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# LIPID COMPOSITION OF *LIMULUS* PHOTORECEPTOR MEMBRANES

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## SUMMARY

The lipid composition has been determined for rhabdomeric photoreceptor membranes of *Limulus*, and these data are compared with those from photoreceptor membranes of albino rats. The comparison is of interest because the membranes of these two photoreceptor cells regulate ionic transport differently during the response to illumination.

1. Phospholipid class composition of *Limulus* is similar, but not identical, to that of rats. The major differences are a greater percentage of sphingomyelin in *Limulus* and a greater percentage of phosphatidylethanolamine in the rat.

2. Ethanolamine plasmalogens, not observed in rat photoreceptor membranes, are present in *Limulus* photoreceptor fractions.

3. The level of cholesterol in *Limulus* is higher than that usually reported for vertebrate rod outer segments.

4. The predominant polyunsaturated fatty acids of *Limulus* photoreceptor membrane phospholipids are 20 : 4($n-6$) and 20 : 5($n-3$) with only traces of 22 : 6($n-3$). This is in sharp contrast with the large percentages of 22 : 6($n-3$) found in rat photoreceptors.

5. The fatty acid distributions of both membrane systems are highly unsaturated, but the ratio of ($n-3$) to ($n-6$) polyunsaturates is only 1.7 for *Limulus* as compared to 4.6 for rat.

## INTRODUCTION

Photoreceptor cells have evolved along several independent pathways, with respect to both morphological organization [1–3] and functional properties [4–6]. Of these, the invertebrate rhabdomeric retina provides a particularly interesting functional contrast to the vertebrate rod retina. Although it is generally believed that $Na^+$ transport is involved in both cases, rhabdomeric and rod membranes appear to employ different electrical strategies to encode visual information. When vertebrate photoreceptors respond to light, membrane conductance decreases [7, 8]; the opposite result is observed in rhabdomeric photoreceptors where membrane conductance increases in response to illumination [9, 10].

By definition, membrane conductance is the change in ionic current per unit change in membrane potential difference. Apparently, during the light response, rod plasma membranes switch off $Na^+$ transport while rhabdomeric plasma membranes switch it on. That these transport differences result from differences in the properties of the membrane systems, rather than from differences in either the direction or the magnitude of change in the membrane potential, follows from the fact that the ionic current changes are normalized with respect to the potential change in the conductance measures. These considerations lead to the conclusion that both membrane systems selectively "recognize" $Na^+$, but in response to illumination the rod and rhabdomeric membranes regulate $Na^+$ transport in different ways.

Given the transport differences, comparative data from rod and rhabdomeric membranes should prove useful for the general problem of how membranes control ionic transport. Visual membranes, at least those of vertebrate rods, have the advantage that membrane composition appears to be relatively simple. On a dry weight basis, membrane fractions of vertebrate rod outer segments are about 50 % protein and 50 % lipid. The glycoprotein rhodopsin accounts for about 80–90 % of the total membrane-bound protein [11], phospholipids account for 80–85 % of total outer segment lipid [12], and there are 60–65 mol of phospholipid per mol of rhodopsin in rat outer segments (Dudley, P. A., unpublished). Phospholipids, as well as proteins, appear to play a functional role in the light response of rod outer segment membranes. In solubilized membrane fractions, phospholipids are required for stability of the rhodopsin chromophore in darkness and for regeneration of the rhodopsin chromophore after bleaching [13–15]. Moreover, in vivo, phospholipid components contribute to the normal process of membrane synthesis [16] and to the electrical response properties of the rod photoreceptor cell [17, 18].

The photopigments of vertebrate rod outer segment membranes have shown very little variation from species to species. By contrast, considerable variation has been observed in the behaviour of photopigments from rhabdomeric eyes [19–24]. However, Hubbard and Wald [25] have shown that the digitonin-solubilized photopigment of *Limulus* behaves very much like the solubilized rhodopsins of vertebrate rods. Consequently we selected *Limulus* photoreceptor membranes for the comparative lipid analysis in an effort to minimize photochemical differences between the comparative systems. A preliminary report of the lipid composition of *Limulus* photoreceptor membranes was presented at an annual meeting of ARVO [26].

## METHODS

*Limulus*, averaging 10–15 cm across the carapace, were supplied by Gulf Specimen Co., P. O. Box 237, Panacea, Florida 32345. Lateral eyes were excised under minimal red light, retinas were stripped from the cornea, immersed in 42 g of purified sucrose (Schwartz-Mann) made up to a final volume of 100 ml with 66 mM $PO_4^{3-}$ buffer (pH 7.0) at 0 °C, and homogenized glass on glass. Homogenized retinas were centrifuged for 10 min at $3000 \times g$, followed by 60 min at $40\,000 \times g$. (The initial low-$g$ field improved yields substantially.) The supernate was resuspended in 42 % sucrose, and centrifuged again for 60 min at $40\,000 \times g$. The upper 10 ml of the latter supernate wasresuspended in 35 ml of 66 mM $PO_4^{3-}$ buffer (pH 7.0). After centrifugation for 30 min at $27000 \times g$, the supernatant was decanted and the pellet was resuspended in

buffer. The latter wash procedure was repeated once.

Spectral properties of the membrane fractions were scanned with a Cary 118C (Benolken, R. M. et al., unpublished). The ratio of the absorbance at 278 nm ($A_{278}$) relative to the absorbance of photopigment ($A_{photo}$) provided an estimate of spectral purity of the membrane fractions. The $A_{278}/A_{photo}$ ratio could be measured with good precision in emulphogene extracts after pre-extraction with digitonin, and the value of the ratio was less than 3.0. This compares quite favorably with the spectral purity of comparable vertebrate outer segment fractions. The $A_{278}/A_{photo}$ ratio could not be determined with precision in our digitonin extracts, but we estimate the ratio was probably no greater than 5.0.

Membrane lipids were extracted in 2 : 1 chloroform/methanol and phospholipids were separated by two-dimensional thin-layer chromatography [27]. After separation, phospholipids were estimated quantitatively by the method of Rouser et al. [28]. Methyl esters of the fatty acids were assayed quantitatively [27] with gas-liquid chromatography, and cholesterol was determined after the method of Zak et al. [29]. Fatty acid nomenclature is as follows: the first number specifies the number of carbon atoms, the number after the colon specifies the number of double bonds, and the number after $n-$ specifies the position of the first double bond relative to the $(n-1)$ position defined by the terminal methyl carbon.

## RESULTS

The phospholipid class composition of the *Limulus* photoreceptor membrane fraction was determined from duplicate two-dimensional thin-layer chromatograms for each of three membrane preparations, where each membrane preparation was isolated from 400 freshly excised eyes. 100 eyes were used for spectral purity estimates on two of the preparations, and usual values of pigment absorbance per eye were observed. Approx. 100 eyes from the third membrane preparation were used for duplicate cholesterol assays. The phospholipid class composition summarized in Table I is expressed as average mol percentage (mol%) plus or minus one standard

TABLE I

PHOSPHOLIPID CLASS COMPOSITION OF PHOTORECEPTOR MEMBRANE FRACTIONS

| Phospholipid class | Limulus* mol% ± S.D. | Rat** mol% |
|---|---|---|
| Origin | 2.8 ± 1.5 | 0.1 |
| Lysophosphatidylcholine | 1.4 ± 0.6 | 1.1 |
| Sphingomyelin | 13.7 ± 0.7 | 3.9 |
| Phosphatidylinositol | 4.7 ± 1.3 | 2.2 |
| Lysophosphatidylethanolamine | 2.2 ± 2.4 | |
| Phosphatidylserine | 9.4 ± 1.0 | 12.6 |
| Phosphatidylcholine | 39.6 ± 1.5 | 41.0 |
| Phosphatidylethanolamine | 23.7 ± 1.3 | 36.6 |
| Solvent front | 2.2 ± 0.4 | 2.6 |
| % recovery of lipid phosphorus | 99.7 | 98.5 |

* From duplicate, two-dimensional, thin-layer plates of three photoreceptor membrane preparations ($n = 6$). Each membrane preparation was isolated from 400 freshly excised eyes.

** From Anderson and Maude [30].

TABLE II

FATTY ACID DISTRIBUTIONS OF PHOSPHOLIPID CLASSES OF PHOTORECEPTOR MEMBRANES OF *LIMULUS* AND RAT

| | Phosphatidyl-ethanolamine | | Phosphatidyl-choline | | Phosphatidyl-serine | | Phosphatidyl-inositol | | Sphingomyelin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Limulus* (mol%±S.D.) | Rat** (mol%) | Limulus* (mol%±S.D.) | Rat** (mol%) | Limulus* (mol%±S.D.) | Rat** (mol%) | Limulus*** (mol%±S.D.) | Rat (mol%) | Limulus* (mol%±S.D.) | Rat |
| 14 : 0 | | | | 0.8 | | | | | | |
| 15 : 0 | | | | 0.3 | | | | | | data not available |
| 16 : 0 DMA† | 1.4±0.3 | | | | 1.1±0.8 | | 1.3±1.6 | | | |
| 16 : 0 | 2.2±0.4 | 6.7 | 9.8±2.2 | 34.7 | 3.1±1.9 | 2.3 | 8.8±7.0 | 14.4 | 67.3±3.9 | |
| 16 : 1 | 0.9±0.7 | | 3.7±1.0 | | 0.8±0.5 | | 1.0±0.8 | | 3.3±1.3 | |
| 17 : 0 | 0.5±0.5 | | 1.3±0.4 | | 1.2±0.9 | | | 1.8 | | |
| 17 : 1 | | | 1.3±1.0 | | 0.2±0.2 | | | | | |
| 18 : 0 DMA† | 12.0±1.2 | | | | | | | | | |
| 18 : 0 | 11.7±1.3 | 33.9 | 6.3±2.3 | 22.0 | 37.5±5.2 | 38.9 | 26.3±5.5 | 34.8 | 10.0±3.8 | |
| 18 : 1 ($n-9$) | 4.3±1.0 | 3.9 | 29.5±3.7 | 17.1 | 22.8±3.5 | 2.5 | 23.0±1.6 | 7.0 | 2.9±0.8 | |
| 18 : 2 ($n-6$) | 1.8±0.6 | | 2.7±1.1 | 0.6 | 5.6±3.2 | | 2.1±0.9 | 2.6 | 0.3±0.3 | |
| 18 : 3 ($n-3$) | 0.8±0.8 | | 2.3±1.8 | | 3.9±3.7 | | 1.0±0.7 | | 0.4±0.7 | |
| 20 : 0 | 11.6±1.5 | | | | 0.1±0.2 | | | | 1.8±1.2 | |
| 20 : 4 ($n-6$) | 12.1±0.8 | 8.1 | 14.0±2.9 | 4.1 | 15.1±3.6 | 2.3 | 29.2±8.8 | 26.2 | 1.1±1.1 | |
| 20 : 5 ($n-3$) | 40.5±3.6 | | 27.6±6.3 | | 7.2±3.0 | | 8.0±1.2 | | 0.2±0.3 | |
| 22 : 0 | | | | | | | | | 11.5±3.8 | |
| 22 : 4 ($n-6$) | | 1.9 | | | 0.8±0.13 | 3.3 | | | | |
| 22 : 5 ($n-6$) | | | 0.6±0.6 | | 0.4±0.3 | | | | | |
| 22 : 6 ($n-3$) | | 45.2 | 0.8±0.8 | 20.4 | 0.2±0.3 | 50.5 | | 10.7 | | |

* From duplicate analyses of three photoreceptor membrane preparations of 400 eyes each ($n = 6$).

** From Anderson and Maude [30].

*** From single analyses of three photoreceptor membrane preparations of 400 eyes each ($n = 3$).

† Dimethyl acetals derived from acid methanolysis of plasmalogens.

deviation (S.D.) with the number of determinations ($n$) equal to six. For comparison, Table I also includes comparable data from the same laboratory on rod outer segment fractions from freshly excised rat retinas [30].

Overall, the phospholipid class compositions of *Limulus* and rat photoreceptor membrane fractions do not appear very different. The greatest percentage differences were observed for sphingomyelin and phosphatidylethanolamine. Total phospholipid averaged 6.5 μg (with a range from 6.0 to 6.8 μg) or 8 nmol per *Limulus* eye. On the basis of a duplicate assay on one preparation, the mol ratio of cholesterol to phospholipid was 0.20. Hence, on the average, total lipid of the *Limulus* membrane fraction was no less than 7.0 μg per eye.

The fatty acid distributions of the major phospholipid classes of *Limulus* and rat photoreceptor membranes are shown in Table II. Comparisons of the *Limulus* and rat data indicate several striking differences in the fatty acid distribution of the rhabdomeric and rod membrane fractions, and this is particularly true of the distributions of the long-chain polyunsaturated fatty acids. The rat photoreceptor membranes contain unusually high percentages of the long-chain polyunsaturate 22 : 6($n-3$), while $C_{22}$ polyunsaturates are virtually absent from *Limulus* photoreceptor membranes. The dominant polyunsaturate of rat phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine is 22 : 6 ($n-3$), and the dominant polyunsaturate of rat phosphatidylinositol is 20 : 4 ($n-6$). The dominant polyunsaturate of *Limulus* phosphatidylethanolamine and phosphatidylcholine is 20 : 5 ($n-3$), and the dominant polyunsaturate of *Limulus* phosphatidylserine and phosphatidylinositol is 20 : 4($n-6$). The ($n-3$) and ($n-6$) polyunsaturates also distribute differently for *Limulus* and rat membranes. After weighting the fatty acid percentages for relative contributions from the phospholipid classes (exclusive of sphingomyelin), the average ratio of ($n-3$) to ($n-6$) polyunsaturates is 4.6 for rat and only 1.7 for *Limulus*. The polyunsaturates of phosphatidylserine provide the most striking example of the differences in the ($n-3$) and ($n-6$) distributions. Whereas 22 : 6($n-3$) is the major fatty acid of rat phosphatidylserine, ($n-3$) polyunsaturates are a relatively minor component of *Limulus* phosphatidylserine.

The fatty acids of *Limulus* and rat photoreceptor membranes also exhibit general similarities. Both membrane systems contain unusually high percentages of polyunsaturated fatty acids. After weighting for the percentage contributions within phospholipid classes (including sphingomyelin for *Limulus*), the sum of ($n-3$) and ($n-6$) polyunsaturates taken together was 41 % for *Limulus* and 42 % for rat. Moreover, in rat rod cells there is good evidence for in vivo functional equivalence of saturated and monounsaturated ($n-9$) fatty acids [18], and the profiles of the saturates plus monoenes taken together were quite comparable for photoreceptor membrane fractions of *Limulus* and rat.

## DISCUSSION

The polyunsaturate profiles probably represent the most significant lipid differences between rat and *Limulus* photoreceptor membranes. $C_{22}$ polyunsaturates, which predominate in rat rod outer segments, contain one more double bond than the corresponding $C_{20}$ polyunsaturates which predominate in *Limulus* membranes. Fatty acid conformation is dependent upon double-bond configuration, and although the

precise packing characteristics of the membrane fatty acids are not known, the more unsaturated fatty acids of rat outer segment membranes should not pack as tightly or as uniformly as the more saturated *Limulus* fatty acids. Consequently, fluid domains should be favored in the rat membranes [31, 32]. At least two additional lipid characteristics favor more fluid properties in rat outer segment membranes. For given chain length, the observed $(n-3)$ polyunsaturates are more unsaturated than the corresponding $(n-6)$ polyunsaturates; the $(n-3)/(n-6)$ ratio was 4.6 for rat and only 1.7 for *Limulus*, and hence the more unsaturated $(n-3)$ fatty acids are heavily favored in rat outer segment membranes. Also, the mol ratio of cholesterol to phospholipid is less in rat than in *Limulus*, and this too should selectively favor fluid domains in the rat membranes. That vertebrate outer segment membranes exhibit fluid properties in situ is indicated by the rapid mixing of bleached and unbleached patches of outer segment disc membranes [33–35]. Since bleached and unbleached are states of the membrane-bound photopigment rhodopsin, the rapid mixing of membrane patches has been interpreted as indicating rapid diffusion of rhodopsin in fluid disc membranes. Comparable experiments have not yet been reported for a rhabdomeric eye, but the fatty acid data suggest that diffusion coefficients should be substantially less for rhabdomeric membranes of *Limulus*.

Independent evidence indicates that the functional properties of rat photoreceptor cells are sensitive to the polyunsaturates in outer segment membranes. Rats cannot synthesize either $(n-3)$ or $(n-6)$ fatty acids de novo and hence the $(n-3)$ or $(n-6)$ polyunsaturate composition can be modified by appropriate dietary manipulations of essential precursors. Along with alterations in membrane polyunsaturates, which were observed after deprivation of $(n-3)$ and $(n-6)$ precursors, alterations were also observed in the spatial pattern of disc membrane renewal [16] and in the distribution of ionic currents generated by illumination of rat photoreceptor cells [17]. Moreover, the electrical response of the rod cell discriminates selectively between purified precursors of $(n-3)$ ,$(n-6)$ and $(n-9)$ fatty acids [18]. Yet rhodopsin was not measurably altered by these fatty acid substitutions [17].

A saturated $C_{18}$ fatty aldehyde*, derived from ethanolamine plasmalogens, makes up 12 % of the *Limulus* phosphatidylethanolamine fraction. Plasmalogens, which are abundant in neural tissue generally [36], have not been detected in substantial amounts in rod outer segments of normal rats** or other vertebrates. The $C_{18}$ fatty aldehyde may represent an important difference between the photoreceptor membranes of *Limulus* and normal rat, or it may be the result of moderate contamination of the photoreceptor fraction by membranes from the neural plexus of the *Limulus* retina.

The question of contamination of the photoreceptor fraction by other retinal tissue is difficult to resolve definitively, especially given the relatively small amount of membrane material per *Limulus* eye. The purity criterion used for our cell fractionation procedures was a minimum value of the ratio of protein absorbance at 278 nm to photopigment absorbance at $\lambda_{max}$. The absorbance ratio of the preparations reported

* Alk-1-enyl side chains of ethanolamine plasmalogens were converted to dimethyl acetals during acid methanolysis of phosphatidylethanolamine.

** The saturated $C_{18}$ fatty aldehyde, from ethanolamine plasmalogens, has been observed in rod outer segments after rats were subjected to prolonged deprivation of $(n-3)$ and $(n-6)$ fatty acid precursors [17].

here was less than that of our earlier preparations by several orders of magnitude, clearly indicating substantial enrichment of photoreceptor membranes in the later preparations. Present values of the absorbance ratio (see Methods) are comparable to those reported for rod outer segment fractions prepared by similar procedures, and the available evidence suggests that contamination of the *Limulus* photoreceptor membrane fractions was probably of the same order of magnitude as the contamination of rod outer segment preparations. In any event, the more interesting comparisons between the *Limulus* and rat data should be relatively insensitive to contamination of the *Limulus* fractions. The virtual absence of 22 : 6($n$—3) in the *Limulus* membrane fraction cannot be explained by the addition of moderate levels of contaminants. Similarly, the differences in the ($n$—3)/($n$—6) ratio appear to be too large to be accounted for by the magnitude of contamination which would be compatible with the observed absorbance ratios.

The phospholipid data reported for photoreceptor membrane fractions of rhabdomeric retinas from squid [37] and *Limulus* (this report) differ substantially. Phosphatidylethanolamine is the predominant phospholipid class of squid and Calliphora [37, 38], while phosphatidylcholine is predominant in *Limulus* and vertebrate photoreceptor membranes. Phosphatidylinositol was not detectable ($< 0.5\,\%$) in squid membranes, although it was a significant component of the *Limulus* membranes. The reported fatty acid profiles for squid [37] and *Limulus* also differ markedly. The squid profile is a continuum of fatty acids ranging in chain length from $C_9$ to $C_{24}$, and over 19 % of the squid fatty acids were shorter than $C_{16}$. The shortest chain length observed in *Limulus* was $C_{16}$. Additionally, 22 : 6 ($n$—3) comprises almost 10 % of total squid fatty acids, while 22 : 6 ($n$—3) was present in only trace amounts in *Limulus* photoreceptor membranes. It is possible that the differences in lipid profiles reported for squid and *Limulus* reflect differences in technique between laboratories, but if these differences are real the lipid profiles of rhabdomeric photoreceptors exhibit greater species variability than has been observed thus far in vertebrate outer segment membranes.

It would be premature to speculate at length about how the lipid differences between the rhabdomeric and outer segment membranes contribute to the observed transport difference of the membranes, since factors other than membrane lipid composition probably also contribute to these transport differences. For example, the normal ionic strengths of the extracellular surrounds are very different for *Limulus* and rod outer segment membranes, and the photopigment properties are not as similar as originally expected (Benolken, R. M. et al. ,unpublished). Additional uncertainties arise from the fact that plasma membranes constitute only 1–2 % of total outer segment membranes in the rat retina with disc membranes making up 98–99 %. Available ionic transport data generally apply to the characteristics of plasma membranes, whereas the lipid composition data apply to total outer segment membrane fractions in which disc membranes predominate. Unfortunately, to date, efforts to isolate purified fractions of plasma membranes have been unsuccessful.

Perhaps the lipid composition of plasma and disc membranes are not very different, because the disc membranes appear to be formed by pinching off invaginated regions of the basal plasma membrane [39]. From this observation and an assumption that lipids are tightly bound once incorporated into outer segment membranes, it could be argued that identical lipid compositions should be expected for plasma and

disc membranes. However, the argument is not compelling because replacement of phospholipid components has been observed in outer segment membranes [40–42]. Also, differences between the transport characteristics of plasma and disc membranes have been suggested [4]. Falk and Fatt [43] propose that the conductance of disc membranes increases in response to light although, as these authors point out, this interpretation depends in a crucial way upon how impedance components are assigned to an electrical model of their outer segment preparation. At the present time a number of important questions relating to the similarities and differences between plasma and disc membranes remain unresolved, and even less is known about possible differences between plasma and rhabdomeric membranes of *Limulus* photoreceptor cells.

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Zinkler [44] recently reported fatty acid distributions of phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine from retinas of the moth *Deilephila elpenor*. Chain lengths ranged from $C_{16}$ to $C_{20}$, and the major polyunsaturates were 18:3 and 20:5.

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