RESONANCE RAMAN STUDIES OF THE PRIMARY PHOTOCHEMICAL EVENT IN VISUAL PIGMENTS

B. ATON, A. G. DOUKAS, D. NARVA, R. H. CALLENDER, *Physics Department, City College of the City University of New York, New York 10031*

U. DINUR, *Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel*

B. HONIG, *Department of Physiology and Biophysics and Department of Physics, University of Illinois, Urbana, Illinois 61801 U.S.A.*

ABSTRACT Resonance Raman multicomponent spectra of bovine rhodopsin, isorhodopsin, and bathorhodopsin have been obtained at low temperature. Application of the double beam "pump-probe" technique allows us to extract a complete bathorhodopsin spectrum from the mixture in both protonated and deuterated media. Our results show that the Schiff base of bathorhodopsin is fully protonated and that the extent of protonation is unaffected by its photochemical formation from either rhodopsin or isorhodopsin. The Raman spectrum of bathorhodopsin is significantly different than that of either parent pigment, thus supporting the notion that a geometric change in the chromophore is an important component of the primary photochemical event in vision. A normal mode analysis is carried out with particular attention devoted to the factors that determine the frequency of the C═N stretching vibration. We find that the increased frequency of this mode in protonated relative to unprotonated Schiff bases is due to coupling between C═N stretching and C═N—H bending motions, and the shift observed upon deuteration of the Schiff base can also be understood in these terms. Various models for the primary event are discussed in light of our experimental and theoretical results.

The initial event in visual excitation involves the photochemical conversion of the pigment rhodopsin into its primary photoproduct. This is generally associated with a species known as bathorhodopsin, which is the first stable photoproduct at 77°K (Yoshizawa and Wald, 1963). The thermal decay of bathorhodopsin at higher temperatures initiates a sequence of chemical reactions that lead ultimately to the permeability changes in the photoreceptor membrane that constitute visual excitation. The identity of bathorhodopsin is thus of extreme interest since it provides the driving force for all of the subsequent events involved in this process.

Bathorhodopsin is a common intermediate between rhodopsin, whose chromophore is an 11-*cis* protonated Schiff base of retinal, and isorhodopsin, which is an artificial pigment based on the corresponding 9-*cis* isomer. For this and other reasons, it has been argued that the chromophore of bathorhodopsin is in a transoid conformation and that the primary event involves a *cis-trans* isomerization about the 11-12 double bond (Hubbard and Kropf, 1958; Yoshizawa and Wald, 1963; Rosenfeld et al., 1977; Honig et al., 1979*a*). The actual structure of the chromophore of bathorhodopsin is not known, nor is there any detailed information as to

---

Dr. Narva is a National Institutes of Health postdoctoral Fellow (1976–1979).

Dr. Aton's present address is Department of Biochemistry, University of Virginia, Charlottesville, Va. 22901.

Dr. Dinur's present address is Department of Chemistry, Harvard University, Cambridge, Mass. 02138.

additional changes in the chromophore-protein complex that might accompany isomerization. However, there is good evidence based on picosecond measurements at low temperatures that a proton is translocated during the formation of bathorhodopsin (Peters et al., 1977; Applebury et al., 1978). As a result, a number of models for the primary event have been proposed in which a change in position of the Schiff base proton is postulated to be the major difference between rhodopsin and bathorhodopsin (van der Meer et al., 1976; Peters et al., 1977; Favrot et al., 1979; Harosi et al., 1978).

In this paper we use resonance Raman spectroscopy to characterize the chromophore of bathorhodopsin. Of particular importance is to determine whether the state of protonation of the Schiff base nitrogen is affected by the rhodopsin-bathorhodopsin transformation. In addition, since Raman profiles tend to be rather sensitive to chromophore conformation, a complete Raman spectrum should in principle provide useful information as to the geometric changes accompanying the primary photochemical event. To obtain the Raman spectrum of bathorhodopsin, we extend the measurements of Oseroff and Callender (1974) on photostationary state mixtures of rhodopsin, bathorhodopsin, and isorhodopsin. These photostationary states may be obtained by irradiating rhodopsin at liquid nitrogen temperatures; the relative concentrations of the three species may be varied by using the two laser beam "pump-probe" technique. Some features of the bathorhodopsin spectrum have been previously reported (Oseroff and Calender, 1974; Sulkes et al., 1978). Here, by using computational techniques to subtract out the Raman profiles of rhodopsin and isorhodopsin, we can obtain a complete Raman spectrum of bathorhodopsin. A similar technique has recently been used by Eyring and Mathies (1979) to isolate the bathorhodopsin spectrum.

Changes in the C=N stretching frequency in deuterated solutions have been regarded as the most reliable measure of whether the Schiff base is protonated. Frequency shifts of ~ 25 cm^{-1} have been obtained (e.g., Callender and Honig, 1977), and this value corresponds remarkably well with that predicted on the basis of reduced mass considerations where the mass of the hydrogen is added to that of the nitrogen and the C=N bond is treated independently of the rest of the molecule. The success of this simple treatment is somewhat surprising, since the assumptions are clearly gross oversimplifications. Indeed, Marcus et al. (1979) have recently found that ^{15}N substitution in bacteriorhodopsin decreases the frequency of the C=N stretch by only 15 cm^{-1}. In view of the central importance generally ascribed to the C=N stretching mode, we have carried out a normal mode analysis of unprotonated, protonated, and deuterated Schiff bases so as to understand the origin of the various frequency shifts in greater detail.

Our experimental and theoretical results indicate that the proton of both rhodopsin and bathorhodopsin is covalently bound to the Schiff base nitrogen. This result is inconsistent with all models of the primary event that involve a change in the state of protonation of the Schiff base nitrogen. Moreover, the resonance Raman spectrum of bathorhodopsin is considerably different than that of rhodopsin, thus suggesting that the chromophores of the two pigments are in different conformations.

METHODS AND MATERIALS

Rod outer segment membrane fragments were isolated from dark adapted bovine retinal as described previously (Ebrey, 1971). Solubilized rhodopsin was obtained by extracting membrane fragments in

3.8% Ammonyx-LO (Onyx Chemical Company, Jersey City, N.J.) and 0.01 M imidazole buffer (pH 7.0) essentially using the procedure of Applebury et al. (1974). However, after the solubilized membrane fragments were applied to a column, a stepwise elution consisting of 100 ml of 35 mM Pi followed by 100 ml of 100 mM Pi was performed instead of the continuous gradient of Pi. The solubilized rhodopsin was then concentrated by vacuum dialysis. Rhodopsin was deuterated by first diluting the concentrated sample with 99.8% D_20 (Bio-Rad Laboratories, Richmond, Calif.) and then dialyzing against the D_20 for 24 h with two changes of dialysate, followed by reconcentration.

The concentrated 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 homemade liquid nitrogen cold-finger dewar and the temperature was maintained at 80°K. The laser Raman spectra were obtained using krypton (Coherent Radiation, Palo Alto, Calif.; model 52) and argon ion lasers (Spectra-Physics Inc., Mountain View, Calif.; model 165), Spex 1401 double monochrometer (Spex Industries, Inc., Metuchen, N.J.), and a cooled RCA 31034A photomultiplier (RCA Solid State, Somerville, N.J.) and stored in a PDP 8/E minicomputer (Digital Equipment Corp., Marlboro, Mass.). The complete system is more fully described in Callender et al. (1976). Band assignments are accurate to within $\pm$ 3 cm^{-1}. While the subtraction procedures were performed on the raw data (see below), all presented data has been averaged with a three point (out of 512 points) simple

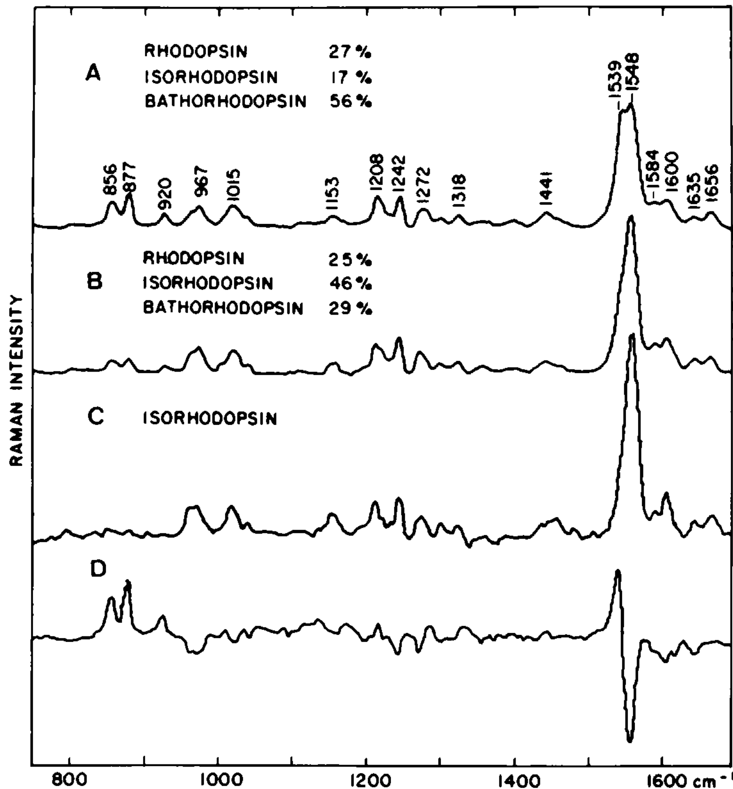

FIGURE 1 The Raman data and one subtraction step leading to the bathorhodopsin spectrum. All spectra were taken with a 476.2-nm probe, at 7.3 cm^{-1} resolution, and at a sample temperature of 80° K. The compositions are given in the figure (see text for method). (*A*) 476.2-nm probe beam alone. (*B*) Simultaneous 580-nm pump beam applied at seven times pump/probe ratio. (*C*) Simultaneous 568.2-nm pump beam at 25 times pump/probe ratio. (*D*) $A - 1.08\ B$. *A*, *B*, and *C* are scaled for the same input probe power; since *D* is a different spectrum and relatively small, we have increased its scale by a factor of three for graphical purposes.

summation procedure to increase the final signal to noise. All labeled peaks were clearly present in the raw data.

As described in detail by Oseroff and Callender (1974), an essential feature of the experimental procedure is to stimulate the Raman scattered light with a fixed single wavelength at fixed power (the "probe" beam), while simultaneously irradiating the sample with a second spacially overlapping laser beam (the "pump" beam), where wavelength and power can be varied. The purpose of the pump beam is to modify the photostationary state of the sample so that the Raman scattered light reflects only changes in component concentration and not changes in Raman cross sections. The measured composition and pump-probe wavelength and relative power used in our present analysis are given in the figures. In addition, one further measurement was performed using a 476.2-nm probe and a 590-nm pump (at 22 times the probe power). This produced sample concentrations of rhodopsin, isorhodopsin, and bathorhodopsin of 0.58, 0.30, and 0.12%, respectively. Use of this data in terms of the analysis below gave the same bathorhodopsin spectrum.

The relative amounts of rhodopsin, isorhodopsin, and prelumirhodopsin of the irradiated sample were determined in separate experiments. The method is similar to that employed by Oseroff and Callender (1974). 1.5 ml of the sample used for the Raman measurements (but not as concentrated) was placed in a 30-ml test tube immersed in liquid nitrogen and irradiated with single and double beams with the same relative power levels as used in the Raman measurements. Care was taken in the double beam experiment to insure that the two beams were coaxial. The beam was expanded to cover the total sample and all sides were irradiated for more than ½ h. After the irradiation the sample was warmed to room temperature and the absorption spectrum recorded using an ultraviolet-visible spectrophotometer (GCA McPherson Corp., Acton, Mass., model EU-707-D). The sample was subsequently bleached at room temperature and the spectrum of the bleached sample taken. The difference spectra were analyzed as follows. The decrease in absorbance of the 485–500 band relative to the control was taken to correspond to the amount of the sample converted to bathorhodopsin during irradiation. In addition, the relative amounts of rhodopsin and isorhodopsin were determined by deconvoluting the difference spectra into the components of rhodopsin and isorhodopsin using a least-square fitting procedure. From variations of the sample composition determined in this way over a large number of separate runs, we estimate an accuracy of ±5% of each component composition. No difference in composition was found in protonated and deuterated samples. It should be pointed out that the consistency of the percent compositions can be checked to some extent against predictions from the known absorption structure of the tube pigments and against changes in the observed strengths of the obtained Raman bands (that have already been assigned to one of the three pigments) as a function of pigment concentration. We found a high degree of internal consistency.

RESULTS

Obtaining the Bathorhodopsin Spectrum

Fig. 1 shows Raman spectra produced by probe laser irradiation at 476.2 nm with and without additional coincident pump irradiation at different laser frequencies. The photostationary state compositions under these conditions are also given in Fig. 1. The spectra of Fig. 1 *A* and *B* are identical to those previously reported by Oseroff and Callender (1974), although obtained here at higher resolution. As pointed out by Oseroff and Callender (1974), it is not possible to obtain a sample composed of pure rhodopsin or bathorhodopsin, because the absorption bands of these pigments overlap. Thus, in general, the sample will contain substantial amounts of all these pigments. However, irradiation near 568.2 nm produces almost pure isorhodopsin, since its absorption is significantly smaller than rhodopsin and bathorhodopsin at this wavelength (Oseroff and Callender, 1974; Eyring and Mathies, 1979). Thus, it is possible to obtain a spectrum of essentially (95%) pure isorhodopsin, as shown in Fig. 1 *C*. It is interesting to note that the Raman spectrum of isorhodopsin in Fig. 1 *C*

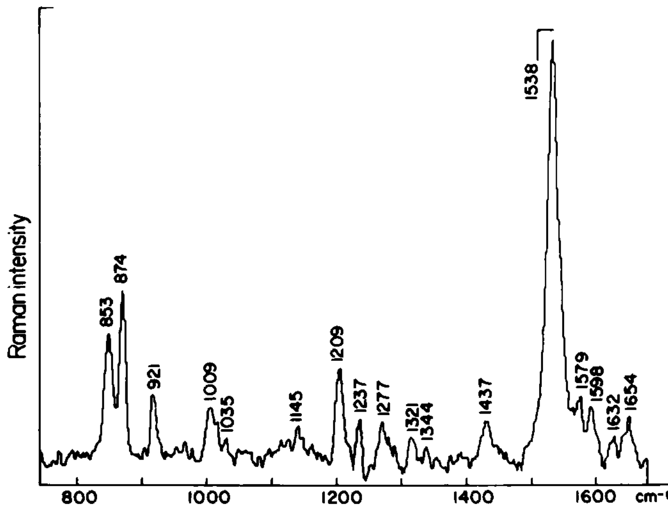

FIGURE 2 The bathorhodopsin spectrum. Represents (Fig. 1 *D*) + 0.25 (Fig. 1 *C*). See text.

stimulated by 476.2-nm light is similar but not identical as to those previously reported, which employed irradiation at longer wavelengths (Oseroff and Callender, 1974; Mathies et al., 1976). This shows that the shape of the Raman profile can be wavelength-dependent with respect to the absorption maximum which, of course, is not unexpected under resonance conditions. This is why the Raman spectra presented here are all taken with the same laser wavelengths. Deconvoluting composite resonance Raman spectra with individual species spectra taken at another wavelength may introduce errors.

The Raman spectrum of bathorhodopsin can be obtained from the spectra of Fig. 1. Briefly, subtraction of an appropriate fraction of the spectrum of Fig. 1 *B* from that of Fig. 1 *A* leaves a spectrum composed of a positive contribution from bathorhodopsin and a negative contribution from isorhodopsin, with the contribution from rhodopsin canceling out. This results in the spectrum of Fig. 1 *D*. By adding an appropriate amount of the isorhodopsin spectrum of Fig. 1 *C*, we can isolate the bathorhodopsin Raman spectrum of Fig. 2. The procedure is described in detail below.

The Raman scattered light, I_s, can be written as

$$I_s = \sigma_R C_R I_o + \sigma_I C_I I_o + \sigma_B C_B I_o, \qquad (1)$$

where the subscripts *R*, *I*, and *B* stand for rhodopsin, isorhodopsin, and bathorhodopsin, respectively; σ_R, etc., are the respective Raman cross sections; C_R, etc., are the respective pigment concentrations; and I_o is the irradiating probe laser power. The total number of molecules irradiated is constant in the series of three experiments given by Fig. 1 *A–C* so that we may view the pigment concentrations, C_R, etc., as percent concentrations, with the appropriate numbers given in Fig. 1; then I_s is the Raman scattered light given in terms of percent concentrations of the three pigments. Let I_s^A be the scattered light for the data in Fig. 1 *A*. Using superscripts *B* and *C* for the data in Fig. 1 *B* and *C*, we obtain

$$I_s^A = \sigma_R(0.27)\, I_o + \sigma_I(0.17)\, I_o + \sigma_B(0.56)\, I_o, \qquad (2)$$

$$I_s^B = \sigma_R(0.25)\, I_o + \sigma_I(0.46)\, I_o + \sigma_B(0.29)\, I_o, \tag{3}$$

$$I_s^C = \sigma_I(1.00)\, I_o, \tag{4}$$

so that the bathorhodopsin spectrum of Fig. 2 is given by

$$I_s^{\text{Batho}} = I_s^A - (1.08)\, I_s^B + 0.33\, I_s^C = (0.25)\, \sigma_B I_o. \tag{5}$$

Fig. 1 *D* shows $I_s^A - (1.08)I_s^B$ multiplied by a factor of three (for graphical purposes) relative to Fig. 1 *A–C*.

Although this procedure isolates the bathorhodopsin spectrum, it should be noted that assay procedures used here and by Oseroff and Callender (1974) and more recently by Eyring and Mathies (1979) determine the relative pigment concentrations, C_R, etc., to no better than $\pm 5\%$. Thus, significant remnants, on the order of ± 10–15%, of other pigments can contribute to the bathorhodopsin spectrum. In testing various worst-case possibilities by using pigment concentrations at the limits of experimental error we found that the bathorhodopsin spectrum was insensitive to the uncertainties in pigment concentration, although there was a small uniform increase or decrease in intensity of the other Raman bands relative to the ethylenic band at 1,538 cm^{-1}. The only exceptions are the bands at 965 (not labeled) and 1,145 cm^{-1}, whose intensities range from essentially zero to a somewhat larger value than presented in Fig. 2. The cause for the uncertainty appears to be that these bands are relatively weak in bathorhodopsin but strong in rhodopsin and isorhodopsin, so that the apparent intensities are extremely sensitive to small changes in rhodopsin and isorhodopsin concentration.

Overview of the Spectral Features

There are a number of striking features of the bathorhodopsin spectrum given in Fig. 2. The intense low-frequency lines below 900 cm^{-1} that were identified previously are evident, and are present in no other pigment (Oseroff and Callender, 1974). The most easily interpretable result of our measurement concerns the C═N vibration at 1,654 cm^{-1}. In a previous study it was shown that the C═N vibration is shifted to 1,630 cm^{-1} in deuterated samples of photostationary state mixtures of rhodopsin, isorhodopsin, and bathorhodopsin at 80° K (Oseroff and Callender, 1974). However, a specific deuterium shift for each separate pigment could not be demonstrated with certainty. Fig. 3 shows the Raman profile above 1,500 cm^{-1} for isorhodopsin (Fig. 3 *C*) and bathorhodopsin (Fig. 3 *D*) in deuterated medium together with the composite spectra (Fig. 3, *A* and *B*) measured under the same conditions as for Fig. 1 *A* and *B*, respectively. As is clear from the figure, the C═N band at ~1,654 cm^{-1} disappears upon deuteration of both pigments. In both bathorhodopsin and isorhodopsin there is a significant increase in intensity near 1,630 cm^{-1} corresponding to the C═N stretching frequency of the deuterated molecule. In rhodopsin[1] the shift from 1,654 to 1,630 cm^{-1} is also quite distinct. These results provide strong support for the conclusion reached previously by a number of groups (Oseroff and Callender, 1974; Sulkes et al., 1978; Eyring and Mathies, 1979) that the state of protonation of bathorhodopsin is the same as that of rhodopsin and isorhodopsin.

The fingerprint region from 1,100 to 1,300 cm^{-1}, which is generally sensitive to chromo-

[1]Narva, D., and R. H. Callender. Submitted for publication.

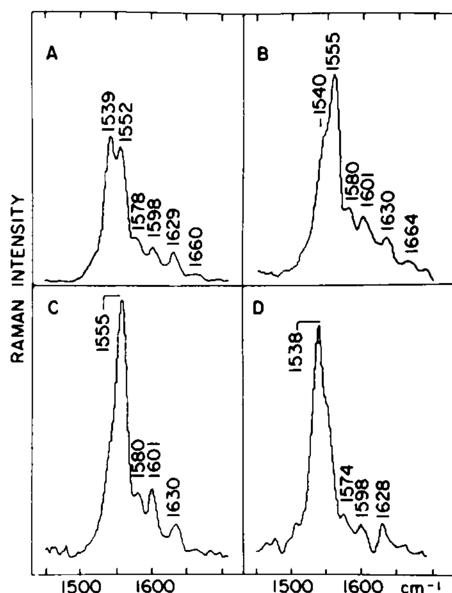

FIGURE 3 Raman spectra for deuterated rhodopsin. Conditions are identical to Fig. 1 *A*, *B*, and *C*, and Fig. 2 for *A*, *B*, and *C* are scaled for the same input proble power; since *D* is different spectrum and relatively small, we increased its scale by a factor of three for graphical purposes.

phore conformation, does not allow a clear cut identification of the isomeric form of the chromophore of bathorhodopsin. However, the existence of a strong line near 1,200 cm^{-1} with weaker lines near 1,237 and 1,277 cm^{-1} is a pattern characteristic of only the all-*trans* isomer for protonated Schiff bases (Mathies et al., 1977; Doukas et al., 1978). The additional weak line at 1,321 cm^{-1} is not present in the all-*trans* model compound but appears in metarhodopsin I (Doukas et al., 1978), whose chromophore clearly has an all-*trans* conformation. Comparisons of this sort are subject to some uncertainty, because measurements are performed at different wavelengths and on compounds with different λ_{max}. However, this pattern in the fingerprint region is apparent for the all-*trans* protonated Schiff base measured at 647.1 nm (Doukas et al., 1978) and at 514.5 nm (Mathies et al., 1977) and for metarhodopsin I (λ_{max} = 478 nm, measured at 476.5 nm; Doukas et al., 1978). Our data suggest, then, that bathorhodopsin has a *trans*-like conformation.

Normal Mode Analysis; the C=N Stretching Frequency

The primary goal in carrying out a normal mode study was to understand the factors that determine the frequency of the C=N stretching vibration in different molecules. Two experimental observations concerning this mode are in fact rather difficult to understand on intuitive grounds. First, it is generally the case that protonation increases the frequency of the C=N stretch (Heyde et al., 1971), even though the C=N force constant is expected to decrease upon protonation. This expectation is, for retinal Schiff bases, based on the observation that protonation increases the extent of π-electron delocalization, thus weakening the double bonds (e.g., Honig et al., 1976). The effect is obvious for the C=C stretching vibration (Doukas et al., 1978), which decreases in frequency upon protonation, but is also

consistent with the increased length of the C═N bond in the protonated species. This difficulty has been recognized previously for retinals (Blatz and Mahler, 1975) and for Schiff bases that are not associated with extended π-electron systems (Elguero et al., 1967). Another factor, the increased effective mass of the nitrogen resulting from protonation, should also tend to decrease the vibrational frequency, as occurs, for example, when a proton is replaced by a deuteron.

A second problem concerns the magnitude of observed reduction in vibrational frequency that occurs upon deuteration. The shift is $\sim$25 cm^{-1} (from 1,655 to $\sim$1,630 cm^{-1}) in pigments and model compounds. This value is almost exactly what is predicted when assuming that the mass of the proton (or deuteron) is simply added to that of the nitrogen and that the C═N bond vibrates independently, so that the entire frequency shift arises from a change in the reduced mass (Oseroff and Callender, 1974). However, both of these assumptions, though perhaps reasonable first approximations, are clearly not strictly valid. For example, the C═N bond should be strongly coupled to the various C═C chain vibrations, and this lends to the expectation that the observed deuterium effect will be considerably smaller than the value calculated for the hypothetical diatomic harmonic oscillator. In fact, ^{15}N substituted Schiff bases of retinal are shifted by only $\sim$15 cm^{-1} relative to the ^{14}N compounds (Marcus et al., 1979) and here the effect is exclusively due to a change in mass.

To understand the factors that give rise to these effects, we have carried out a normal mode analysis of several molecules of increasing complexity. The normal mode analysis (program no. 177 of the Quantum Chemistry Program Exchange, Chemistry Department, Indiana University) allows us to define a set of force constants as input and to obtain as output the vibrational frequencies and normal modes of a particular molecule. By treating the force constants as parameters and studying the frequencies that are obtained, it is possible to gain an intuitive feeling as to the factors that determine the observed frequencies and to the ways that the various modes interact with one another.

We first consider the hypothetical diatomic molecule C═N and the triatomic molecule C═N—H. These allow us to study the Schiff base mode alone without the complications introduced by the rest of the polyene chain. Our approach will be to study the effect of various internal modes on the frequency of the C═N stretching vibration. Since we are seeking primarily qualitative insights the exact values of the parameters we choose are not important. For the purpose of the discussion of these simple molecules, we use the force constants determined for the longer polyenes (see below and Table I). However, even major changes in the force constants have little influence on the relative effects, which are of interest.

For the diatomic molecule C═N, a stretching force constant of 8.5 mdyn/Å yields a vibrational frequency of 1,494 cm^{-1}. Lowering this force constant to 8.1 mdyn/Å (consistent with the weaker C═N bond in the protonated species) would decrease the frequency of the C═N stretch to 1,458 cm^{-1} in the triatomic molecule if there were no interactions with other vibrations (Table II). If we now allow the C═N stretch to interact only with the N—H stretch, the frequency is reduced to 1,451 cm^{-1} for the protonated species and to 1,441 cm^{-1} for the deuterated species. Thus, at this level of approximation the C═N stretching frequency of the protonated molecule is far below that of the unprotonated molecule, which is the "intuitive expectation" discussed above, and is clearly the inverse of what is observed. Note as well that deuteration introduces only a 10-cm^{-1} reduction in frequency. If we now

TABLE I
FORCE CONSTANTS

Type	Value	Comment
C=N stretch	8.1 mdyn/Å	Protonated Schiff base
C=N stretch	8.5 mdyn/Å	Unprotonated Schiff base
C=C stretch	7.1 mdyn/Å	—
C–C stretch	5.0 mdyn/Å	—
C–H stretch	5.0 mdyn/Å	—
C–C=C bend	0.675 mdyn Å/rad^2	—
N–C–C bend	0.675 mdyn Å/rad^2	—
C=N–C bend	0.675 mdyn Å/rad^2	—
C=N–H bend	0.82 mdyn Å/rad^2	—
C=N/C=N–H	−0.2 mdyn/rad	stretch/bend interaction*
C=N/C–C=N	0.6 mdyn/rad	stretch/bend interaction*
C–C/C–C=C	0.6 mdyn/rad	stretch/bend interaction*
C=C/C=C ·	−0.3 mdyn/Å	stretch/stretch interaction‡
C=N/C=C	−0.3 mdyn/Å	stretch/stretch interaction‡

*The stretch/bend interaction is for common atoms.
‡Nearest neighbor stretch/stretch double bond interaction.

include the C=N—H bending coordinate in the normal mode analysis the situation is markedly improved. The vibrational frequency of the protonated molecule is higher than that of the unprotonated molecule, agreeing with the experimental observation. The deuterium effect is now 43 cm^{-1} (1,522–1,479 cm^{-1}) which is somewhat too large.

It is easy to understand these results on a qualitative basis. The C=N stretch at 1,458 cm^{-1} interacts with the C=N—H bend at ~1,250 cm^{-1}, and this increases the frequency of the

TABLE II
CALCUATED C=N STRETCHING FREQUENCIES

Force constants used	C=N stretching frequency		
	Unprotonated	Protonated	Deuterated
		(*centimeters^{-1}*)	
C=N			
C=N stretch	1,494	—	—
C=N–H			
C=N stretch	1,458	—	—
C=N stretch N–H stretch	—	1,451	1,441
Stretch constants and C=N–H bend	—	1,498	1,465
Entire force field	—	1,522	1,479
Retinal Schiff base			
Entire force field except C=N/C=N–H interaction	1,624	1,642	1,625
Entire force field	1,624	1,659	1,633
Retinal Schiff base (^{15}N)			
Entire force field	1,617	1,640	1,622

The C=N stretching force constants are 8.5 and 8.1 mdyn/Å for the unprotonated and protonated cases, respectively.

more energetic vibration. The effect of the C=N—D bending motion is much smaller because the extent of interaction decreases with increasing separation of the vibrational frequencies, and the C=N—D bending vibration has a frequency of only 947 cm^{-1}. Thus we have the important result that protonated Schiff bases have higher C=N stretching frequencies than unprotonated Schiff bases, because the frequency is "pushed up" by interaction with the C=N—H bend.

It is important to realize that there are no other factors that can, for these simple molecules, influence the observed frequencies. Thus, the increased frequency of the protonated C=N stretch relative to the unprotonated stretch can only arise from the C=N—H bending mode. This result is completely independent of the set of force constants chosen to represent the molecule. It should also be emphasized that the shift due to deuteration is not primarily a reduced mass effect, as has been previously assumed (Oseroff and Callender, 1974). What still remains to be demonstrated is that the importance of the bending mode is retained for the longer polyenes and that other modes (such as C=C stretching vibrations that interact with the C=N stretch) don't alter the qualitative conclusions reached for the simpler molecules.

A more realistic description of the normal modes of retinal Schiff bases may be obtained from analyzing the idealized polyene Schiff base of Fig. 4. We have determined an approximate force field, given in Table I, which produces a reasonable set of frequencies for the molecule. The C=C stretching and bending force constants and interaction are similar to those used by other workers who have studied polyene chains (e.g., Gavin and Rice, 1971; Inagaki et al., 1975) but have been modified somewhat so as to produce C=C vibrations in the range observed for retinals (1,500–1,610 cm^{-1}). The C=N stretching force constant was chosen so as to reproduce the 1,624-cm^{-1} frequency of the Schiff base of retinal and was lowered from 8.5 to 8.1 mdyn/Å to reflect the weakening of this bond in the protonated species. We have used a C=N—H bending constant equal to that used by Gavin and Rice (1971) for the C=C—H (and close to that used by Rabolt et al., 1977, in their analysis of polypeptides). As in the case of the triatomic molecule, the C=N—H bending mode clearly plays a major role in determining the frequency of the C=N stretch. The frequency is increased from 1,624 cm^{-1} in the unprotonated molecule to 1,642 cm^{-1} in the protonated molecule, despite a decrease in the stretching force constant. Moreover, a deuteration effect of 17 cm^{-1} (1,642–1,625 cm^{-1}) is obtained. To reproduce the experimental results, we have added a C=N/C=N—H interaction constant similar to that used by other workers. The resulting frequencies are 1,624, 1,659, and 1,633 cm^{-1} for the unprotonated, protonated, and deuterated Schiff bases, respectively.

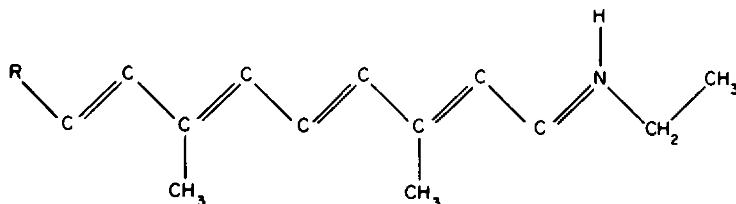

FIGURE 4 The simplified retinal used in the theoretical normal mode calculations. Mass values used: C = 12, N = 13, H = 1, D = 2, R = 123, CH_2 = 14, and CH_3 = 15. Bond lengths used (in ångströms): C—C of 1.47, C=C of 1.33, C—H of 1.0, C=N of 1.33, N—C of 1.47, N—H of 1.0. Molecules were assumed planar with bond angles of 120°.

Notice that the shift between the unprotonated and protonated Schiff base, as well as the magnitude of the deuterium effect, are smaller than for the simple diatomic and triatomic molecules. This is because the C=N stretch is now coupled strongly to the C=C stretch of the adjacent double bond and to a lesser extent to the next bond along the chain. Thus the mode is no longer a pure diatomic vibration, and as a result it interacts less strongly with the C=N—H bend.

To subject our analysis to an additional test we have carried out calculations on two molecules where specific substitution effects on the C=N stretching frequency have been isolated. Marcus et al. (1979) found frequency shifts of 12 and 13 cm^{-1}, respectively for ^{15}N-enriched Schiff bases and protonated Schiff bases of retinal. We calculate 7 and 19 cm^{-1} shifts with the force field of Table I in reasonable agreement with these results. It should be pointed out that the ^{15}N enrichment should have produced a shift equal to or greater than deuterium substitution if the reduced mass was the major factor involved in both cases. That the frequency shift is greater when the proton is replaced by a deuteron is in itself evidence for the role of the C=N—H bending mode in determining the C=N frequency (Marcus et al., 1979).

The important effect of the C=N—H bend on the C=N stretch seems well-established. The surprising frequency increase in going from the unprotonated to the protonated Schiff base, as well as the large deuteration effect, arise naturally from a reasonable choice of force constants, and moreover, both phenomena can be understood qualitatively, as discussed above. We wish to reemphasize that we have been primarily concerned with a qualitative understanding of the factors that determine the C=N stretching frequency. A quantitative description of the normal modes of retinal Schiff bases would require a larger body of experimental data than is currently available.

DISCUSSION

Comparison with Other Studies

Eyring and Mathies (1979) recently reported a bathorhodopsin spectrum using techniques similar to ours. They used a 585-nm probe wavelength, which has the advantage of being in closer resonance with the absorption maximum of bathorhodopsin relative to the absorption maxima of rhodopsin and isorhodopsin. Thus, under these conditions, the resonance enhancement factor of bathorhodopsin would be expected to be larger than that of the other two pigments, as contrasted with our own study, where a 476-nm probe beam was used. However, it was necessary for Eyring and Mathies (1979) to subtract a spectrum of rhodopsin obtained at room temperature from a low temperature spectrum of a mixture of rhodopsin and bathorhodopsin. This procedure introduces particular uncertainties in their results which are different than those involved in our own analysis. It is thus reassurring that the two bathorhodopsin spectra are so similar, particularly because they are measured at different wavelengths. The only significant differences are that there is less intensity in the 1,145- and 1,237-cm^{-1} bands in the spectrum of Eyring and Mathies (1979) than in ours, while the 1,224- and 1,166-cm^{-1} bands apparent in their spectra are very weak or non-existent in our data. It is not clear whether these differences simply reflect a wavelength dependence of the Raman cross sections or errors in the parameters used in each case to extract the

bathorhodopsin spectrum. At least part of the discrepancy may be due to experimental error, since in our analysis intensity at 1,145 cm^{-1} was particularly sensitive to the uncertainties in pigment concentration, while Eyring and Mathies (1979) report similar uncertainties for the 1,224- and 1,242-cm^{-1} bands.

A recent study of Sulkes et al. (1978) reported composite spectra of bathorhodopsin, isorhodopsin, and rhodopsin measured with a 514-nm probe beam. These workers did not extract a bathorhodopsin spectrum but did assign a number of lines to bathorhodopsin that agree with our own assignments and those of Eyring and Mathies (1979). However, the lines at 1,237 and 1,321 cm^{-1} were not attributed to bathorhodopsin, nor was any intensity in the 1,100–1,200-cm^{-1} region.

The State of Protonation of the Schiff Base

Recently, a number of models for the primary event in vision have appeared in which the extent of protonation of the Schiff base nitrogen is an important parameter (van der Meer et al., 1976; Peters et al., 1977; Favrot et al., 1979; Harosi et al., 1978). It was proposed that the Schiff base proton is only hydrogen-bonded to the nitrogen in rhodopsin and that full protonation of the Schiff base occurs only during the formation of bathorhodopsin. This suggestion has gained support from ^{13}C nuclear magnetic resonance (NMR) experiments that were interpreted to demonstrate that rhodopsin contains an unprotonated Schiff base (Shriver et al., 1977) (although recently it has been shown that NMR data are consistent with a protonated chromophore; Honig et al., 1979*b*). The standard interpretation of Raman experiments has been specifically questioned in several studies and the possibility raised that the proton is transferred as a consequence of the Raman measurement, thus precluding detection of the unprotonated Schiff-base vibration in rhodopsin itself (Favrot et al., 1979; Harosi et al., 1978). It is therefore of interest to consider the arguments that relate to the state of protonation of the Schiff base in some detail.

The first line of evidence is based on the frequency of the C=N stretching vibration. Protonated Schiff bases in solution have C=N vibrations of ~1,655 cm^{-1}, and Raman bands of approximately this frequency have been observed for rhodopsin and isorphodopsin at both room temperature and 77° K (Heyde et al., 1971; Oseroff and Callender, 1974; Mathies et al., 1976; Callender et al., 1976). However, arguments based on band frequencies alone are not unequivocal (Oseroff and Callender, 1974; Honig and Ebrey, 1974; Callender and Honig, 1977; Favrot et al., 1978) since the protein modifies the electronic structure of the chromophore; this might have significant effects on vibrational frequencies. For this reason, it has been important to study the effects of deuteration on the Schiff base stretching mode.

Oseroff and Callender (1974) demonstrated that the frequency shift seen upon deuteration of model chromophores closely matched that of deuterated rhodopsin. On the other hand, the actual measurement was done on a photostationary state mixture containing a large fraction of bathorhodopsin and not specifically on the individual pigments. The data of Fig. 3 *C* show conclusively that there is a large deuterium effect on the C=N stretching frequency of isorhodopsin, whose magnitude is almost identical to that seen in model protonated Schiff bases. Using similar techniques, we have shown that the deuteration effect on the Schiff base mode is the same for rhodopsin (footnote 1). Figs. 2 and 3 *D* also show that the state of protonation of bathorhodopsin is the same as that of isorhodopsin and rhodopsin. All three

pigments have essentially the same C=N stretching frequency; moreover, bathorhodopsin, isorhodopsin, and rhodopsin exhibit the same deuteration effect.

Our results strongly suggest that the Schiff base proton is strongly bound to nitrogen in all three pigments. First, as mentioned above, model protonated Schiff bases (where the proton is covalently bound to the nitrogen) exhibit the same C=N stretching frequency and deuterium shift as do the various pigments. Second, our normal mode analysis shows that the large frequency shift seen upon deuteration can only be understood if the proton is covalently bound to the nitrogen so that a large C=N—H bending force constant can be assumed. Furthermore, as we have discussed above, the bending mode is responsible for shifting the C=N stretching frequency of protonated Schiff bases to values greater than that of unprotonated Schiff bases. If the proton were not tightly bound to the nitrogen, a much lower frequency would have been observed for the pigments. Finally, the magnitude of the deuterium shift expected from a hydrogen-bounded proton can be estimated from the effects of deuteration on the normal vibrations of helical peptides where a hydrogen bond of the form C=O---H—N is formed. Shifts on the order of 5 cm^{-1} are seen in the carbonyl stretching frequency (Rabolt et al., 1977), which is much smaller than the 25-cm^{-1} shifts in the C=N frequency seen in visual pigments and protonated Schiff bases.

Another strong experimental demonstration of the state of protonation of the Schiff base comes from the work of Mathies et al. (1977), who showed that the Raman spectrum in the fingerprint region of rhodopsin and isorhodopsin, respectively, was remarkably similar to that of model 11-*cis* and 9-*cis* protonated Schiff bases. Thus there are three independent lines of evidence based on the Raman data alone that seem to require that the Schiff base of rhodopsin, isorhodopsin, and bathorhodopsin is fully protonated. The same conclusion is appropriate for metarhodopsin I, which is formed thermally from bathorhodopsin (Doukas et al., 1978).

The validity of interpreting resonance Raman experiments in the standard way has recently been questioned by Sandorfy and co-workers (Favrot et al., 1979; Harosi et al., 1978), and it is important to consider their objections in some detail. These workers have raised the interesting possibility that photochemistry occurs as a consequence of the Raman experiment, as the incident beam is in resonance with the excitation energy of the chromophore. In this case the emitted photon would result from a photochemically generated excited state of a new species, and not from the Raman process. However, there is evidence that this is not occurring in our own measurements and in those of other workers who have studied visual pigments. First, the implication of this objection to the Raman measurements is that the final state reached is not a vibrationally excited state of the ground electronic state of rhodopsin, as is normally assumed, but rather vibrational states of bathorhodopsin which is formed as a result of the photochemistry. Recall, however, that Raman experiments measure the difference in energy between the incident and emitted photon. Thus, for example, a Raman line at, say, 1,000 cm^{-1} as is typically observed, implies a 1,000-cm^{-1} energy difference between the incident and emitted photon. However, the bathorhodpsin ground state has been shown to be over 13 kcal/mol (4,550 cm^{-1}) above the ground state of rhodopsin (Rosenfeld et al., 1977). Thus, the difference in energy between the absorbed and emitted photon would have to be $>$4,550 cm^{-1} if the ground state of bathorhodopsin were reached, and this is well out of our observed spectral range.

A second argument can be based on the fact that three pigments (rhodopsin, isorhodopsin, and bathorhodopsin) are present in samples at 77° K, and three distinct and different Raman spectra have now been associated with each pigment: the present results and those of Eyring and Mathies (1979) for the bathorhodopsin Raman spectrum; previous results on rhodopsin (Mathies et al., 1976; Callender et al., 1976) and isorhodopsin (Oseroff and Callender, 1974; Mathies et al., 1976). If in the process of the Raman measurement, one species were converted to another, it would not be possible to isolate three clearly separate spectra.

We conclude, therefore, that the standard interpretation ascribed to the Raman experiments is correct. Thus, the Raman data required that the chromophore of rhodopsin is a fully protonated Schiff base of 11-*cis* retinal and that the degree of protonation is not changed in the photochemical formation of bathorhodopsin and in the subsequent thermal formation of metarhodopsin I. Models (van der Meer et al., 1976; Peters et al., 1977; Favrot et al., 1979; Harosi et al., 1978) that assume that the Schiff base proton is only hydrogen-bonded to the nitrogen in rhodopsin or that postulate significant changes in its position as a primary event appear, then, to conflict with the Raman measurements.

A Model for the Primary Photochemical Event

As discussed above, the Raman spectrum of bathorhodopsin cannot be unambiguously interpreted in terms of a particular *cis* or *trans* isomer. However, the large differences between the Raman spectra of rhodopsin and bathorhodopsin indicate that some form of geometric isomerization has accompanied the primary photochemical event. The chromophore bathorhodopsin clearly does not have the conformation of the *all-trans* protonated Schiff base in solution, although there are close similarities to an *all-trans* model compared in the fingerprint region (see above), a spectral region sensitive to isomeric form. Additionally, given the other evidence that some form of isomerization involving torsional motion about the 11–12 double bond has taken place, and since by the metarhodopsin I stage the chromophore is close to the true *all-trans* conformation (Doukas et al., 1978), a strained "transoid" structure, which has been previously suggested (Callender and Honig, 1977; Rosenfeld et al., 1977; Aton et al., 1978), seems most plausible. A structure twisted about one or more single bonds is a likely possibility. In any case, the Raman data clearly indicate that a major geometric change in the chromophore accompanies the photochemical formation of bathorhodopsin, and this should be accounted for in any model of the primary event.

A second constraint provided by the Raman data is that the Schiff base in both rhodopsin and bathorhodopsin is fully protonated. It is necessary, however, to account for the important finding of Peters et al. (1977) that a proton transfer event is associated with the formation of bathorhodopsin. It is thus necessary to seek a model that accounts for some form of isomerization as well as proton transfer not directly involving the Schiff base.

We have recently proposed such a model that makes use of the likelihood that a negatively charged amino acid forms a salt bridge with the positively charged nitrogen of the retinyllic chromophore (Honig et al., 1979*a*). The photochemical event is a *cis-trans* isomerization in visual pigments and *trans-cis* isomerization in bacteriorhodopsin, which in each case cleaves the salt bridge and thus separates charge in the interior of the protein. We propose that this is how the energy of a photon is transduced into chemical free energy of the primary photoproduct. The increase in energy and the spectral red shift that characterize the primary

photochemical events are natural consequences of the separation of charge. Proton-dependent transients detected with picosecond techniques (Peters et al., 1977) are shown to correspond to ground-state relaxation processes involving protons on the opsin rather than on the chromophore.

This work was supported in part by the National Science Foundation (PCM 77-06728, and PCM 79-02683), by the National Institutes of Health (EY03142), and by a City University Professional Staff Congress-Board of Higher Education Faculty Award, and by the Kultusministerium des Landes Niedersachsen for Göttingen-Jerusalem Exchange Program.

Received for publication 26 March 1979 and in revised form 28 July 1979.

REFERENCES

APPLEBURY, M. L., K. PETERS, K. and P. RENTZEPIS. 1978. Primary Intermediates in the photochemical cycle of bacteriorhodopsin. *Biophys. J.* **23**:375.

APPLEBURY, M. L., D. M. ZUCKERMAN, A. A. LUMOLA, and T. M. JOVIN. 1974. Rhodopsin, purification recombination with phospholipids assayed by the metarhodopsin I-metarhodopsin II transition. *Biochemistry.* **13**:3448.

ATON, B., R. H. CALLENDER, and B. HONIG. 1978. Photochemical *cis-trans* isomerization of bovine rhodopsin at liquid helium temperatures. *Nature (Lond.)* **273**:784.

BLATZ, P. E., and J. H. MAHLER. 1975. Effect of selected anions and solvents on the electronic absorption nuclear magnetic resonance and infrared spectra of the *N*-retinylidene-*n*-butylammonium cations. *Biochemistry.* **14**:2304.

CALLENDER, R. H., and B. HONIG. 1977. Resonance Raman studies of visual pigment. *Annu. Rev. Biophys. Bioeng.* **6**:33.

CALLENDER, R. H., A. DOUKAS, R. CROUCH, and K. NAKINISHI. 1976. Molecular flow resonance Raman effect from retinal and rhodopsin. *Biochemistry.* **15**:1621.

DOUKAS, A. G., B. ATON, R. H. CALLENDER, and T. EBREY. 1978. Resonance Raman studies of bovine metarhodopsin I and II. *Biochemistry.* **17**:2430.

EBREY, T. 1971. The use of ammonyx LO in the purification of rhodopsin and rod outer segments. *Vision Res.* **11**:1007.

ELGUERO, J., R. GILL, and R. JACQUIER. 1967. Sur l'identification por infrarouge des groupes immonium. *Spectrochim. Acta Part A. Mol. Spectrosc.* **22**:383.

ERYING, G., and R. MATHIES. 1979. Resonance Raman studies of bathorhodopsin: evidence for a protonated Schiff base linkage. *Proc. Natl. Acad. Sci. U.S.A.* **76**:33.

FAVROT, J., J. M. LECLERCQ, R. ROBERGE, C. SANDORFY, and D. VOCELLE. 1979. Intermolecular interactions in visual pigments: the hydrogen bond in vision. *Photochem. Photobiol.* **29**:99.

GAVIN, R. M., and S. A. RICE. 1971. Correlation of pi-electron density and vibrational frequencies of linear polyenes. *J. Chem. Phys.* **55**:2675.

HAROSI, F. I., J. FAVROT, J. M. LECLERCQ, D. VOCELLE, and C. SANDORFY. 1978. Photochemistry of visual pigments: an interpretation of spectral changes in terms of molecular associations and isomerization. *Rev. Can. Biol.* **37**:257.

HEYDE, M. E., D. GILL, R. G. KILPONEN, and L. RINAI. 1971. Raman spectra of Schiff bases of retinal (models of visual photoreceptors). *J. Am. Chem. Soc.* **93**:6776.

HONIG, B., and T. G. EBREY. 1974. The structure and spectra of the chromophore of the visual pigments. *Ann. Rev. Biophys. Bioeng.* **3**:151.

HONIG, B., A. D. GREENBERG, U. DINUR, and T. G. EBREY. 1976. Visual pigment spectra: implications of the retinal Schiff base. *Biochemistry.* **15**:4593.

HONIG, B., T. EBREY, R. H. CALLENDER, U. DINUR, and M. OTTOLENGHI. 1979*a*. Photoisomerization and charge separation. A model for light energy transduction in visual pigments and bacteriorhodopsin. *Proc. Natl. Acad. Sci U.S.A.* **76**:2503.

HONIG, B., U. DINUR, K. NAKANISHI, V. BALOGH-NAIR, M. A. GAWINOWICZ, N. ARNABOLDI, and M. MOTTO. 1979*b*. An external point charge model for wavelength regulation in visual pigments. *J. Am. Chem. Soc.* **101**:7084.

HUBBARD, R., and A. KROPF. 1958. The action of light on rhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **44**:130.

INAGAKI, F., M. TASUMI, and T. MIYAZAWA. 1975. Vibrational analysis of polyene chains. *J. Raman Spectrosc.* **3**:335.

MARCUS, M. A., A. T. LEMLEY, and A. LEWIS. 1979. Implication of modelling the chromophore of rhodopsin and bacteriorhodopsin with resonance raman spectra of retinal schiff bases. *J. Raman Spectrosc. 8:22.*

MATHIES, R., T. B. FREEDMAN, and L. STRYER. 1977. Resonance Raman studies of the conformation of retinal in rhodopsin and isorhodopsin. *J. Mol. Biol.* **109**:367.

MATHIES, R., A. R. OSEROFF, and L. STRYER. 1976. Rapid-flow resonance Raman spectroscopy of photolabile molecules: rhodopsin and isorhodopsin. *Proc. Natl. Acad. Aci. U.S.A.* **73**:1.

OSEROFF, A. R., and R. H. CALLENDER. 1974. Resonance Raman spectroscopy of rhodopsin in retinal disk membranes. *Biochemistry.* **13**:4243.

PETERS, K., M. L. APPLEBURY, and P. M. RENTZEPIS. 1977. Primary photochemical event in vision: proton translocation. *Proc Natl. Acad. Sci. U.S.A.* **74**:3119.

RABOLT, J. F., W. H. MOORE, and S. KRIMM. 1977. Vibrational analysis of peptides, polypeptides, and proteins. 3. α-poly (L-alanine). *Macromolecules.* **10**:1065.

ROSENFELD, T., B. HONIG, M. OTTOLENGHI, and T. G. EBREY. 1977. *Cis-trans* isomerization in the photochemistry of vision. *Pure Appl. Chem.* **49**:341.

SHRIVER, J., G. MATEESCU, G., R. FAGER, D. TORCHIA, and E. W. ABRAHAMSON. 1977. Unprotonated chromophore-protein bond in visual pigments from 13C-NMR spectra. *Nature (Lond.)* **270**:271.

SULKES M., A. LEWIS, and M. A. MARCUS. 1978. Resonance Raman spectroscopy of squid and bovine visual pigments: the primary photochemistry in visual transduction. *Biochemistry.* **17**:4712.

VAN DER MEER, K., J. J. C. MULDER, and J. LUGTENBERG. 1976. A new facet in rhodopsin photochemistry. *Photochem. Photobiol.* **24**:363.

YOSHIZAWA, T., and G. WALD. 1963. Prelumirhodopsin and the bleaching of visual pigments. *Nature (Lond.).* **197**:1279.