Determination of the Chromophoric Binding Site in Native Bovine Rhodopsin*

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ABSTRACT: Two different laboratories have recently shown that when the vertebrate (bovine) visual pigment rhodopsin is illuminated in the presence of the reducing agent $NaBH_4$, the retinylidene chromophore is reductively affixed to the ϵ-amino group of a lysyl unit in the backbone protein of opsin. In our laboratory we have demonstrated a different binding site for the retinylidene chromophore in native (unilluminated) vertebrate (bovine) rod outer segments and rhodopsin micelles.

In contrast to the *illuminated* rhodopsin, native rod outer segments or rhodopsin detergent micelles in the dry or wet state can be extracted with methanol or acidic methanol to remove the chromophore quantitatively as *N*-retinylidenephosphatidylethanolamine. Similar results were obtained with methanol extracts of heat-denatured, acid-buffered (pH 4.5) native rod outer segments or rhodopsin micelles that had been reduced with $NaBH_4$. Since isolation of this chromophore can be carried out under acid conditions that precludes any imine exchange, we conclude that the prosthetic chromophore in native bovine rhodopsin exists as a chromolipid Schiff base, *N*-retinylidenephosphatidylethanolamine.

In 1950 Collins and Morton tentatively proposed that the retinal chromophore in visual pigments was conjugated to the protein moiety of opsin through an amino group. Morton and Pitt (1955, 1957) deduced that the nitrogen atom was quaternary suggesting the linkage was a protonated Schiff base. Krinsky (1958), on the other hand, based on his identification of substantial quantities of aminophospholipids in vertebrate rods suggested a possible lipid binding site for the chromophore in native rhodopsin. In support of this he cited an early observation of Wald (1937–1938) in which he extracted from rhodopsin in organic solvents a retinal complex with acid–base indicator properties.

Recently Bownds and Wald (1965) and Akhtar *et al.* (1967) reported the reductive affixation of the chromophore on illumination of aqueous, digitonin micellular suspensions of bovine rhodopsin. Both groups (Bownds, 1967; Akhtar *et al.*, 1968) later reported that the retinylidene group was covalently bound at the metarhodopsin$_{380}$ II stage to the ϵ-amino group of a lysyl unit in the backbone protein. However, as the reductive affixation was performed in the pH range 6.0–8.0, where imine exchange and Schiff base formation readily occur for retinal derivatives (Morton and Pitt, 1955), there appears to be some question as to whether the ϵ-amino group of lysine is, in fact, the chromophore binding site at metarhodopsin$_{380}$ II. But an even less justified inference has been made that the chromophore binding site in *native* rhodopsin is the same lysyl group identified in metarhodopsin$_{380}$ II.

In an earlier note (Poincelot *et al.*, 1969) we reported our finding that in *native* rhodopsin (as CTAB[1] micelles or in the rod outer segments) the chromophore is bound not to the protein but rather to the lipid phosphatidylethanolamine. We also verified the amino group of a lysyl unit in the backbone protein as the chromophore binding site in metarhodopsin$_{380}$ II under acidic conditions which normally preclude imine exchange. Furthermore we demonstrated that the retinylidene chromophore underwent imine exchange from lipid to protein in the thermal reaction metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II.

The present paper sets forth in detail the experimental evidence for the lipid binding site of the chromophore in native rhodopsin. A second paper (Kimbel *et al.*, 1970) describes the experiments substantiating the chromophore transfer from lipid to protein in the process metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II. A third paper (Poincelot and Abrahamson, 1970a) deals with the phospholipid content of native rhodopsin and also with the lipid extractability of metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II rod outer segments.

Experimental Procedures

Preparation and Isolation of Native Rod Outer Segments. Dark-adapted bovine retinas were obtained frozen from the Hormel Institute, Austin, Minn., and were stored at −20°. All procedures were performed at 4° under dim red light (7.5-W white lamp filtered through Wratten Safelight Series 1 filter, Eastman Kodak, Rochester, N. Y.). Frozen retinas (200) were ground in a mortar until they were soupy in consistency. They were mixed with 200 ml of 0.1 M phosphate buffer at pH 7. This resulting solution was homogenized for 5 min in a Potter-Elvehjem homogenizer, the resulting homogenate being centrifuged at 75*g* for 15 min. Careful decantation of the supernatant was followed by its centrifuga-

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[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: CTAB, cetyltrimethylammonium bromide; *N*-RPE, *N*-retinylidenephosphatidylethanolamine; *N*-RH_2PE, *N*-retinylphosphatidylethanolamine; *N*-RPE, *N*-perhydroretinylethanolamine; *N*-RH_2PS, *N*-retinylphosphatidylserine.

tion at 27,000*g* for 15 min. The resulting precipitate was dispersed in 100 ml of 40% (by weight) sucrose in 0.1 M phosphate buffer (pH 7) by homogenization in the Potter-Elvehjem apparatus for 5 min. This dispersion was centrifuged at 27,000*g* for 15 min and followed by dilution of the separated supernatant to four times its volume with phosphate buffer. Centrifugation at 12,000*g* for 15 min resulted in a precipitate which was washed three times with 20 ml of distilled water by repeated homogenization and centrifugation at 12,000*g* for 15 min. The wet rod outer segments were usually lyophilized (microscopically homogeneous, 1250×) overnight to dryness in the dark (yield 0.2 g). When partial delipidation was desired, the rod outer segments were immediately extracted with three 20-ml volumes of hexane (0°). This was accomplished by treatment in the Potter-Elvehjem homogenizer followed by centrifugation at 12,000*g* for 15 min. The rod outer segments were used immediately for further extractions after pouring off the last volume of hexane.

Preparation of Bovine Rhodopsin. Wet rod outer segments derived from 200 retinas (prior to lyophilization) were dark extracted at 4° with 60 ml of 2% CTAB in 0.1 M phosphate buffer (pH 7) by stirring for 30 min. Any insoluble residue was removed by centrifugation at 12,000*g* for 15 min followed by Millipore syringe filtration (pore size 0.22 μ). Dark lyophilization overnight produced a powder which was extracted with hexane in the same manner as the rod outer segments. This procedure removed excess CTAB and then the rhodopsin was used immediately for further extractions.

Spectra. Solution spectra were taken on a Cary 14 R double-beam recording spectrophotometer in two matched 1-cm light-path cells at 20°. Spectra of powders were taken with a cell-space total diffuse reflectance accessory.

Extractions were carried out in darkness at 4° and under N_2. Solvent (30 ml) was added to the freshly prepared rod outer segments or rhodopsin as (one-fourth of amount prepared from 200 retinas) CTAB micelles. This was homogenized for 3 min in the Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 12,000*g* for 15 min with the supernatant being poured off. Residues were then similarly reextracted twice. Extracts were stored under N_2 at −20°. Extractions were done using deoxygenated Spectroscopic grade hexane, methanol, and 0.1–$10^{-3.5}$ M dry HCl gas in methanol.

Reductions of the C=N linkage of native Schiff bases were carried out in methanol or methanol $10^{-3.5}$ M in HCl gas (total extract of rod outer segments or rhodopsin from approximately 50 retinas, *i.e.*, 90 ml) using 30 mg of $NaBH_4$ at room temperature for methanol and 50 mg for acidified methanol. Catalytic hydrogenation of the polyene chain of the retinyl moiety of synthetic and native Schiff bases was performed over palladium black in methanol or methanol $10^{-3.5}$ M in HCl gas. This solution was stirred while H_2 was bubbled into it for 2 hr. The resulting product was filtered to remove catalyst and stored under N_2 at 4°. $NaBH_4$-reduced extracts were rotary evaporated to a small volume and partitioned between H_2O–$CHCl_3$–CH_3OH (1.0:2.0:1.0, v/v). The $CHCl_3$ organic layer was removed, leaving behind extraneous ions in the other layer, and concentrated for thin-layer chromatography.

Heat Denaturation of Rod Outer Segments and Rhodopsin. Rod outer segments (from 100 retinas) were homogenized in 0.1 M pH 4.5 citric acid–phosphate buffer (McIlvaine, 1921) and then centrifuged at 12,000*g* for 15 min. This process was repeated twice. After this washing, one-half of the rod outer segments was dark extracted at 4° with 2% CTAB in the same McIlvaine's buffer (15 ml) and then centrifuged at 12,000*g* for 15 min, the precipitate being discarded. A spectrum of each (rod outer segments and rhodopsin) was taken to assure that the material remained unbleached (no loss of spectral λ_{max} at 500 nm). Both were thermally dark denatured (66°) at pH 4.5 (McIlvaine's buffer, 30 ml) for 15 min with stirring, in the presence of 50 mg of $NaBH_4$ or later addition within a few minutes. After thermal denaturation one cannot regenerate rhodopsin upon adding 11-*cis*-retinal as is the case with photolytically denatured opsin (Hubbard, 1958). A check was run upon the pH to ensure that it remained at 4.5. The reduced rod outer segments and rhodopsin were then lyophilized, followed by methanol extraction described previously under extractions. A spectrum was taken and then the extracts were partitioned between the aqueous and organic phases of H_2O–$CHCl_3$–CH_3OH (1.0:2.0:1.0, v/v) with a spectrum of each phase being taken. The amount of reduced Schiff base was determined at the λ_{max} at 325 nm using an ϵ value of 50,000 (Bownds, 1967).

Chromophore Determination. Lyophilized rod outer segments or rhodopsin were weighed and a known amount removed in the dark. This was stirred in 0.1 M pH 7 phosphate buffer which was made 0.2 M in hydroxylamine and then bleached. After lyophilization the retinene oxime was removed by extraction with ethanol and the amount was determined based on an extinction coefficient (ϵ 60,000) at 355 nm for retinylidene oxime (Wald and Brown, 1953–1954; Pitt *et al.*, 1955). Methanol or (neutralized) acidified methanol extracts were treated with hydroxylamine to convert the Schiff base into the oxime (Akhtar *et al.*, 1968) which was determined spectrally and compared with the total amount present.

Hydrolysis of Extracts. Extracts containing the reduced Schiff base were rotary evaporated to near dryness without application of heat; 6 ml of 2 N NaOH was added and the mixture was frozen under vacuum to −76°. The tube was sealed under vacuum and heated at 100° for 6 hr, whereupon it was centrifuged and the supernatant was removed. This was used directly for amino acid analysis and was extracted with $CHCl_3$ for thin-layer or gas chromatography.

Amino Acid Analysis. Analyses were done on a Beckman amino acid analyzer Model 120C.

Thin-Layer Chromatography. All thin-layer chromatography was done on commercially available silica gel G plates (Brinkmann Instruments). Analytical plates (5.0 × 20.0 cm) were developed in unlined, cylindrical, ground glass sealed tanks (23.0 × 6.0 cm) while preparative plates (20.0 × 20.0 cm) were developed in unlined tanks (29.0 × 9.5 × 28.0 cm) which were flushed with N_2 before each run at 4°. Standard and sample spots were analyzed on wet plates for fluorescence by irradiation under ultraviolet light and treated with iodine. Specific tests for phosphorus, free amino groups, and retinal were also utilized (Waldi, 1965). Spots were applied under N_2, but never allowed to dry during the process.

Plasmalogen Determination. Methanol extracts of partially delipidated rod outer segments or rhodopsin (reduced by $NaBH_4$) were applied in the lower right-hand corner of silica gel G plates (20 × 20 cm) and developed in 10:1 $CHCl_3$–CH_3OH (see preceding section on thin-layer chromatography).

Phospholipids remained at the origin while *N*-RH_2PE and retinol were found by fluorescence at R_F ($\times 100$) values of 22 and 88, respectively. Plates were dried under N_2 and then held over concentrated HCl (50–60°) at a 15-cm distance for 5 min, whereupon they were rotated 90° clockwise and developed twice in the same direction with 95:5 hexane–diethyl ether (Schmid and Mangold, 1966; Schmid *et al.*, 1967). After drying, the plates were sprayed with an ethanol–HCl solution of 2,4-dinitrophenylhydrazine to detect free aldehyde (Waldi, 1965) at R_F ($\times 100$) 51.

Gas chromatography was carried out on a Varian Aerograph Model 600C using a 5 ft $\times$ $^1/_8$ in. column packed with 5% SE-30 on acid-washed, 100–120 mesh Chromosorb W (Supelco). The carrier gas was N_2 (15 ml/min) and the detection was by flame ionization using H_2 (15 ml/min). Temperature of the column and detector was maintained at 245° while the injector was set at 340°.

Synthesis of all-trans-N-Retinylphosphatidylethanolamine. Phosphatidylethanolamine (Calbiochem grade A, 0.1 mmole) was dissolved in absolute methanol to which was added under N_2 *all-trans*-retinal (Distillation Products Inc.) in the amount of 0.03 mmole. These were allowed to react in the dark under N_2 for 1 hr. Rotary evaporation was used to concentrate the solution (without heat) to a small volume (about 1 or 2 ml) to which under N_2 was added 0.1 g of $NaBH_4$. A spectrum before and after reduction was taken in methanol and acidified methanol. A spectral maximum at 365 nm which shifted to 445 nm upon acidification with a shoulder peak at 397 nm was observed, indicative of Schiff base behavior (Pitt *et al.*, 1955; Ball *et al.*, 1949). After $NaBH_4$ reduction of this *N*-RPE to *N*-RH_2PE, a λ_{max} was observed at 325 nm. Thin-layer chromatography in 10:1 $CHCl_3$–CH_3OH (F. J. M. Daemen, personal communication, 1969) showed two spots and a faint trace near the solvent front. Tests for phosphorus, free amino groups, fluorescence under ultraviolet light, retinyl derivatives, and standards showed the component at R_F ($\times 100$) 0 to be phosphatidylethanolamine, that at R_F 22 to be *N*-RH_2PE, and that at 88 to be retinol. The component at R_F 22 was examined under ultraviolet light on the wet plate. Fluorescence appeared after a few minutes of irradiation and the spot tested positive for phosphorus and retinyl derivatives but negative for free amino groups. Elution of this compound with 2:1 $CHCl_3$–CH_3OH for spectral analysis yielded a solution whose absorption spectrum showed a λ_{max} at 325 nm. *N*-RH_2PE was stored under N_2 at −20° and used within 24 hr.

Synthesis of 11-cis-N-RPE. Synthesis was performed in the same manner as the all-*trans* form, except for the use of 11-*cis*-retinal (Distillation Products Inc.). Caution had to be taken to ensure that all operations were carried out under dim red light and N_2. Spectra were also taken. No noticeable differences in the spectrum were observed as compared with the all-*trans* form. In the acidified form of 11-*cis*-*N*-RPE, a shoulder is also seen at 397 nm.

Synthesis of all-trans-N-RH_2PS. Phosphatidylserine (Applied Science) was dissolved in 0.1 N methanolic NaOH and then *N*-RH_2PS was formed in the same manner as *N*-RH_2PE. The same solvent system (as for *N*-RH_2PE) was used, the only difference being that *N*-RH_2PS had an R_F ($\times 100$) of 7.

Synthesis of N-Retinylethanolamine. *all-trans*-Retinal (1 mmole) was added under N_2 to 250 mmoles of ethanolamine (purified by vacuum distillation). Stirring (darkness) under N_2 for 2 hr over activated Zeolite 4A (dehydrating reagent) completed the reaction. A small aliquot was removed and dissolved in methanol. An ultraviolet–visible spectrum was taken before and after acidification with a few drops of HCl. Infrared spectra were taken after the methanol was removed by N_2 evaporation. $NaBH_4$ (0.3 g) was stirred into the reacted mixture after addition of 10 ml of methanol, whereupon an infrared and ultraviolet–visible spectrum was taken of this mixture. The methanolic solution was then diluted with two volumes of deoxygenated, distilled water and extracted with three 20-ml portions of hexane to remove retinol. Following this the aqueous portion was extracted with three 20-ml portions of $CHCl_3$. The $CHCl_3$ extract was rotary evaporated to near dryness without application of heat and then extracted with water to remove any ethanolamine that was carried over. The yellow, oily residue was vacuum dried at −76° over P_2O_5 and paraffin shavings for 24 hr and then sealed under vacuum. The elemental analysis and molecular weight was calculated as: C, 80.09; H, 10.71; N, 4.25; mol wt 329. Found: C, 78.80; H, 10.85; N, 4.16; mol wt 322. Attempts at crystallization were unsuccessful. The end product in all instances was a yellow oil which was stored under vacuum at −20°.

N-Retinylideneethanolamine (basic form) had a λ_{max} at 365 nm and was infrared characterized by the absence of the C=O stretching frequency of retinal at 1664 cm^{-1} with concomitant appearance of a sharp peak at 1620 cm^{-1}, indicative of the C=N stretching frequency. Acidification shifted the λ_{max} to 445 nm and the C=N stretching frequency to 1650 cm^{-1}, characteristic of $C{=}N^+$. Reduction blue shifted the λ_{max} to 325 nm with a loss of the C=N stretching frequency (Witkop, 1954; Kovacic, 1967; Leonard and Paukstelis, 1963).

Synthesis of N-Retinyllysine. Lysine monohydrochloride (0.5 g from Nutritional Biochemicals Corp.) was dissolved in 10.0 ml of H_2O and the pH was increased to 9.5 with 2.0 N NaOH. To this was added 50 mg of *all-trans*-retinal in 20 ml of ethanol. A color change was noted and the λ_{max} after standing 1 hr was 365 nm. One drop of concentrated HCl added to that part of the solution used for spectral measurement shifted this to 445 nm.

To the solution was added 100 mg of $NaBH_4$. After bubbling had ceased, the λ_{max} was 325 nm. The reaction mixture was then concentrated by rotary evaporation and applied to 20 $\times$ 20 cm preparative thin-layer chromatography plates (Brinkmann Instruments), and developed in unlined grease-sealed tanks with methyl ethyl ketone–pyridine–H_2O–acetic acid (70:15:15:2). The component band at R_F ($\times 100$) 33 which fluoresced under ultraviolet light had a positive retinyl derivative test as well as a free amino group test. The component was removed and eluted with ethanol–concentrated NH_4OH (50:50), yielding a filtered solution whose spectrum had a λ_{max} at 325 nm. The other two bands were identified as lysine (R_F ($\times 100$) 0) and retinol (R_F ($\times 100$) 100). *N*-Retinyllysine was stored dry under vacuum at −20°.

Synthesis of N-Retinylserine. A 1:3 millimolar ratio of retinal and serine was dissolved in 10 ml of absolute methanol with the retinal being dissolved first; 20 ml of 0.1 N ethanolic NaOH was added. This was done under red light and the mixture was dark reacted for 6 hr. A spectrum at that time showed a λ_{max} at 365 nm which shifted to 445 nm upon addition of one drop of concentrated HCl. $NaBH_4$ (0.2 g) was

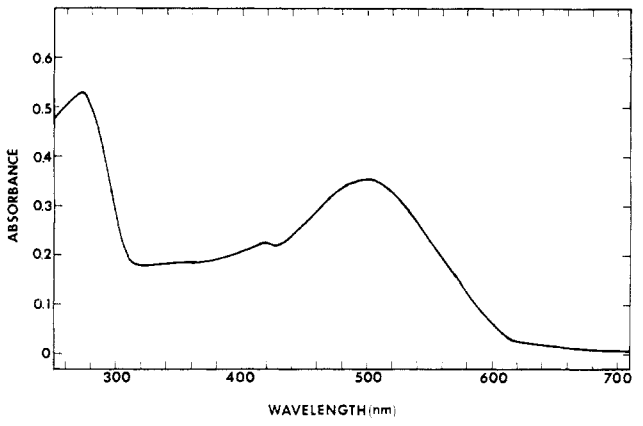

FIGURE 1: Powder spectrum of native rod outer segments taken after dark lyophilization.

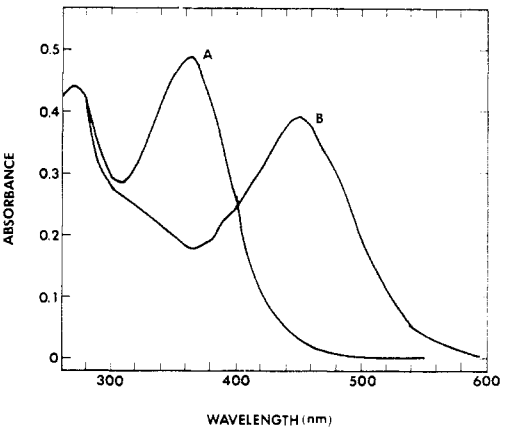

FIGURE 2: Solution spectra of alkaline (A) and acidic (B) forms of the *N*-retinylidene Schiff base extracted in methanol.

added and stirred under N_2 yielding finally a solution with a λ_{max} at 325 nm. The solution was rotary evaporated to near dryness without heat and diluted with two volumes of water, then extracted first with hexane and secondly with $CHCl_3$ as was *N*-retinylethanolamine. This $CHCl_3$ extract was applied to preparative (20 × 20 cm) thin-layer chromatographic plates (Brinkmann Instruments) under a gentle stream of N_2. Plates were developed in absolute methanol with component bands at R_F (×100) 0 (trace) and 28 and 98 (trace). These were identified through fluorescence under ultraviolet light, tests for free amino groups, retinyl derivatives, and standards as serine, *n*-retinylserine, and retinol, respectively. The component band at R_F (×100) 28 was eluted with 2:1 $CHCl_3$–CH_3OH and had a λ_{max} at 325 nm. It was stored under vacuum at −20°.

Synthesis of N-Perhydroretinylethanolamine. *N*-Retinylethanolamine (1 mmole) was dissolved in 30 ml of absolute ethanol and 0.1 g of palladium black was added. The system was flushed with N_2 and stirred. H_2 was then gently bubbled into the solution and the N_2 was turned off. Stirring and bubbling of H_2 was continued for 2 hr. A color change from yellow to colorless was observed. The H_2 was shut off and the system was purged with N_2. The solution was filtered to remove the catalyst and rotary evaporated without heat to 1 or 2 ml. Methanol (deoxygenated by N_2) was used to elute the sample after application to a Sephadex LH-20 column (23 × 2 cm). Samples (5 ml) were collected and monitored by gas chromatography. Fractions 8–11 were the peak fractions and came off at a molecular weight of approximately 350. Ethanolamine came off in later fractions as verified by standard ethanolamine samples applied to the column. Fractions 9 and 10 were dried by rotary evaporation and under vacuum for 24 hr over P_2O_5 and paraffin shavings. *Anal.* Calcd: C, 77.81; H, 13.35; N, 4.12. Found: C, 79.93; H, 12.25; N, 5.10. Attempts at further purification by crystallization were not successful.

Preparation of N-PRE from Rod Outer Segments or Rhodopsin. Extracts in methanol or methanol $10^{-3.5}$ M in HCl gas were first reduced with 30 mg of $NaBH_4$ and then hydrogenated as in the above procedure. The resulting solution after filtration was then alkaline hydrolyzed as described earlier and the methanol extract of this hydrolysate (1–2 ml after rotary evaporation without heat) was purified on Sephadex LH-20 as for synthetic *N*-PRE in the above procedure.

Schiff Base Model Studies. All work was carried out under N_2 at 4° and care was taken to avoid introduction of water into the systems. *N*-Retinylidenehexylamine (0.5 μmole; see *N*-RH_2PE for synthesis method) was diluted to 90 ml in $10^{-3.5}$ M HCl in anhydrous methanol. To this solution was added 10 μmoles of aniline and, in a similar experiment, 10 μmoles of hydroxylamine (free base). A spectrum was taken prior to and after the addition of the amines. A spectral examination was repeated on these mixtures after a 45-min interval. These same experiments were also carried out in anhydrous methanol (no HCl gas). Schiff base formation with 10 μmoles of phosphatidylethanolamine and 0.5 μmole of retinal was also attempted in 90 ml of $10^{-3.5}$ M HCl in anhydrous methanol.

Results

Examination of dark lyophilized or partially delipidated rod outer segments and rhodopsin (as the CTAB micelle) by ultraviolet–visible spectroscopy confirmed that the spectrum of the native pigment was not affected in the process. The absorbance maximum at 500 nm, characteristic of the native state, was preserved in both systems. Two λ_{max} at 278 and 500 nm and a small shoulder at 410 nm were observed for rod outer segments (Figure 1) whereas λ_{max} at 278 and 500 nm and a small (345 nm) β peak were observed for rhodopsin (at CTAB micelles). This small shoulder at 410 nm in rod outer segments can be attributed to traces of hemoglobin contamination; however, this is not spectrally detectable in CTAB micelles of rhodopsin.

Methanol dark extracted a yellow component from wet or lyophilized rod outer segments or rhodopsin micelles with a λ_{max} at 275 and 365 nm. This red-shifted to 445 nm upon acidification (Figure 2). Reduction with $NaBH_4$ blue-shifted the λ_{max} to 325 nm. Chromophore determination showed that more than 90% of the retinal had been removed.

Imine exchange was found to occur readily in methanol. *N*-Retinylidenehexylamine has a λ_{max} at 360 nm as opposed to 385 nm for *N*-retinylideneaniline. When aniline was added to *N*-retinylidenehexylamine, we observed a broadening of the

TABLE I: Fluorescent Compounds Observed in Reduced Methanol and Acidified Methanol ($10^{-3.5}$ M HCl gas) Dark Extracts of Native Rod Outer Segments and Rhodopsin.

Sample	R_F (×100) Values of Fluorescent Spots Solvent I[a]	II[b]	III[c]	IV[d]
Reduced CH_3OH extract				
Rod outer segments	69, 92	79, 100	72, 97	17, 81
Rhodopsin	67, 94	81, 100	73, 98	22, 89
Reduced acidified CH_3OH extract				
Rod outer segments	68, 92	79, 99	73, 98	20, 82
Rhodopsin	69, 92	81, 99	74, 98	25, 87
CH_3OH extract				
Rod outer segments[e]	68, 92[f]	76, 100[f]	70, 96[f]	23, 83[f]
Rhodopsin[e]	66, 95[f]	81, 100[f]	74, 98[f]	26, 88[f]
NRH_2PE	69	79	72	22
NRH_2PS				7
Retinol	93	100	97	88

[a] $CHCl_3$–CH_3OH–28% NH_4OH (70:30:4, v/v). [b] Methyl ethyl ketone–pyridine–water–acetic acid (70:15:15:2, v/v). [c] $CHCl_3$–CH_3OH–acetic acid (70:27:3, v/v). [d] $CHCl_3$–CH_3OH (10:1, v/v). [e] Reduced before extraction after being thermally dark denatured at pH 4.5. [f] Faint.

peak at 360 to 385 nm. Addition of hydroxylamine to methanolic solutions of *N*-retinylidenehexylamine caused a blue shift of the peak from 365 to 355 nm, the λ_{max} of *N*-retinylideneoxime.

When thermally denatured rod outer segments and rhodopsin were reduced in the dark at pH 4.5 in an aqueous system with $NaBH_4$ and lyophilized, the methanol extract had a λ_{max} at 325 nm. When this was partitioned between H_2O–$CHCl_3$–CH_3OH (1.0:2.0:1.0, v/v), the 325-nm absorbance remained with the $CHCl_3$ phase (Figure 3). Determination of the chromophore showed more than 85% was removed. A check on pH showed no change after the reduction took place.

Extraction of wet or dry rod outer segments or rhodopsin in the dark with methanol containing HCl gas yielded a solution having λ_{max} at 275, 397, and 445 nm (Figure 4), which accounted for more than 90% of the chromophore. The spectral ratio of the absorbance at 397 nm to that at 445 nm varied from 2.5 for extraction of lyophilized rod outer segments (no hexane delipidation) with 0.1 M HCl gas in methanol to 1.1 for extraction of hexane delipidated lyophilized or wet rod outer segments with $10^{-3.5}$ M HCl gas in methanol. Addition of $NaBH_4$ caused the disappearance of these peaks (397 and 445 nm) and a new peak appeared at 325 nm.

Formation of *N*-RPE did not occur in acidified methanol ($10^{-3.5}$ M in HCl gas), even after standing for several hours under N_2 at 4°. Furthermore, imine exchange in acid methanol ($10^{-3.5}$ M HCl) between protonated *N*-retinylidenehexylamine and aniline or hydroxylamine was not evident after 45 min.

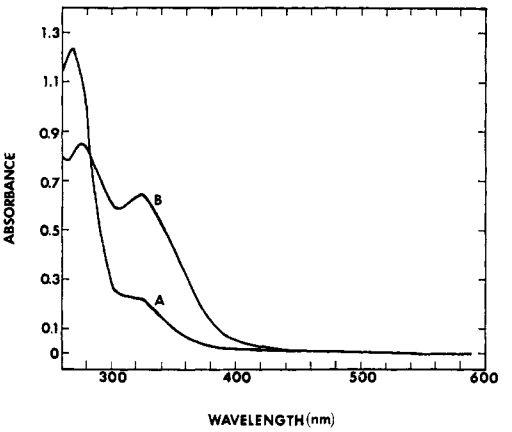

FIGURE 3: Solution spectra of the organic soluble *N*-retinyl compound isolated from heat-denatured, acid-buffered rod outer segments (A) and CTAB–rhodopsin (B).

No change in the λ_{max} of protonated *N*-retinylidenehexylamine (λ_{max} 445 nm) was observed as would be noticed if protonated forms of *N*-retinylideneaniline (λ_{max} 500 nm) or *N*-retinylideneoxime (λ_{max} 420 nm) were formed.

Thin-layer chromatography on all the methanol or acidified methanol extracts after reduction with $NaBH_4$ was carried out in four different solvent systems and examined for fluorescence under ultraviolet light (Table I). Both retinol and *N*-RH_2PE exhibited fluorescence. Extremely concentrated solutions were needed for thin-layer chromatography, especially in the case of rod outer segments, as even after delipidation by hexane there is 1 mole of the chromophore *N*-RPE to approximately 70 moles of lipid (Poincelot and Abrahamson, 1970a). However, very great sensitivity is possible under examination with ultraviolet light.

Plasmalogens, detected by the presence of a fatty aldehyde released by acid hydrolysis of the α,β-unsaturated ether linkage, were found to be associated with the phospholipid fraction but not with *N*-RH_2PE.

Upon alkaline hydrolysis and $CHCl_3$ extraction of the ($NaBH_4$) reduced methanol or acidified methanol extracts,

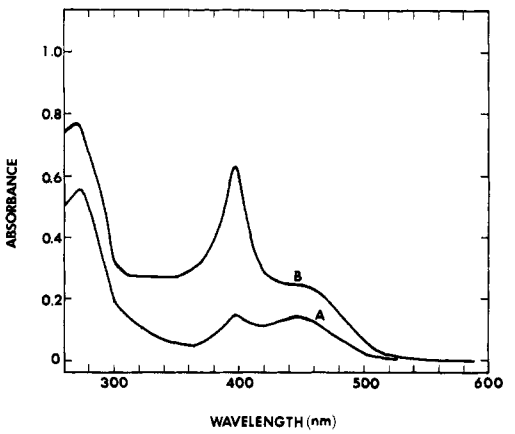

FIGURE 4: Solution spectrum of the *N*-retinylidene Schiff base extracted in methanol containing $10^{-3.5}$ M HCl gas (A) and 0.1 M HCl gas (B).

TABLE II: Thin-Layer Chromatography on Chloroform-Soluble Products in the Hydrolysate of Reduced Methanol or Acidified Methanol Extracts.

Sample	R_F Values (×100) for Fluorescent Retinyl Derivatives Solvent I[a]	II[b]	III[c]
Rhodopsin	27, 91	61, 100	58, 97
Rod outer segments	24, 90	62, 100	54, 96
N-Retinylethanolamine	27	63	57
N-Retinylserine	20	55	50
N-Retinyllysine	0	34	0
Retinol	95	100	96

[a] Methanol. [b] Methyl ethyl ketone–pyridine–H_2O–acetic acid (70:15:15:2, v/v). [c] $CHCl_3$–CH_3OH–acetic acid (70:27:3, v/v).

TABLE III: Gas Chromatographic Results of Perhydroretinyl Hydrolysates Derived from Reduced Acidified Methanol Extracts.

	Retention Time (min)		
Rod outer segments	2.6	2.9	13.8
Synthetic *N*-PRE	2.7	3.0	13.8

thin-layer chromatography was carried out using *N*-retinyllysine, *N*-retinylserine, *N*-retinylethanolamine, and retinol as standards. Plates were examined for fluorescence under ultraviolet light (Table II). Amino acid analyses of the hydrolysates before $CHCl_3$ extraction showed very slight traces of amino acids or none at all.

When the acidified methanol extracts ($10^{-3.5}$ M HCl gas) were treated to yield *N*-PRE and subjected to gas chromatography along with synthetic *N*-PRE, a similar gas chromatograph for each resulted (Table III).

Discussion

The absorption spectra of methanol extracts of wet or lyophilized rod outer segments and rhodopsin micelles showed them to contain a compound demonstrated to be a Schiff base of retinal by the characteristic change in λ_{max} from 365 to 445 nm upon protonation by acid (Figure 2). A further shift to 325 nm upon reduction by $NaBH_4$ was also observed, characteristic of reduced retinylidene Schiff bases, as observed in all our *N*-retinyl model Schiff bases. It is significant that greater than 90% of the chromophore was present in the methanol extract. These observations are inconsistent with the notion that in the native state retinal is bound to a lysyl unit (Bownds, 1967; Akhtar *et al.*, 1968) of a protein of approximate molecular weight 28,600 (Shields *et al.*, 1967; Heller, 1968; Shichi *et al.*, 1969). One would not expect a protein of this molecular weight to be soluble in methanol. A Schiff base complex of retinal with a phospholipid, however, would most likely be soluble in these solvents.

Our model studies with Schiff bases demonstrate that their formation and imine exchange can occur in methanol. In aqueous systems exchange is possible in mildly alkaline media, but not under acidic conditions (Morton and Pitt, 1955). From this we reasoned that no imine exchange should take place in acidified methanol. Using *N*-retinylidenehexylamine as a model at a concentration approximating that found for *N*-RPE in our acidified methanol extracts, we observed no imine exchange in acidic methanol ($10^{-3.5}$ M HCl) upon addition of 10 μmoles of aniline or hydroxylamine. This 20-fold excess is equivalent to the molar ratio of phosphatidylethanolamine to retinal in hexane delipidated rod outer segments (Poincelot and Zull, 1969; Poincelot and Abrahamson, 1970a). This lack of exchange seems, at first glance, hard to reconcile with the notion that a protonated Schiff base might be expected to be more labile to attack by nucleophilic reagents than the free base. The stability of the former in acid solutions arises from the fact that the excess hydrogen ions present effectively tie up the nucleophilic reagents, rendering them inactive. From these studies we concluded that a Schiff base isolated in acidified methanol, as with aqueous acid media (Morton and Pitt, 1955), had to be present as such originally.

Accordingly, when we extracted wet or lyophilized rod outer segments or rhodopsin with acidified methanol (0.1–$10^{-3.5}$ M in HCl gas) in the dark (Figure 4), we obtained a protonated Schiff base (445 nm). This was true for rod outer segments prior to and after hexane delipidation. An additional peak was observed at 397 nm which was blue-shifted to 365 nm upon alkalinization and to 325 nm upon $NaBH_4$ reduction as did the peak at 445 nm. This absorption maximum at 397 nm, favored by increasing acidity, may be attributed to an excimer of *N*-RPE. Extracts of partially delipidated rod outer segments with $10^{-3.5}$ M HCl gas in methanol yielded a spectrum having the most protonated Schiff base and the least "397" component, and accounted for better than 90% of the chromophore. Any delay in preparing and extracting rod outer segments or rhodopsin caused a decreased chromophore yield.

Methanol causes disruption of rod outer segments and rhodopsin micelles, destroying the spectral integrity at 500 nm. Presumably this occurs as a consequence of denaturation of the backbone protein. Had the chromophore been bound to the protein, one would have expected the acid conditions to have prevented the migration to phosphatidylethanolamine. A remote possibility exists that exchange did occur even under these conditions, possibly favored by the solubility of *N*-RPE in the acid methanol. To eliminate this possibility, rod outer segments or rhodopsin were thermally dark denatured and reduced in an acidic aqueous buffer and then extracted with methanol. The solution contained a reduced Schiff base (λ_{max} 325 nm) (Figure 3) which accounted for greater than 85% of the chromophore. Partition of this Schiff base between H_2O–$CHCl_3$–CH_3OH (1.0:2.0:1:0, v/v) showed it to migrate with the $CHCl_3$ organic phase. A Schiff base complex of the chromophore with lysine on the backbone protein would certainly not show such behavior, but the chromophore complexed to a phospholipid would.

Amino acid analyses on all the preceding nonaqueous extracts showed essentially no amino acids present and

particularly no lysine. Serine and ethanolamine, however, were found in amounts more than sufficient to accommodate the chromophore. One could conclude from this that the extracts contained a Schiff base of retinal with a phospholipid, either phosphatidylethanolamine or phosphatidylserine.

Thin-layer chromatography of the reduced ($NaBH_4$) Schiff base derived from methanolic or thermally denatured rod outer segments and rhodopsin (as CTAB micelles) identified it as *N*-RH_2PE (Table I). Hydrolysis of this compound followed by thin-layer chromatography showed the expected *N*-retinylethanolamine (Table II), which upon further reduction (H_2, Pd–charcoal) was identified by gas chromatography as *N*-PRE (Table III). *N*-Retinylphosphatidylserine and *N*-retinyllysine were definitely ruled out. Spots corresponding to *N*-retinylphosphatidylethanolamine were all fluorescent, phosphorus positive, and free amino group negative.

Since ethanolamine plasmalogen has been found to constitute 10% of the phosphatidylethanolamine fraction (Borggreven *et al.*, 1970), one has to consider the possibility of *N*-RH_2PE being in the plasmalogen form. The vinyl ether (α,β-unsaturated ether) groups of plasmalogens are very labile to dilute acids, yielding fatty aldehydes. Therefore, the lyso form of *N*-RH_2PE would be found in acid methanol and the intact form in methanol. Upon thin-layer chromatography these two would not yield the same R_F values. The fact that they do argues against any *N*-RH_2PE being in the plasmalogen form. As further proof, we examined methanol extracts containing *N*-RH_2PE and phospholipids for free aldehydes. Indeed, the phospholipid portion yielded free aldehyde, but none was observed from *N*-RH_2PE.

In an accompanying paper (Kimbel *et al.*, 1970) we have shown, upon reduction ($NaBH_4$) of illuminated rod outer segments or rhodopsin micelles in acidic aqueous buffer (pH 4.5), as opposed to reduction after heat denaturation, that the bulk of the chromophore was not extractable in organic solvents. Instead the chromophore was found after alkaline hydrolysis as *N*-retinyllysine. Similar observations were made on acid–methanol extractions of dark-adapted and illuminated rod outer segments or rhodopsin, for in the latter the chromophore was not removed in the organic solvent, but was subsequently recovered as *N*-retinyllysine after alkaline hydrolysis. Thus under identical solution conditions, rod outer segments and rhodopsin in the native state yield *N*-RH_2PE upon heat or methanolic denaturation accompanied by reduction ($NaBH_4$), yet upon illumination and reduction or illumination, acidic methanol denaturation, and reduction yield *N*-retinyllysine after alkaline hydrolysis. It is indeed difficult to reconcile this behavior with a binding site in native rhodopsin other than phosphatidylethanolamine.

The zwitterionic character strongly suggests that *N*-RPE exists in native rhodopsin as the protonated form (Figure 5), a point confirmed by recent work (Hubbard, 1969; Daemen and Bonting, 1969). That this protonated *N*-RPE chromophore complex is essential to the spectral integrity of rhodopsin is supported by our inability to prepare a completely lipid free preparation with a λ_{max} at 500 nm (Poincelot and Abrahamson, 1970a). Although rhodopsin preparations retaining their spectral integrity with as few as 3 moles of lipid (one phosphatidylethanolamine and two phosphatidylserine) have been made in our laboratory (F. J. M. Daemen, unpublished results, 1969), we have been unable to confirm

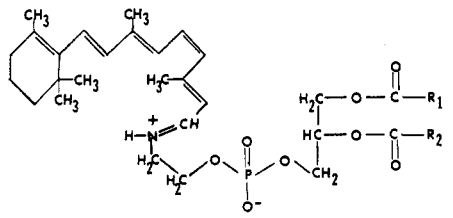

FIGURE 5: Structure of protonated *N*-retinylidenephosphatidylethanolamine. R_1 and R_2 represent C_{15} to C_{21} chains containing zero to six double bonds (Poincelot and Abrahamson, 1970b).

the report of Heller (1968) of a spectrally intact, lipid-free rhodopsin. We must stress, however, that although the chromophore is covalently bound to phosphatidylethanolamine, the evidence to date indicates the presence of the backbone protein to be essential to the maintenance of the spectral integrity of rhodopsin. Evidently, there is some intricate, biochemical relationship between the chromophore and the backbone protein which fits the spectrum to its physiological function (Wiesenfeld and Abrahamson, 1968).

In summary, we can remove the chromophore as *N*-retinylidenephosphatidylethanolamine from rod outer segments and rhodopsin micelles in almost quantitative amounts under neutral and acidic conditions in methanol and as *N*-retinylphosphatidylethanolamine from ($NaBH_4$) reduced, thermally dark denatured rod outer segments and rhodopsin micelles under aqueous acidic conditions. A CTAB extraction of the rod outer segments or rhodopsin residue left after a methanol or acidic methanol treatment showed essentially no chromophore remaining in the residue. It follows from its quantitative presence in the methanol or acidic methanol extracts that the chromophore in native rhodopsin and rod outer segments must exist as *N*-RPE, barring some unlikely condition in the rod outer segments or rhodopsin micelle that would effect imine exchange of the retinylidene moiety to phosphatidylethanolamine under acidic conditions from some other initial binding site. We conclude, therefore, that the retinylidene chromophore in native rhodopsin is present as *N*-retinylidenephosphatidylethanolamine.

Added in Proof

Recently in a short communication, Akhtar and Hirtenstein (1969) presented results in agreement with those reported in our earlier note (Poincelot *et al.*, 1969), which have been elaborated in this paper and the following one (Kimbel *et al.*, 1970). The supporting evidence, which these authors present, verifies the role of phosphatidylethanolamine in the native chromophoric binding site as *N*-retinylidenephosphatidylethanolamine, as well as the subsequent transfer of the chromophore to the ϵ-amino group of a lysyl unit in the backbone protein.

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