Resonance Raman Spectroscopy of Rhodopsin in Retinal Disk Membranes†

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ABSTRACT: Low-temperature resonance Raman spectroscopy has been used to study the conformation and interactions of retinal within its opsin binding site in disk membrane vesicles formed from bovine retinal rod outer segments. At 80°K, laser irradiation within the visible absorption band produces well-defined photostationary states containing only rhodopsin, isorhodopsin, and bathorhodopsin. A double wavelength, pump-probe technique has been devised to distinguish scattering from these three components. In addition to the conventional Raman "probe" laser beam, a "pump" beam at a different wavelength is used to modify the composition of the photostationary state. The fixed wavelength probe holds the resonant enhancement factors constant so that changes in the spectra that are induced by the pump beam are directly related to the composition of the photostationary state. The Raman data demonstrate that, *in situ,* retinal and opsin are joined by a protonated Schiff base. Deuteration of the Schiff base causes a predictable reduction in the stretching frequency from 1655 to 1630 cm^{-1}. The C=C stretching frequency of the retinal chain decreases from about 1563 in *trans*-retinylideneethanolamine to 1551 in isorhodopsin to 1539 cm^{-1} in bathorhodopsin, in direct proportion to the magnitude of the bathochromic shift. The spectrum of bathorhodopsin, which contains relatively intense bands at 856, 877, and 920 cm^{-1}, and weak scattering near 1160 cm^{-1}, is markedly different from that of *all-trans* retinylidene solutions. The data suggest that the formation of bathorhodopsin from rhodopsin or isorhodopsin may not involve a simple cis-trans isomerization about the 11-cis or 9-cis double bond.

Rhodopsin, the visual pigment in the disk membranes of vertebrate retinal rod cells, is composed of the chromophore 11-*cis*-retinal linked by a Schiff base to the membrane glycoprotein opsin (Wald, 1967; Hubbard and Kropf, 1958; Kropf and Hubbard, 1958; Bownds, 1967). The binding site within the opsin molecule accommodates both 11-*cis*- and 9-*cis*-retinal (Wald, 1967; Hubbard and Kropf, 1958; Kropf and Hubbard, 1958), the latter forming isorhodopsin. The absorption bands of the resulting pigments are considerably red-shifted from those of the free chromophores, and from protonated or unprotonated Schiff bases formed with retinal in organic solvents (Abrahamson and Wiesenfeld, 1972). An understanding of the retinal–opsin interactions responsible for the color of the visual pigments, and for the coupling between absorption of light and the hyperpolarization of the rod cell plasma membrane, requires information about the structure of the retinal–opsin complex, and about the conformational changes in the chromophore which accompany visual excitation.

Resonance Raman spectroscopy (Albrecht, 1969; Behringer, 1967) is uniquely suited to probe the *in situ* conformation of retinal. The resonance enhanced spectra are due only to vibrational modes of the chromophore, and of adjacent opsin residues which couple to the visual absorption band, while other membrane components do not significantly contribute to the observed scattering. The technique has potentially wide application to colored, macromolecular systems, and resonance enhanced Raman spectra have recently been obtained from several biologically interesting molecules including free retinal (Rimai *et al.,* 1971; Gill *et al.,* 1971; Heyde *et al.,* 1971), hemoglobin (Strekas and Spiro, 1972b; Spiro and Strekas, 1972, 1974; Brunner *et al.,* 1972), cytochrome *c* (Yamamoto *et al.,* 1973; Strekas and Spiro, 1972a; Brunner, 1973; Collins *et al.,* 1973), and digitonin extracts of bovine rhodopsin at 4° (Lewis *et al.,* 1973).

We have used low-temperature (80°K) resonance Raman spectroscopy to study the interactions between retinal and opsin in disk membrane vesicles from bovine rod outer segments during the initial stage of visual excitation. Except at low temperatures, laser irradiation of rhodopsin rapidly produces a quasi-photostationary state mixture which can include rhodopsin, isorhodopsin, and all the thermally allowed metastable intermediates in the bleaching sequence. The composition of the sample will depend in detail on the absorption cross section, quantum yield, and kinetic lifetime of each component relative to the photon flux of the incident beam. As a consequence, the observed Raman spectra will be a sensitive function of the exact experimental configuration, particularly at temperatures near 0° (A. Doukas, R. H. Callender, and A. R. Oseroff, manuscript in preparation). At 80°K, however, laser irradiation produces a photostationary state containing only rhodopsin, isorhodopsin, and bathorhodopsin[1] (Yoshizawa and Wald, 1963; Yoshizawa, 1972). Bathorhodopsin (λ_{max} 543 nm) is the earliest bleaching intermediate observed at room temperature (Busch *et al.,* 1972). It is a common photoproduct of both rhodopsin and isorhodopsin, and upon absorbing a photon can revert to either of the parent species (Yoshizawa and Wald, 1963; Yoshizawa, 1972). The relative concentrations of the three components in the photostationary state depend only on the wavelength(s) of the incident illumina-

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[1] Also called prelumirhodopsin.

tion (Yoshizawa and Wald, 1963) and are independent of other experimental factors including photon flux.

Interpretation of resonance Raman scattering from a multiple component, photostationary system can be complex. The wavelength dependence of the resonance-enhanced Raman cross section of a molecule is not a simple function of its adsorption profile (Fenstermacher and Callender, 1974; Tasumi *et al.*, 1973). It may vary for different vibrational modes (Spiro and Strekas, 1974) and may scale differently for each of the components of the photostationary state. We have therefore devised a double-beam technique, in which a relatively weak "probe" beam is used to excite the Raman scattering while an additional "pump" beam at a different wavelength is used to modify the photostationary state. This procedure has the advantage that the fixed probe wavelength holds the resonance enhancement factors constant, so that pump beam induced changes in the spectrum are a direct representation of changes in the composition of the photostationary state.

Materials and Methods

Bovine retinas were purchased from Hormel and stored at −20° until ready for use. Pellets of rod outer segment (ROS) vesicles were isolated as described previously (Waggoner and Stryer, 1971) and stored at −20°. The ROS pellets were not sonicated. Deuterated ROS samples were prepared by washing the ROS pellets twice with D_2O, resuspending the pellet in 20 ml of 0.1 M D_2O–phosphate buffer (pD ~6.5), storing overnight at 4°, and repelleting. Lipid-free rhodopsin was prepared by chromatography on hydroxylapatite (Bio-Rad) (Hong and Hubbell, 1972) and precipitated by dialysis overnight against cold 0.067 M phosphate buffer (pH 6.5). The rhodopsin precipitate was recovered by centrifugation at 30,000*g* for 30 min. All operations were carried out under dim red light.

all-trans-Retinal was purchased from Eastman Chemical Co. Protonated retinylideneethanolamine was prepared by treating a 25-fold molar excess of ethanolamine in the dark for 2 hr with a 25 mM solution of the appropriate retinal isomer in absolute ethanol. The Schiff base solution (0.2 ml) was acidified with one drop of concentrated aqueous HCl. Absorption measurements showed λ_{max} 443 nm. Deuterated retinylideneethanolamine was prepared as above except C_2H_5OD (Thompson Packard) and concentrated DCl in D_2O (Thompson Packard) were used.

The pelleted rhodopsin was deposited in a concave depression in a temperature-controlled copper sample holder arranged for 90° scattering from the face of the sample. The specimen was mounted in a Janis Super Vari-Temp liquid helium dewar and cooled from the front surface by a stream of cold helium gas. We found no change in the spectra at temperatures between 30 and 120°K, and the low-temperature spectra reported in this paper were taken at about 80°K.

Laser Raman spectra were obtained with a Spex 1401 double monochrometer, a cooled ITT FW130 phototube, and photon counting electronics interfaced with a PDP-8 computer. A Coherent Radiation Model 52 krypton ion laser, a Spectra Physics Model 165 argon ion laser, and a Spectra Physics Model 370 tunable dye laser were used to produce monochromatic radiation between 458 and 650 nm. The laser beams were moderately focused on the sample at power levels between 5 and 50 mW; no dependence on laser power was observed. The spectrometer was calibrated from known frequencies of the Ar^+ and Kr^+ discharge lines; line assignments are accurate to ±3 cm^{-1}. The frozen samples produced a broad fluorescence background (typical half-width greater than 1000 cm^{-1}) whose average level was about four times greater than the Raman signal. The fluorescence from the lipid-free rhodopsin preparations was significantly lower than that of the ROS preparations. Except where indicated, the background was removed from the data by computing the Fourier transform of the raw data and the background points, and taking an inverse transform of the subtracted Fourier coefficients. The spectra presented in the paper consist of digitally pooled data from multiple runs, and all identified lines have been observed in at least four measurements.

Two laser beams were used simultaneously in some of the experiments, one beam acting as a "probe" to excite the Raman scattering and the second at a different wavelength acting as a "pump" to modify the photostationary state of the sample. The beams, produced by separate lasers at right angles to each other, were made coaxial by a beam splitter at 45° to both beams and passed through aperatures. The aperatures ensured that the sample areas illuminated by each of the two beams were identical, by constraining the beams to be coaxial and of the same diameter. Both parameters were tested by examining the beam profiles after several meters path length. Relative and absolute beam powers were measured just before the sample using a calibrated Eppley thermopile.

Separate absorption measurements were carried out to determine the photostationary steady-state equilibria established by single and double beam irradiation. Thin pellets containing about 6 mg of ROS vesicles in 30-ml Corex test tubes were completely immersed in liquid nitrogen. Using expanded beams about 1 cm in diameter, both sides of each of the pellets were uniformly irradiated with about 200 mW of laser power, and were assayed as a function of time, as described below. For laser wavelengths below 370 nm, equilibrium was reached in less than 15 min. At 604.9 nm both rhodopsin and isorhodopsin have very small absorbances, so the time to reach equilibrium was much longer, on the order of 90 min. Because the beam was focused in the Raman experiments, the photon flux was 10^3 greater than in these absorption measurements, so that the corresponding time to equilibrium was much less than 1 min. All Raman samples were irradiated for 10 min prior to collecting spectral data.

To determine the relative amounts of rhodopsin, isorhodopsin, and bathorhodopsin in the irradiated samples, the rhodopsin pellets were warmed to room temperature in the dark, and dissolved in a solution of 1% cetyltrimethylammonium bromide in 0.067 M phosphate buffer (pH 6.5) containing 0.2 M hydroxylamine. A Cary 15 recording spectrophotometer was used to measure absorption spectra before and after the sample was bleached by exposure to room light. The difference spectra were resolved into contributions from rhodopsin and isorhodopsin (Hubbard and Kropf, 1958; Kropf and Hubbard, 1958) and compared with control preparations which had been immersed in liquid nitrogen in the dark. The decrease in absorbance in the 485–500-nm band relative to the controls was taken to correspond to the amount of sample converted to bathorhodopsin during irradiation.

Results

An Overview of the Raman Spectra. Figure 1 shows Raman spectra excited at 514.5, 604.9, and 568.2 nm; the photostationary state compositions at these wavelengths are

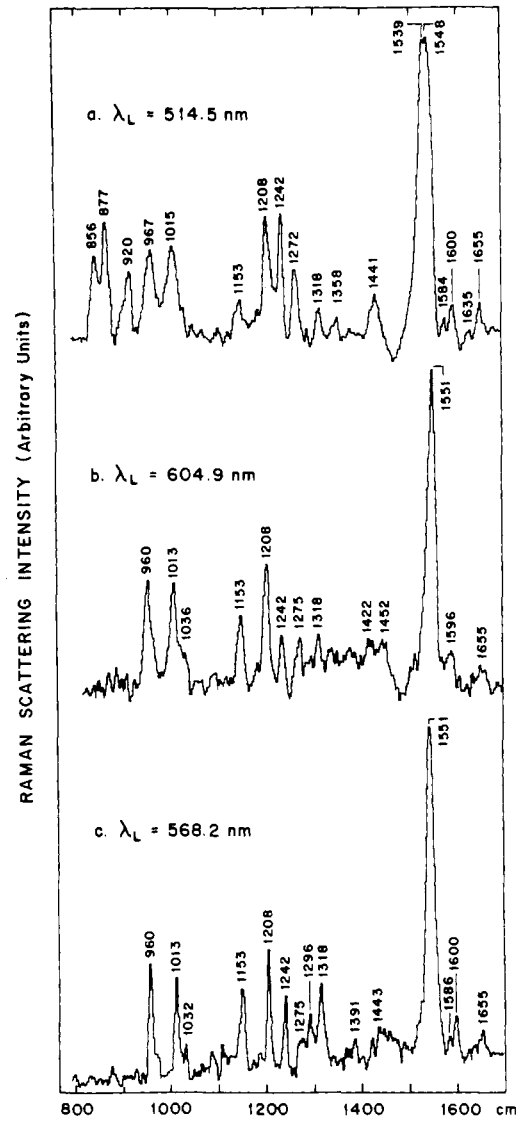

FIGURE 1: Raman spectra taken at 80°K of (a) ROS vesicles using 514.5-nm irradiation, (b) purified rhodopsin at 604.9 nm, and (c) ROS vesicles at 568.2 nm. The spectral resolution in all cases is 8 cm^{-1}. The data have been approximately normalized to the prominent peak near 1550 cm^{-1} and the fluorescence background scattering removed (see text). The relative concentrations of rhodopsin, isorhodopsin, and bathorhodopsin are given in Table I.

given in Table I. We found that the spectrum produced at a given wavelength was independent of prior irradiation at other wavelengths, as predicted by the interconvertibility of the absorption spectra (Yoshizawa and Wald, 1963; Yoshizawa, 1972). In general, the photostationary state contains contributions from at least two of the three possible components. Overlapping absorption bands (Yoshizawa, 1972) preclude photoproduction of either pure rhodopsin or bathorhodopsin. It is only near 570 nm where the absorption of isorhodopsin is significantly smaller than either rhodopsin or bathorhodopsin (Yoshizawa, 1972) that it is possible to prepare almost pure isorhodopsin. As a consequence, the 568.2-nm spectrum is the least complex of the three presented in Figure 1.

From measurements of retinal isomers (Rimai *et al.*, 1971; Gill *et al.*, 1971) and their Schiff bases (Heyde *et al.*, 1971) general assignments of three regions of the spectrum have been made. (1) The Schiff base stretching mode occurs near 1625 or 1650 cm^{-1}, depending on whether the

TABLE I: Per Cent Composition of Rhodopsin, Isorhodopsin, and Bathorhodopsin in the Photostationary States Prepared by Irradiation of ROS Vesicles at 80°K.

Laser (nm)		Rho-	Iso-	Batho-
Probe	Pump	dopsin[a]	rhodopsin[a]	rhodopsin[a]
514.5		23	24	53
604.9		30	70	0
568.2		<2	>98	0
568.2	488[b]	33	45	22
476.2		21	16	63
476.2	580[c]	25	49	26

[a] Compositions are averages of three or more measurements and are accurate to ±5%. [b] The pump/probe power ratio is 2.6/1. [c] The pump/probe power ratio is 7/1.

linkage is unprotonated or protonated, respectively (Heyde *et al.*, 1971). All three spectra in Figure 1 show a distinct line at 1655 cm^{-1}. (2) The ethylenic band, the most intense line in the spectrum, is primarily due to a C=C stretching mode of the polyene chain (Rimai *et al.*, 1971; Gill *et al.*, 1971; Heyde *et al.*, 1971). It lies at 1551 cm^{-1} for isorhodopsin, and is not appreciably shifted by the additional scattering from rhodopsin, Figure 1b. The inclusion of bathorhodpsin at 514.5 nm, however, generates the additional line near 1539 cm^{-1} shown in Figure 1a. (3) The 800-1500-cm^{-1} region, containing the so-called "fingerprint" bands (Rimai *et al.*, 1971), consists chiefly of admixtures of C—C stretching and C—H bending modes, which have been found to depend strongly on the isomeric form of the retinal (Rimai *et al.*, 1971; Gill *et al.*, 1971). A line near 1010 cm^{-1} has been assigned to a stretching mode between the methyl groups and the polyene chain, and that near 1440 cm^{-1} to C-H bending within the methyl groups (Rimai *et al.*, 1971; Gill *et al.*, 1971), but other normal mode identifications have not yet been made. Note that most of the lines in the isorhodopsin spectrum have a sharpness characteristic of a single-species spectrum, while the two composite spectra are distinctly broadened.[2]

Schiff Base Region. The close correspondence between the positiun of the C=N stretching frequency of both protonated retinylideneethanolamine (Figure 2c) and protonated retinylidenehexylamine (Heyde *et al.*, 1971) and the 1655 cm^{-1} in the ROS spectra in Figures 1 and 2a suggest, but do not prove, that retinal is joined to opsin by a protonated Schiff base. If the Schiff base is protonated, however, the frequency of the stretching mode will decrease if the proton is exchanged for a deuteron. The deuterated stretching frequency, ν_D, can be easily estimated. Assuming that the stretching mode is decoupled from the rest of the molecule, and that the force constant (*i.e.*, the C=N bond strength) is not affected by the substitution, then $\nu_D = \nu_H(\mu_H/\mu_D)^{1/2}$, where ν_H is the protonated stretching frequency and μ_H and μ_D are, respectively, the reduced mass of the HC=NH$^+$ and HC=ND$^+$ systems (Herzberg,

[2] To reduce background fluorescence the data at 604.9 nm were taken from a lipid-free rhodopsin preparation (Hong and Hubbell, 1972). The effect of delipidation on the low-temperature Raman spectra does not appear to be large, since the spectrum of this sample at 514.5 nm was virtually identical with that of an ROS sample.

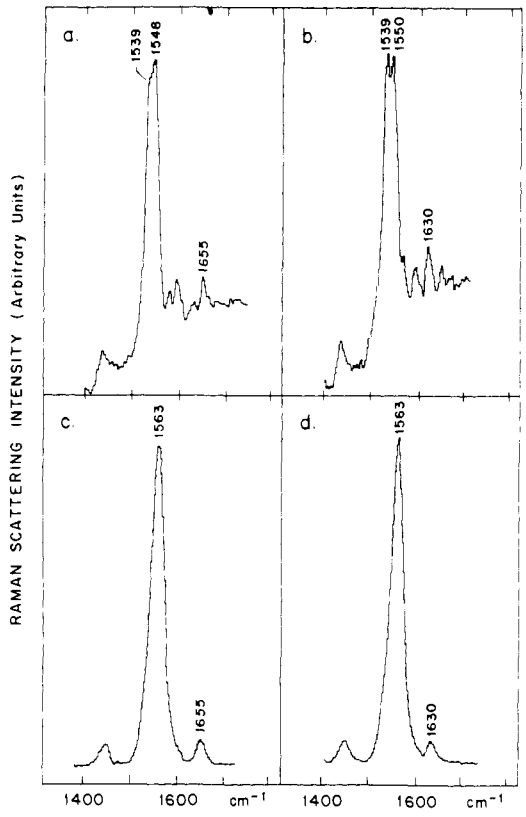

FIGURE 2: Raman spectra of (a) ROS vesicles at 80°K using 514.5-nm irradiation. Resolution is 8 cm^{-1} and the broad fluorescence background structure has *not* been removed; (b) deuterated ROS vesicles under the same conditions as in a; (c) protonated *all-trans*-retinylidenethanolamine in ethanol at room temperature using 647.1-nm irradiation; resolution is 8 cm^{-1}; (d) deuterated *all-trans*-retinylidenethanolamine in deuterated ethanol under the same conditions as in c.

1950). For ν_H = 1655 cm^{-1}, we calculate that ν_D will decrease to about 1633 cm^{-1}.

We measured the Raman spectra from deuterated ROS vesicles and deuterated *trans*-retinylideneethanolamine in CH_3CH_2OD and compared them to the corresponding protonated preparations. Figure 2a shows the 514.5-nm spectrum of the Schiff base region of ROS vesicles; the deuterated ROS spectrum is given in Figure 2b. The corresponding spectra for the protonated and deuterated model compounds taken with 647.1-nm irradiation appear in Figures 2c and 2d. In both the deuterated ROS vesicles and the model compounds the lines previously at 1655 cm^{-1} have shifted to about 1630 cm^{-1}, in good agreement with the calculations.[3] To give some indication of the contribution from fluorescence, the broad background structure has not been removed from the ROS data (compare Figures 1a and 2a).

Ethylenic Lines. The double beam experiment shown in Figure 3 demonstrates that the 1539-cm^{-1} line is indeed due to bathorhodopsin, and it also provides a striking example of resonance effects on the Raman scattering cross section. Figure 3 shows the scattering from a 568.2-nm probe beam in the absence (Figure 3a) and the presence (Figure 3b) of a 488-nm pump. Without the pump, the scattering is due only to isorhodopsin. The pump beam shifts the equilibrium away from isorhodopsin toward the formation of bathorhodopsin and rhodopsin (*cf.* Table I). Because the 568.2-nm probe is close to the absorption maximum of bathorhodopsin (and far from that of rhodopsin and isorhodopsin), bathorhodopsin scattering is preferentially enhanced. Despite the fact that the pump produces only about 22% bathorhodopsin, the 1539-cm^{-1} line becomes the dominant feature in the spectrum, and the integrated Raman intensity increases by more than a factor of two.

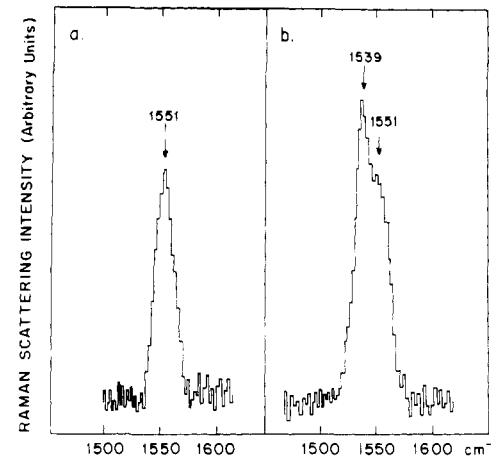

FIGURE 3: Raman spectra at 80°K of ROS vesicles taken with 568.2-nm irradiation in (a) the absence and (b) the presence of a coaxial 488-nm pump beam. Pump/probe power ratio is 2.6/1. The two spectra have been scaled identically. Resolution is 5 cm^{-1}. See Table I for sample compositions.

The 800–1500-cm^{-1} Region. A comparison of the intermediate frequency region of isorhodopsin with the other spectra in Figure 1 suggests that the three species may share some spectral features such as the scattering near 960, 1013, 1600, and possibly 1208 cm^{-1}, since changes in the photostationary state lead only to broadening and sometimes small centroid shifts of these lines. It is also apparent, however, that there are significant differences between Figures 1a and 1c.

We used double beam experiments to explore some of the differences between bathorhodopsin, rhodopsin, and isorhodopsin. Figures 4a and 4b show Raman scattering at 476.2 nm in the absence and presence of á 580-nm pump. As specified in Table I, the pump beam decreases the bathorhodopsin concentration by a factor of 2.4 ± 0.2, while rhodopsin and especially isorhodopsin increased their relative concentrations. The major spectral change in Figure 4b, as compared to Figure 4a, is a *decrease* in the intensities of the lines at 856, 877, and 920 cm^{-1} by a factor of 2.5 ± 0.1 which *quantitatively* matches the decrease in bathorhodopsin. Note that the intensities of most of the other lines in Figure 4b have increased, consistent with the greater absorption and enhancement of rhodopsin and isorhodopsin, as compared to bathorhodopsin, at 476.2 nm.

The converse experiment using 488-nm pump and 568.2-nm probe excitation is shown in Figure 5. The 488-nm pump beam causes an increase in both bathorhodopsin and rhodopsin. For the 568.2-nm probe, the bathorhodopsin scattering is again preferentially enhanced, and dominates the spectrum. In this case (Figure 5a), the intense 856-, 877-, and 920-cm^{-1} lines appear, while the relative scattering at 1153 cm^{-1} is somewhat reduced.

The two double-beam experiments clearly demonstrate that the three lines between 850 and 920 cm^{-1} are due only

[3] The close agreement between the Schiff base stretching frequency of the ROS samples and the retinylidene solutions is, in part, fortuitous. The Schiff base frequency in retinylidene is solvent dependent, ranging from 1646 to 1654 cm^{-1} in previous work (Heyde *et al.*, 1971).

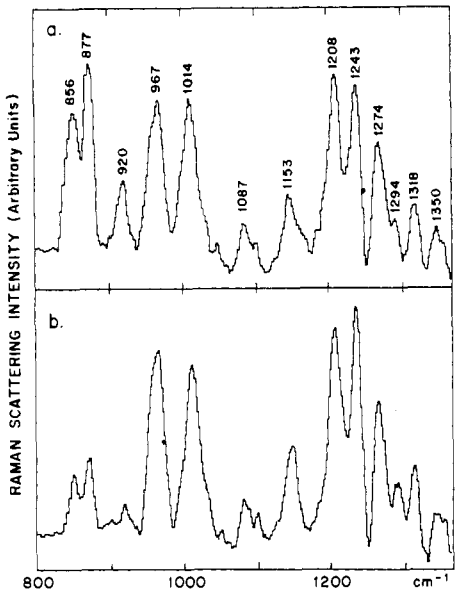

FIGURE 4: Raman spectra at 80°K of ROS vesicles taken with 476.2-nm irradiation in (a) the absence and (b) the presence of a 580-nm coaxial pump beam. The pump/probe power ratio is 7/1. The two spectra have been scaled identically. Resolution is 12 cm^{-1}. See Table I for sample composition.

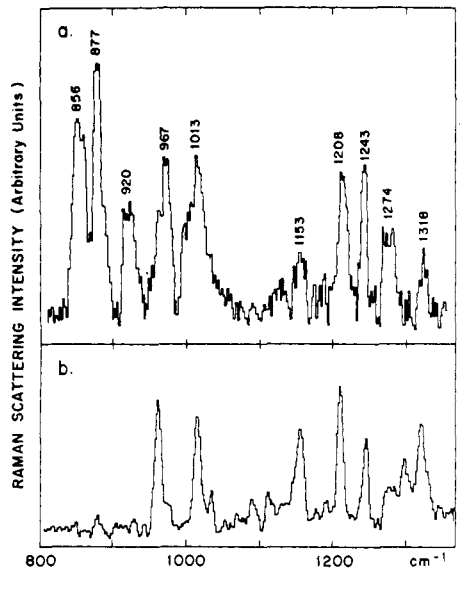

FIGURE 5: Raman spectra at 80°K of ROS vesicles with 568.2-nm irradiation in (a) the presence and (b) the absence of a coaxial 488-nm pump. The pump/probe power ratio is 2.6/1. Resolution is 8 cm^{-1}. The two spectra are normalized to equivalent scattering amplitude at 1208 cm^{-1}. See Table I for sample composition.

to bathorhodopsin. Taken together with the spectra in Figure 1, the data also suggest that the 1153-cm^{-1} line is not present in the bathorhodopsin spectrum but can be assigned to isorhodopsin and probably rhodopsin.

Discussion

The Raman scattering data presented here show that, *in situ*, the linkage between retinal and opsin is a protonated Schiff base,[4] which can therefore account for part of the red-shifted absorption of the visual pigments (Wald, 1967; Hubbard and Kropf, 1958; Kropf and Hubbard, 1958; Abrahamson and Wiesenfeld, 1972). The C=C stretching frequency of the ethylenic mode is well correlated with the position of the absorption maximum of a particular species, decreasing almost linearly from 1563 cm^{-1} in protonated retinylidenethanolamine (λ_{max} 443 nm) to 1551 cm^{-1} in isorhodopsin (λ_{max} 494 nm), to 1539 cm^{-1} in bathorhodopsin (λ_{max} 543 nm). This correlation is consistent with the suggestion that the bathochromic shift is due to increased delocalization of the π electrons of the polyene chain (Abrahamson and Wiesenfeld, 1972; Heyde *et al.*, 1971). The mechanism for this delocalization, which almost certainly involves interactions between retinal and opsin residues at the binding site (Wald, 1967; Hubbard and Kropf, 1958; Kropf and Hubbard, 1958; Abrahamson and Wiesenfeld, 1972), is not yet known. A detailed interpretation of the spectra may provide information concerning the nature of these interactions.

The Raman spectrum of bathorhodopsin is markedly different from that of rhodopsin and isorhodopsin. It is also very different from the spectra of all-trans model compounds. Thus, while bathorhodopsin has little scattering between 1100 and 1200 cm^{-1}, *all-trans*-retinal (Rimai *et al.*, 1971), protonated *all-trans*-retinylidenehexylamine (Heyde *et al.*, 1971), and protonated *all-trans*-retinylideneethanolamine (unpublished data) all contain an intense band near 1160 cm^{-1}. The bathorhodopsin spectrum also shows major bands at 856, 877, and 920 cm^{-1}, while the all-trans model compounds have only very weak scattering near 856 and 877 cm^{-1}, and evidently none near 920 cm^{-1} (Rimai *et al.*, 1971; Gill *et al.*, 1971; Heyde *et al.*, 1971; Oseroff and Callender, unpublished data).

These data suggest that the formation of bathorhodopsin is not a simple cis–trans isomerization of the chromophore. It is likely that the retinal assumes some intermediate conformation which is dependent on the presence of opsin and which may not be equivalent to the conformations which exist in solutions. This conclusion is supported by our recent measurements of quasi-photostationary state rhodopsin preparations near 0° (A. Doukas, R. H. Callender, and A. R. Oseroff, manuscript in preparation). The three lines between 855 and 920 cm^{-1} are not observed, even though the scattering contains contributions from bleaching intermediates formed subsequent to bathorhodopsin, which are presumably closer to a trans conformation (Wald, 1967; Hubbard and Kropf, 1958; Kropf and Hubbard, 1958; Abrahamson and Wiesenfeld, 1972). The kinetic measurements of Busch *et al.* (1972) are also consistent with an intermediate conformation since the less than 6-psec formation of bathorhodopsin seems to preclude the extensive rotation necessary for a complete cis–trans isomerization of a double bond. The formation of a common batho intermediate from 9-*cis*- and 11-*cis*-retinal (both chromophores may well be 12-s-cis) (Honig and Karplus, 1971) without complete isomerization about the unequivalent double bonds presents an intriguing structural problem which cannot be resolved at the present time.

Acknowledgments

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[4] Lewis *et al.* (1973) reached a similar conclusion from Raman measurements of digitonin extracts of bovine rhodopsin at 4°.

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CORRECTION

"Troponin, Tropomyosin, and Actin Interactions in the Ca^{2+} Regulation of Muscle Contraction," by James D. Potter* and John Gergely, Volume 13, Number 13, June 18, 1974, page 2697.

Figure 3 was reproduced poorly, and detail was lost. The figure is reproduced below.

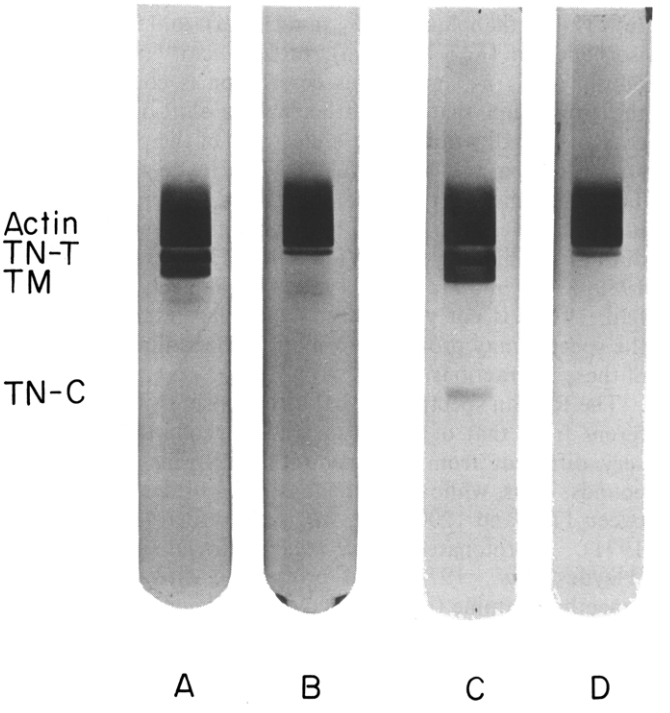

FIGURE 3: Interaction of TN-T and CT with F-actin and F-actin–TM. Proteins were mixed in a solution containing 10 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 0.01 mM $CaCl_2$, and either 0.4 M KCl (A,B) or 0.1 M KCl (C,D). (A) F-actin, TM, and TN-T; (B) F-actin and TN-T; (C) F-actin, TM, and CT; (D) F-actin and CT. Protein concentrations: F-actin, 0.66 mg/ml; TM, 0.16 mg/ml; TN-T, 0.09 mg/ml; and CT 0.13 mg/ml. The CT complex was prepared as described in the legend to Table I. The samples were centrifuged and the pellets were subjected to gel electrophoresis as described in the legend to Figure 1.