.9 ± 0.4
Sphingomyelin	1.3 ± 0.1
Phosphatidylserine	4.0 ± 0.4
Phosphatidylcholine	32.2 ± 0.4
Phosphatidylethanolamine	30.6 ± 0.6
Unknown phospholipids	0.5 ± 0.2
Total phospholipid	74.2 ± 1.4
Free fatty acids	8.6 ± 1.5
Cholesterol	17.2 ± 0.1
Total neutral lipid	25.8 ± 1.4



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Lysophosphatidylcholine	1.7 ± 0.2
Lysophosphatidylethanolamine	3.9 ± 0.4
Sphingomyelin	1.3 ± 0.1
Phosphatidylserine	4.0 ± 0.4
Phosphatidylcholine	32.2 ± 0.4
Phosphatidylethanolamine	30.6 ± 0.6
Unknown phospholipids	0.5 ± 0.2
Total phospholipid	74.2 ± 1.4
Free fatty acids	8.6 ± 1.5
Cholesterol	17.2 ± 0.1
Total neutral lipid	25.8 ± 1.4

phosphatidylethanolamine from the squid retina membrane preparations is given in Fig. 4. Temperature programming facilitated the separation of shorter chain esters yet allowed the elution of the long chain polyunsaturates in less than 35 min. Identification was made on the basis of elution patterns compared to authentic esters before (Fig. 4A) and after (Fig. 4B) catalytic hydrogenation.

The fatty acid compositions of the lipids of squid photoreceptor membranes given in Table II are unlike those reported for any other membrane preparation in that they contain unusually high percentages of long chain polyunsaturated fatty acids. For example, phosphatidylethanolamine and lysophosphatidylethanolamine each contain almost 90% polyunsaturated fatty acids, the most abundant of which is 22:6ω3. Free fatty acids have 75% long chain polyunsaturates, the most abundant of which is 20:5ω3. Phosphatidylcholine and phosphatidylserine contain 58 mol % long chain polyunsaturates, which is the lowest level found in any of the squid retinal lipids.

There are no quantitatively significant amounts of short chain fatty acids (less than 14:0) which Mason et al. [12] reported make up 19% of the total fatty acids of squid photoreceptor membranes.

Discussion

An unexpected finding in this investigation was the unusually large amount of polyunsaturated fatty acids in each of the lipid classes of the squid membranes. To our knowledge, the value of 90% polyunsaturated fatty acids in lysophosphatidylethanolamine and phosphatidylethanolamine is the highest ever reported. In the one other invertebrate photoreceptor membrane preparation that we have analyzed [11], long chain polyunsaturates comprised about 50% of the total fatty acids. Zinkler [10] also found at most 50% polyunsaturates in the moth eye. In all of the vertebrate photoreceptor membranes we have examined, the levels of long chain polyunsaturated fatty acids rarely exceed 50—55% in phosphatidylethanolamine and phosphatidylserine, and the levels in phosphatidylcholine are usually of the order of 25—30%. Therefore, the values we report for squid are higher than ever observed in either vertebrate or invertebrate photoreceptor membranes.

The types of long chain polyunsaturated fatty acids found in the squid photoreceptor membrane preparations are also unlike those of either vertebrate or invertebrate membranes. The major polyunsaturates of the moth eye [10] are 18:3ω3 and 20:5ω3, while 20:4ω6 and 20:5ω3 are the major components of *Limulus* photoreceptor membranes [11]. Neither of these invertebrates contain 22:6ω3, which is the major polyunsaturate of vertebrate photoreceptor membranes [1]. Although vertebrates may contain up to 6—8% 20:4ω6, they usually contain only trace amounts of 20:5ω3. Therefore, prior to our analysis of squid photoreceptors there were clear-cut differences between the types of polyunsaturated fatty acids in vertebrate and invertebrate photoreceptor membranes. However, our finding of large amounts of 20:4ω6, 20:5ω3, and 22:6ω3 in all lipid classes of the squid photoreceptor membranes now prevents any broad generalization regarding the types of fatty acids found in vertebrates and invertebrates.

The high levels of free fatty acids in squid photoreceptor membranes remain an enigma. While they may have resulted from lipolysis of membrane phospholipid, the fatty acid composition shown in Table II argues against this possibility. The free fatty acids contain over 30 mol % 20:5ω3, whereas the potential lypolytic substrates, phosphatidylcholine and phosphatidylethanolamine, contain only 4.6 and 6.5 mol %, respectively, of this fatty acid. Preferential hydrolysis of 20:5ω3 would then be required to produce the high levels seen in the free fatty acids. However, the level of 20:5ω3 in lysophosphatidylethanolamine is the same as in phosphatidylethanolamine, indicating no preferential hydrolysis.

Mason et al. [12] examined the lipids of squid photoreceptor membranes from *L. pealei* and found high levels of short chain polyunsaturated fatty acids, low levels of long chain polyunsaturates, and significant quantities of triglycerides and cholesterol esters. Our results disagree on all four points. Gas-liquid chromatography of native and hydrogenated methyl esters did not show any short chain fatty acids (Fig. 4), and triglycerides and cholesterol esters were not detected in any of the high temperature gas liquid chromatograms of the total neutral lipids from squid photoreceptor membranes (Fig. 3). We can not reconcile the differences between our results and those of Mason et al., except for the possibility that their gas chromatography conditions were not optimum for quantitation of long chain polyunsaturated fatty acids.

Goldsmith and Wehner [19] recently reported that the translational and rotational diffusion of rhodopsin in photoreceptor membranes from the crayfish retina is restricted compared to vertebrate membranes. Unfortunately, no chemical analyses have been carried out on crayfish rhabdoms, and no mobility studies have been done on squid rhodopsin. The mobility of a protein in a bilayer is a function of such factors as cholesterol content, fatty acid composition, phospholipid class composition, surface cations and lipid/protein ratios, as well as the relative proportions of the protein imbedded in the bilayer. If the protein substructure in squid photoreceptor membranes eventually proves to be rigid, the lipid/protein ratios may prove the deciding factor, since the lipid profiles seem to favor the opposite alternative.

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