The early receptor potential, then, may reflect the electrical change accompanying the chromophore transfer.

In conclusion it is worth pointing out that the transfer reaction discussed here is apparently the only established example of a membrane process in which two major constituents are clearly involved, *i.e.*, the prosthetic chromophore is transferred from a specific *lipid* to a specific *protein site*. Should the early receptor potential be shown to be in the mainstream of physiological events, then the transfer of the prosthetic chromophore would appear to be the key molecular event in the generation of the (late) membrane receptor potential.

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Phospholipid Composition and Extractability of Bovine Rod Outer Segments and Rhodopsin Micelles*

Raymond P. Poincelot and E. W. Abrahamson

ABSTRACT: Bovine rod outer segments, after exhaustive phospholipid extraction with hexane or diethyl ether, liberate additional amounts of phospholipid upon photolysis. Examination of rod outer segments at various stages in the photolytic cycle was carried out to determine at what photolytic intermediate(s) this release occurred. In the rod outer segments the largest portion of the phospholipid was liberated in the photolytic process

$$\text{native rhodopsin} \xrightarrow{h\nu} \text{metarhodopsin}_{478}\ \text{I}$$

while in the subsequent thermal process

$$\text{metarhodopsin}_{478}\ \text{I} \longrightarrow \text{metarhodopsin}_{380}\ \text{II}$$

an additional but smaller amount of phospholipid was released. Examination of the released phospholipids showed that in the rod outer segments phosphatidylcholine was tightly bound, while phosphatidylethanolamine constituted the bulk of the loosely bound phospholipids. Rhodopsin isolated by detergent extraction (Emulphogene) from the bovine rod outer segments contained 31 moles of phospholipid/mole of rhodopsin. Separation of the rhodopsin from the detergent by ammonium sulfate fractionation and subsequent hexane extraction of the lyophilized material yielded a native rhodopsin, containing an eightfold molar excess of tightly bound phospholipid, largely phosphatidylethanolamine. These tightly bound phospholipids which apparently were directly and indirectly involved in the chromophoric binding site were subsequently released after photolysis.

Phospholipids have an active role in the chemistry of the visual process through their involvement as *N*-RPE[1] in the binding site and in the lipid to protein chromophore transfer (Poincelot *et al.*, 1970; Kimbel *et al.*, 1970). Previously, it had been suggested that lipids had some indirect function in the visual cycle as there was a release of an extra lipid increment upon bleaching delipidated rod outer segments in the presence of a nondenaturing solvent (Ishimoto and Wald, 1946; Krinsky, 1958). Attempts by Krinsky (1958) to do the same type of experiment with digitonin solutions

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *N*-RPE, *N*-retinylidenephosphatidylethanolamine; PE, phosphatidylethanolamine.

of rhodopsin were not successful. Recently, Shichi *et al.* (1969) have shown that the detergent Emulphogene can be used to solubilize rhodopsin and can be removed from native rhodopsin by treatment with ammonium sulfate. This largely detergent-free rhodopsin precipitate could then be extracted by lipid solvents.

We felt that with this technique it would be instructive to examine differences in phospholipid release from native rhodopsin and the intermediates metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II in rod outer segments and detergent micelles. One would expect to find an increase in phospholipid extractability to occur in the metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II transition, where the chromophore transfers from the lipid to the protein. Furthermore, as a direct consequence of the native binding site being phosphatidylethanolamine, one should not be able to remove all of that phospholipid in native rhodopsin with nondenaturing lipid solvents without destroying its spectral integrity. Complete removal by extraction should be possible either after photolysis or denaturation. With these ideas in mind, the following series of experiments were conducted.

Experimental Procedures

Lyophilized native rhodopsin as well as its metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II intermediates was prepared in rod outer segments and characterized as described previously (Poincelot *et al.*, 1970; Kimbel *et al.*, 1970).

Rhodopsin Prepared from Emulphogene Micelles. All work was done under tungsten white light (7.5 W) filtered through a Kodak series I Wratten filter. Rod outer segments (Poincelot *et al.*, 1970) from 200 retinas were hardened at 0° in 80 ml of 4% aqueous alum for 1 hr. They were washed in distilled water three times by homogenization and centrifugation at 12,000*g* for 15 min. Potter-Elvehjem homogenization with 30 ml of 1% Emulphogene (General Aniline) in phosphate buffer (pH 7, 0.1 M) was followed by gentle stirring of the solution for 30 min at 24°. The solution was then centrifuged at 40,000*g* for 30 min. Clear rhodopsin was drawn off and centrifugation was repeated under the same conditions. The clear supernatant was filtered through a Millipore syringe (pore size 0.22 μ) to remove suspended protein. A spectrum was taken on a Cary 14 recording spectrophotometer, which based on a molar extinction coefficient of 42,000 at 498 nm (Shichi *et al.*, 1969) showed yields of 1.2–2.0 μmoles of rhodopsin. This yielded 25 ml of solution to which was added sufficient ammonium sulfate to give 30% saturation. Centrifugation at 10,000*g* for 20 min left rhodopsin as a red fluff on the surface (Shichi *et al.*, 1969). This solution was poured off and an equivalent amount of 0.1 M phosphate buffer (pH 7) was added. The rhodopsin fluff was then homogenized with the Potter-Elvehjem apparatus. Treatment with 30% saturation of ammonium sulfate, centrifugation, decantation of the solution, and replacement with new buffer was repeated once when native rhodopsin was used for phospholipid analyses and twice for rhodopsin used in phospholipid thin-layer chromatography. The final resulting fluff was homogenized in a Potter-Elvehjem apparatus with 15 ml of 0.1 M phosphate buffer (pH 7) and then centrifuged for 15 min at 12,000*g*. The buffer was poured off and the process was repeated five times. This removed ammonium sulfate and some remaining Emulphogene. This preparation was carefully dark lyophilized to dryness and not allowed to warm above 24°. The powder was chilled to 4° under N_2 and used immediately.

Determination of Rhodopsin and Dry Weight of Outer Segments. A portion of the native lyophilized rod outer segments (0.1 g) or rhodopsin (0.01 g) was carefully weighed under red light to ±0.01 mg and rhodopsin was dissolved with stirring in 10 ml of Emulphogene in pH 7 0.1 M phosphate buffer for 30 min at 24°. This solution was then centrifuged at 40,000*g* for 30 min. Rhodopsin was drawn off and traces left in the tube were carefully washed out with distilled water. The rhodopsin solution was then filtered through a Millipore syringe (pore size 0.22 μ) and any rhodopsin remaining in the syringe was washed through with distilled water. From the absorbance at 498 nm and ϵ 42,000 (Shichi *et al.*, 1969), the molar content of rhodopsin was calculated. From their known weight, the molar content of other samples of rhodopsin was determined.

Extraction Procedure. All solvents were Lipopure grade (Applied Science) and deoxygenated with N_2 prior to use. Extractions were done under N_2 and red light at 4° using hexane or 2:1 $CHCl_3$–CH_3OH (Bligh and Dyer, 1959). Each prechilled sample (4°) was treated with 20-ml portions of solvent for 2 min in a Potter-Elvehjem homogenizer. The material was centrifuged at 12,000*g* for 15 min (0°) and the supernatant was removed. This extraction procedure was repeated twice. Finally, the hexane extract was filtered through the Millipore syringe and both extracts (hexane and 2:1 $CHCl_3$–CH_3OH) were stored under N_2 at −20°. A portion of the native rhodopsin left after hexane extraction was dissolved in 10 ml of 1% Emulphogene in phosphate buffer (pH 7) by stirring in the dark for 30 min at 24°. The slightly cloudy solution was filtered through the Millipore syringe and the spectrum was determined.

Photolysis of Rhodopsin. A tungsten photoflood lamp (150 W) at 20 cm was used as the light source. Illumination of the dry rhodopsin (known weight) in phosphate buffer (pH 7, 0.1 M) proceeded with stirring for 10 min at 24°. During this time, a rapid color change from red to yellow was observed. The solution was then lyophilized to dryness.

Hydrolysis of Phospholipids. Acid hydrolysis in 6 *N* constant-boiling HCl was carried out under vacuum at 110° for 3 hr. Longer time periods did not increase the yield of hydrolysis products.

Analyses. After phospholipid hydrolysis, the resulting ethanolamine and serine were determined on a Beckman Model 120C amino acid analyzer and the choline was determined colorimetrically by formation of a complex between choline and *cis*-aconitic anhydride (Bottcher *et al.*, 1961).

Thin-Layer Chromatography. Silica gel plates (Brinkmann Instruments, 5 × 20 cm, 0.25 mm thick) were run in unlined, grease-sealed tanks (29.0 × 9.5 × 28.0 cm) which were flushed with N_2 before each run at 4°. Extracts were applied under N_2 but not allowed to dry completely during application. The thin-layer plates were developed with I_2 vapor and tests for phosphorus, free amino groups, and choline were applied (Waldi, 1965).

Results

Extraction of four different rod outer segment preparations at various spectral intermediate stages with hexane yielded

TABLE I: Phospholipid Release in Hexane at Various Photolytic Stages of Bovine Rod Outer Segments.

Photolytic Stage of Rod Outer Segments		Dry Weight Per Cent[a] of Rod Outer Segments				
		I	II	III	IV	Mean
Native rhodopsin	PE[b]	4.03	3.71	3.30	3.51	3.64 ± 0.31
	PS[c]	0.83	0.80	0.75	0.87	0.81 ± 0.09
	PC[d]	2.35	2.21	2.43	2.61	2.40 ± 0.29
Metarhodopsin$_{478}$ I	PE	5.77	6.16	5.67	5.85	5.86 ± 0.37
	PS	1.19	1.31	1.21	1.18	1.22 ± 0.10
	PC	3.90	3.75	4.34	3.91	3.98 ± 0.44
Metarhodopsin$_{380}$ II	PE	7.91	8.41	7.64	7.80	7.94 ± 0.57
	PS	1.60	1.69	1.80	1.60	1.67 ± 0.16
	PC	5.61	5.01	5.72	5.23	5.39 ± 0.57

[a] Based on molecular weight of 750. [b] Phosphatidylethanolamine. [c] Phosphatidylserine. [d] Phosphatidylcholine (contains trace amounts of sphingomyelin; Poincelot and Zull, 1969).

the results summarized in Table I. No intermediates beyond metarhodopsin$_{380}$ II were examined.

Spectra of the native rhodopsin in Emulphogene have λ_{max} at 278 and 498 nm; the latter shifts to 370 nm upon photolysis. Average spectral absorbance ratios of $A_{400}:A_{498} = 0.31$ and $A_{278}:A_{498} = 2.85$ are observed. Ammonium sulfate precipitation (twice), lyophilization, and hexane extraction do not destroy the spectral integrity of native rhodopsin, as a sample subjected to these conditions followed by solvation in Emulphogene still has a λ_{max} at 498 nm (Figure 1).

Precipitation of rhodopsin three times with ammonium sulfate caused partial bleaching as indicated by an increase in absorbance at 370 nm. Hexane extracts of this material after photolysis and lyophilization showed the phospholipid composition summarized in Table II. Identification of the substances in the hexane extract showed phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, an unknown, retinol, Emulphogene, and retinal.

Extraction with hexane of light- and dark-adapted rhodopsin, precipitated twice with ammonium sulfate, followed by acid hydrolysis and chemical determination of ethanolamine, serine, and choline yielded the results summarized in Table III.

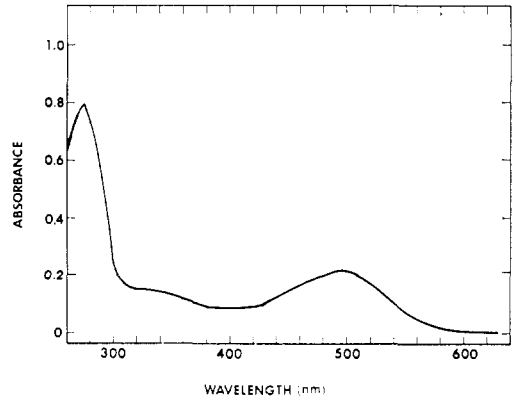

FIGURE 1: Solution spectrum of rhodopsin that was fractionated twice with ammonium sulfate, dark lyophilized, and hexane extracted.

TABLE II: Lipid Content of Hexane Extracts of Photolyzed Rhodopsin.

	R_F Values (× 100) in $CHCl_3$–CH_3OH–28% NH_4OH (70:30:4)			
	I_2	Free Amino[b] Groups	Phosphorus	Choline
Hexane extract[a]	7	7	7	
	27		27	27
	46	46	46	
	59[c]		59	
	93			
	97			
Standards				
Phosphatidylethanolamine	44	44	44	
Phosphatidylcholine	26		26	26
Phosphatidylserine	6	6	6	
Sphingomyelin	12		12	12
N-RPE	71		71	
Emulphogene	97			
Retinol	93			
Retinal	97			

[a] Average R_F values for three separate preparations. [b] Plates were heated to 110° (10 min) prior to ninhydrin spray to drive off concentrated NH_4OH. [c] Not identified.

TABLE III: Phospholipid Extractibility of Dark- and Light-Adapted Rhodopsin.

		Phospholipid[a] and Solvent							
		PE[b]		PS[c]		PC[d]			
Expt		Hexane	$CHCl_3$–CH_3OH (2:1)	Hexane	$CHCl_3$–CH_3OH (2:1)	Hexane	$CHCl_3$–CH_3OH (2:1)	Light–Dark[e]	$Light_{CM}$–$Dark_h$[f]
I	Dark	8.1		1.7		12.6		6.1	9.2
	Light	11.9	14.8	2.9	2.7	13.7	14.1		
II	Dark	8.9		1.9		13.8		4.2	6.8
	Light	12.1	13.7	2.5	2.3	14.2	15.4		
III	Dark	9.1		1.8		13.1		5.9	7.4
	Light	13.0	14.1	2.7	3.0	14.2	14.3		
IV	Dark	7.9		1.6		13.2		6.6	8.5
	Light	12.7	13.2	2.5	3.1	14.1	14.9		
Mean			13.9 ± 1.2		2.8 ± 0.6		14.7 ± 1.0	5.7 ± 1.1	8.0 ± 1.3

[a] Expressed as: moles of phospholipid/mole of rhodopsin. [b] Phosphatidylethanolamine. [c] Phosphatidylserine. [d] Phosphatidylcholine. [e] Total lipid difference between light and dark hexane extracts. [f] Total lipid difference between 2:1 $CHCl_3$–CH_3OH extract and dark hexane extract.

Discussion

Rod outer segments contain 38.2% lipid (dry weight) of which 2.75% is phosphorus (Poincelot and Zull, 1969; Adams, 1967). Therefore the phospholipid content of rod outer segments (assuming average phosphorus content of phospholipids is 4%) is 26.3%. From this and the phospholipid composition (Poincelot and Zull, 1969), it was calculated that the rod outer segments contained 10.1% phosphatidylethanolamine, 1.9% phosphatidylserine, 13.6% phosphatidylcholine, and 0.4% sphingomyelin as compared with 0.103% retinal. This yielded a total phospholipid:retinal molar ratio of 95:1 (106:1, Krinsky, 1958), a phosphatidylethanolamine:retinal molar ratio of 37:1, and a phosphatidylcholine:retinal molar ratio of 49:1.

Hexane extracted about 26% (11 to 30%, Krinsky, 1958) of the phospholipids in native rod outer segments (Table I) to yield the following molar ratios in the rods: phospholipid:retinal 70:1, phosphatidylethanolamine:retinal 24:1, and phosphatidylcholine:retinal 42:1. In rod outer segments at $metarhodopsin_{478}$ I hexane removed 44% of the phospholipid (phospholipid:retinal 53:1) and at $metarhodopsin_{380}$ II it extracted 58% of the phospholipid (phospholipid:retinal 40:1). Phospholipid releases have been observed to increase considerably upon photolysis of exhaustively extracted rod outer segments in the presence of petroleum ether (bp 30–60°) or diethyl ether (Ishimoto and Wald, 1946; Krinsky, 1958). Unfortunately, these releases were not studied quantitatively, so we have no previous data with which to compare our results.

Native rod outer segments after hexane extraction in the dark are enriched in the percentage of phosphatidylcholine as the hexane extract contained approximately 52% phosphatidylethanolamine as opposed to 30% phosphatidylcholine. Hence, the main bulk of phosphatidylcholine seems to be tightly bound while the bulk of the phosphatidylethanolamine is loosely bound. This same result was observed when the lipids of the erythrocytes were extracted (Roelofsen *et al.*, 1964).

Photolytic release of the larger hexane extractable increment of phospholipids occurs (Table I) in

$$\text{native rhodopsin} \xrightarrow{h\nu} \text{metarhodopsin}_{478}\text{ I (rod outer segments)}$$

Isomerization of the chromophore appears to be the only well defined molecular event that occurs in the photolysis cycle preceding the $metarhodopsin_{478}$ I stage. Initially the plane of the retinylidene chromophore is parallel to the plane of the disk membrane, so it would appear to be situated (Schmidt, 1938; Denton, 1959; Liebman, 1962; Wald *et al.*, 1963) in the membrane. Hence the isomerization of the chromophore in the membrane structure of the bovine rod outer segments must initiate some conformational change in the membrane affecting the protein–lipid interaction such that the phospholipid portion at $metarhodopsin_{478}$ I is more exposed to the solvent. In support of this notion it has been recently shown that the kinetics of the $lumirhodopsin_{493} \rightarrow metarhodopsin_{478}$ I in rod outer segments can be interpreted as a second order process in which the chromolipid *N*-RPE has mobility in the lipoprotein membrane (Rapp *et al.*, 1970).

Release of another smaller phospholipid increment at the $metarhodopsin_{478}$ I to $metarhodopsin_{380}$ II rod outer segments stage (Table I) is quite likely associated with the transfer of the chromophore from the lipid to protein (Kimbel *et al.*, 1970). Within the framework of the Danielli–Davson (1935) bilayer membrane theory, in which one has a lipid bilayer sandwiched between two proteins, an arrangement indicated by X-ray studies on frog and bovine rod outer segments (Blaurock and Wilkins, 1969; Gras and Worthington, 1969), one could envision the Schiff base (*N*-RPE) as being encapsulated in a hydrophobic environment. This model has some merits, as at

the phospholipid linkage site in native and metarhodopsin$_{478}$ I rod outer segments, $NaBH_4$ as observed by us has no reductive effect on the Schiff base. Furthermore, 8 M urea over a 24-hr period (24°) has no effect upon the spectral integrity of native rod outer segments (R. P. Poincelot, unpublished data, 1969). When the transfer from lipid to protein occurs, one could expect disruption of this hydrophobic capsule in the transfer of the chromophore to the hydrophilic environment of the protein where it is easily reduced by $NaBH_4$. Indeed $NaBH_4$ effected reduction at the *N*-retinylidinelysine site in rod outer segments (Kimbel *et al.*, 1970). Disruption of the hydrophobic capsule would be expected to alter the protein–lipid interaction, making rod outer segment phospholipids more susceptible to hexane extraction.

These additional photolytic releases of phospholipid are only found when using solvents that do not destroy the spectral integrity of rhodopsin at 500 nm. When solvents such as 2:1 $CHCl_3$–CH_3OH are used, the spectral integrity at 500 nm is destroyed and no additional phospholipids are released upon illumination. Upon photolysis, rhodopsin (in rod outer segments) undergoes a conformational change (Rapp *et al.*, 1970, and unpublished data) which may be regarded as a reversible denaturation; however, the denaturation caused by 2:1 $CHCl_3$–CH_3OH is not reversible. It would appear, then, that photolytically induced conformational changes involve alterations in the interaction of the lipid with the protein, leading to increased extractability of lipids when exposed to nondenaturing solvents. However, 2:1 $CHCl_3$–CH_3OH denatures the lipoprotein, removing the bulk of the lipid without photolysis.

Thin-layer chromatography was carried out on hexane extracts of rhodopsin (Emulphogene micelles). The rhodopsin was precipitated three times with ammonium sulfate to remove essentially all the detergent, as too much Emulphogene in the extract increased the R_F values for the phospholipids. Examination of Table II shows the expected phospholipid composition, with the exception that no sphingomyelin was present in the hexane extract of rhodopsin as was the case in rod outer segments (Adams, 1967; Poincelot and Zull, 1969). This lack of sphingomyelin in rhodopsin has also been observed by Adams (1969). Phosphatidylcholine, phosphatidylserine, retinal, and retinol were also identified in the extracts.

Examination of hexane dark extracts from essentially detergent free native rhodopsin compared with $CHCl_3$–CH_3OH (2:1) extracts (Table III) yielded a major difference with respect to rod outer segments (Table I). The hexane-extracted native rhodopsin was enriched in percentage of phosphatidylethanolamine, whereas the hexane-treated native rod outer segments were enriched in the percentage of phosphatidylcholine. Tightly bound phosphatidylcholine would appear to be associated with the rod outer segment disk membrane structure as indicated by its loose binding to rhodopsin micelles. Tight binding of phosphatidylethanolamine is consistent with its participation in direct and indirect binding of the chromophore. This small amount of tightly bound PE goes unnoticed in the rod outer segments, as there the bulk of this phospholipid is loosely bound. However, in micellular rhodopsin, considerably less PE is present initially and hence the tightly bound PE is more apparent upon hexane extraction.

A difference of 5.7 moles of hexane-extractable phospholipid/mole of rhodopsin between dark- and light-adapted visual pigment was observed (Table III). This extra increment released upon bleaching is approximately 4 moles of phosphatidylethanolamine, 1 mole of phosphatidylserine, and 1 mole of phosphatidylcholine. As in the case of rod outer segments, this difference in phospholipid extractibility is probably associated with the isomerization and subsequent transfer of the chromophore from lipid to protein in the metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II process.

To obtain some notion of the amount of phospholipid intimately associated with and essential to the maintainence of the spectral integrity of native rhodopsin, we subtracted the amount removed in the dark by the nonbleaching hexane from the total amount of phospholipid present as determined by the Bligh and Dyer (1959) procedure with 2:1 $CHCl_3$–CH_3OH (Table III). A total of about 31 moles of phospholipid associated with native rhodopsin was found, 8.0 of which were tightly bound. Five of these tightly held were phosphatidylethanolamine. This would appear to be the phospholipids associated with the binding site. Adams (1969) also found a comparable amount of phosphatidylethanolamine in his partially delipidated bleached rhodopsin. However, his phosphatidylcholine content was higher than ours which could be due to variations in the respective methods. Our native rhodopsin was isolated in Emulphogene and freed from the detergent with ammonium sulfate precipitation whereas Adams' rhodopsin was isolated in digitonin and precipitated with ergosterol. These observed differences in phosphatidylcholine content could quite likely be attributed to differences in the detergent–phospholipid interactions. A stronger binding interaction between Emulphogene and phosphatidylcholine over that for rhodopsin and phosphatidylcholine, with the reverse being true for digitonin micelles of rhodopsin, would explain the observed differences. No difference in the phosphatidylethanolamine content was observed with the two methods, suggesting that it is tightly bound to the binding site region so as to be unaffected by detergent interactions.

A point of some interest is the λ_{max} at 275 nm observed in our methanol and acid methanol extracts of rhodopsin and rod outer segments (Poincelot *et al.*, 1970). We observed that natural bovine phosphatidylethanolamine had a λ_{max} at 275 nm whereas the synthesized dipalmitoyl variety did not. This may mean that a variation in the fatty acid content was responsible for the λ_{max} at 275 nm. Fatty acid analyses on total lipid extracts in rod outer segments and rhodopsin showed large amounts of polyunsaturates, which as the esterified moiety might, through some cooperative effect or partial oxidation, account for a λ_{max} at 275 nm (Poincelot and Abrahamson, 1970). Absorbances from unsaturates have been observed in the 270–280-nm regions with ethanol solutions of animal fats and oils (Brice and Swain, 1945).

Using 28,000 as the molecular weight of opsin (Shields *et al.*, 1967; Heller, 1968; Shichi *et al.*, 1969), and assuming a molecular weight of 750 for a mole of phospholipid, a molecular weight of 51,250 was calculated for the lipoprotein rhodopsin as isolated by Emulphogene. This compares with 40,000 as isolated in digitonin (Hubbard, 1953–1954).

We have prepared essentially detergent-free rhodopsin with 8.0 moles of phospholipid/mole of rhodopsin without altering its spectral maximum at 500 nm. This is evidently the limit of hexane extractability. F. J. M. Daemen, however, in our laboratory (unpublished data, 1969), by treating

rod outer segments with phospholipase C was able to obtain spectrally intact rhodopsin with only 3 moles of phospholipid, 1 of which was phosphatidylethanolamine, the other 2 being phosphatidylserine.

One cannot remove all the phosphatidylethanolamine without disrupting the spectral integrity at 500 nm. Complete removal was possible only with denaturation or extraction after photolysis (Table III), both of which disrupted the spectral integrity. This is clearly to be expected if the chromophore binding site is phosphatidylethanolamine. Heller (1968) has reported a rhodopsin preparation which upon phosphorus analysis would appear to have only 0.5 mole of phospholipid/mole of rhodopsin. However, as pointed out by H. Shichi (personal communication, 1969), this was based on a value of ϵ 23,000, which disagrees with the generally excepted value ϵ 40,600 (Wald and Brown, 1953–1954) or the more recent value ϵ 42,000 (Shichi *et al.*, 1969). If one uses ϵ 42,000 this gives approximately 1 mole of phospholipid/mole of rhodopsin. Shichi upon duplication of Heller's rhodopsin preparation found a value of ϵ 39,700. On close examination of Heller's procedure for phosphorus analysis, it is apparent that if 1 mole of phospholipid/mole of rhodopsin was present in his rhodopsin–CTAB solution, there would be present a 5000:1 molar ratio of CTAB to phospholipid. Under such conditions there is considerable doubt whether one extraction with ethanol–diethyl ether (3:1) would quantitatively remove the phospholipids from CTAB micelles of rhodopsin, particularly in the case of tightly bound phosphatidylethanolamine. This detergent–phospholipid–protein interaction would most likely be difficult to disrupt with ordinary lipid solvents. Furthermore, the ethanol–diethyl ether (3:1) used by Heller is known to only partially extract brain lipids (Mangold, 1965). An analysis of the residue left after extraction, which was not reported by Heller, would have been necessary to assure that no phospholipids were in his rhodopsin preparation.

In conclusion it is apparent that lipids play a significant role in the visual process, not only through binding the prosthetic chromophore (Poincelot *et al.*, 1970) but presumably in modulation of the transmembrane potential (Kimbel *et al.*, 1970) and permeability characteristics.

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