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Chemistry

Rapid-flow resonance Raman spectroscopy of photolabile molecules: Rhodopsin and isorhodopsin

(visual excitation/conformation of retinals/transient kinetics/retinal disc membranes)

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ABSTRACT **We have devised a method for obtaining the resonance Raman spectrum of a photolabile molecule before it is modified by light. The essence of this technique is that the sample is flowed through the light beam at a sufficiently high velocity so that the fraction of photoisomerized (or photodestroyed) molecules in the illuminated volume is very low. This rapid-flow technique has enabled us to measure the resonance Raman spectrum of unphotolyzed bovine rhodopsin in Ammonyx LO detergent solution and in sonicated retinal disc membranes. The major features of these spectra, which are very similar to one another, are the protonated Schiff base line near 1660 cm^{-1}, the ethylenic line at 1545 cm^{-1}, lines due to skeletal modes at 1216, 1240, and 1270 cm^{-1}, and a line due to C-H bending at 971 cm^{-1}. The resonance Raman spectrum of unphotolyzed isorhodopsin formed by the addition of 9-*cis*-retinal to opsin was also measured. The spectrum of isorhodopsin is more complex and differs markedly from that of rhodopsin. In isorhodopsin, the ethylenic line is shifted to 1550 cm^{-1}, and there are six lines between 1153 and 1318 cm^{-1}. The rapid-flow technique described here makes it feasible to control the extent of interaction between light and any photolabile molecule. We present a theory for predicting the effective sample composition in the illuminated volume as a function of the flow rate, light intensity, and spectral characteristics of the photolabile species.**

Resonance Raman spectroscopy takes advantage of the selective enhancement in Raman scattering from vibrations coupled to an electronic transition when the excitation wavelength is within or near the corresponding absorption band (1, 2). This resonance effect makes it possible to specifically monitor the structure of chromophoric sites in macromolecules. Resonance Raman spectroscopy has recently been used to investigate colored metalloproteins (2–5), bacteriorhodopsin (6, 7), rhodopsin (8, 9), and enzymes with extrinsic chromophores (10, 11).

Resonance Raman spectroscopy is a promising technique for elucidating the conformational changes in retinal during the initial stages of visual excitation (8, 9). There is a problem, however, in obtaining the spectrum of a photosensitive molecule such as rhodopsin. Raman scattering is a very weak process (1). Even with resonance enhancement, the probability that a molecule absorbs a photon is orders of magnitude greater than the probability that it scatters a photon by the resonance Raman process. Consider a solution of rhodopsin that is illuminated by a 25 mW beam of 600 nm light focused to a diameter of 40 μm.The rate constant for bleaching rhodopsin is then 7.2×10^3 sec^{-1} (see *Theory*), so that nearly all the rhodopsin in the beam is isomerized within 1 msec. Since a Raman spectrum is typically recorded over a period of many minutes (7, 9), it is clearly not feasible to obtain the resonance Raman spectrum of *unphotolyzed* rhodopsin under these conditions.

We present here a new technique to obtain the resonance Raman spectrum of a photolabile molecule before it is altered by light. To appreciate this approach, it is essential to recognize that resonance Raman scattering does not alter the electronic state of the molecule or cause photoisomerization (1, 2). This makes it possible to devise experimental conditions that minimize the concentration of photoisomerized molecules in the illuminated volume while permitting the detection of Raman scattering. The essence of our experimental technique is that *the sample is rapidly flowed through the light beam so that the transit time of the sample through the illuminated volume is short compared to the photoisomerization time.* For rhodopsin, the transit time must be on the order of 10 μsec. This was achieved by forming a jet stream of rhodopsin with a velocity of about 400 cm/sec and flowing it past a laser beam with a diameter of 40 μm. We present here a theory for this rapid-flow technique and report the resonance Raman spectra of unmodified rhodopsin and of unmodified isorhodopsin.

THEORY

The rate of photoisomerization $k(sec^{-1})$ of a photolabile molecule such as rhodopsin is given by

$$k = I(\lambda)\sigma_A(\lambda)\varphi(\lambda) \quad [1]$$

where $I(\lambda)$ is the light flux (photons cm^{-2} sec^{-1}), σ_A (λ) is the absorption cross-section (cm^2 $molecule^{-1}$), and $\varphi(\lambda)$ is the quantum yield of photoisomerization. For a typical experiment on rhodopsin with a laser power of 25 mW at 600 nm and a beam diameter of 40 μm ($I = 6.01 \times 10^{21}$ photons cm^{-2} sec^{-1}, $\epsilon = 470$ cm^{-1} M^{-1}, and $\varphi = 0.67$), the rate constant for photoisomerization is 7.2×10^3 sec^{-1}. The photoisomerization time τ_i(sec), which is the average time a rhodopsin molecule spends in the beam before it is isomerized, is $1/k$. If we assume that the stream has a uniform velocity v (cm/sec) and the laser beam a uniform intensity inside a square cross-section of length l(cm), then the transit time τ_t (sec) of the molecule in the beam is l/v. The fraction F of rhodopsin that is isomerized while traversing the beam is then

$$F = 1 - e^{-\tau_t/\tau_i} \quad [2]$$

When the extent of isomerization is small ($F \ll 1$), this expression can be expanded to give

$$F = \tau_t/\tau_i = (I\sigma_A\varphi l)/v \quad [3]$$

It is convenient to express F in terms of the laser power P (photons sec^{-1}), which is equal to $I\,l^2$. Also, we convert from σ_A (cm^2 $molecule^{-1}$) to the decadic extinction coefficient ϵ (cm^{-1} M^{-1}), which are related by $\sigma_A = 3.824 \times 10^{-21}$ ϵ.

Then,

$$F = (P\epsilon\varphi 3.824 \times 10^{-21})/lv \quad [4]$$

This expression shows that F, *the photoalteration parameter*, can be made suitably small (say <0.05) by having a sufficiently rapid flow.

We will now turn to a more general and exact derivation for the extent of photoalteration in these experiments. This is necessary because the actual profile of the light beam is a Gaussian function. As a result, the effective sample composition in the illuminated volume is a more complex function than implied by Eq. [2]. The laser beam profile is given by

$$I(x,y) = I_0 \exp[-2(x^2 + y^2)/\omega^2] \quad [5]$$

where I_0 is the peak intensity and ω is the radius of the beam where its intensity is I_0/e^2. The beam power is then $\pi\omega^2 I_0/2$. The coordinate system and experimental geometry are given in Fig. 1. The velocity in the stream was previously assumed to be uniform. However, the velocity profile in a jet stream is not uniform and must lie between the limiting cases of bulk and parabolic flow. We have calculated the effect of a parabolic flow profile on F and have found that it gives less than a 5% change in our experiment. Therefore, it is justified to assume that the flow velocity is independent of the z-axis. When the beam is focused by a long focal length lens to a radius of 20 μm, the radius varies by less than 2% over the width of the jet stream. If the absorbance and scattering of the solution are low, then the laser beam intensity is also independent of the z-axis.

The rate of detected counts S (counts/sec) for a particular Raman line is given by

$$S = \int_V I(r)C(r)\sigma_R D dr \quad [6]$$

where C is the concentration (molecules/cm^3), σ_R is the Raman scattering cross-section (cm^2 molecule^{-1}), and D is the overall detection efficiency. This integral is taken over the illuminated volume. Since $I(r)$ and $C(r)$ are independent of z, this dimension may be integrated out. Also, the form of $I(x,y)$ permits the extension of the limits in the other two dimensions to give

$$S = 2a\sigma_R D \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} I(x,y)C(x,y)dxdy \quad [7]$$

where a is the radius of the jet stream. Only $C(x,y)$ is still needed to complete this expression. We derive an expression for $C(x,y)$ by noting that when the flowing sample is illuminated, a steady state is established such that the concentration of the photolabile species in any differential part of the illuminated volume is constant. This means that the rate of change in concentration due to photoisomerization, which is given by Eq. [1], is balanced by the differential flow of unisomerized molecules into that volume. This steady-state condition is expressed by

$$k(x,y)C(x,y) = -\frac{\partial C(x,y)}{\partial x}v \quad [8]$$

where k is space-dependent because the light intensity is not uniform. Integration of Eq. [8] gives

$$C(x,y) = C_0 \exp\left[-\frac{2F}{\sqrt{\pi}\omega}\int_{-\infty}^{x} e^{-2[x'^2 + y^2]/\omega^2}dx'\right] \quad [9]$$

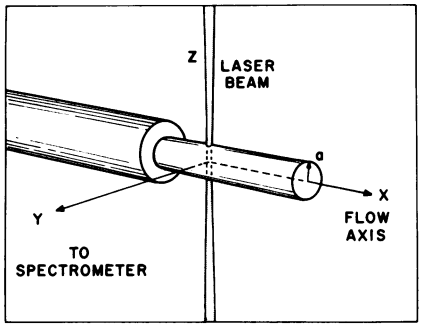

FIG. 1. Geometry of the jet-stream sampling system. The flow axis x, the detection axis y, and the laser beam axis z are perpendicular to each other.

where F, the photoalteration parameter, is equal to

$$F = (P\epsilon\varphi 3.824 \times 10^{-21})/(\sqrt{\pi}\omega v) \quad [10]$$

Note that this is the same as Eq. [4] except that l is replaced by $\sqrt{\pi}\,\omega$, which is the equivalent length of the Gaussian laser beam. Eq. [9] may now be combined with Eq. [7] to give $S(F)$, the scattering rate as a function of F. However, it is more useful to consider S', the *normalized Raman scattering intensity*, which is defined as

$$S' = S(F)/S(0) \quad [11]$$

where $S(0)$ is the scattering that would be observed if the molecule were not photolabile. Upon substituting $s = \sqrt{2}x/\omega$ and $t = \sqrt{2}y/\omega$, the expression for S' becomes

$$S' = \frac{1}{\pi}\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}[\exp - (s^2 + t^2)] \exp - \left\{\sqrt{\frac{2}{\pi}}F\int_{-\infty}^{s}\exp - (s'^2 + t^2)ds'\right\}dsdt \quad [12]$$

Note that this equation for S' depends only on F. *Eq.* [12] *serves as a criterion of the feasibility of obtaining a Raman spectrum of any photolabile molecule.* A plot of S' as a function of F is shown in Fig. 2.

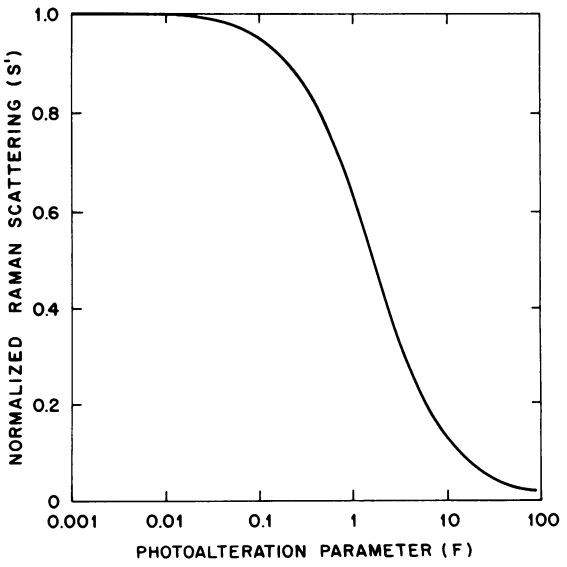

FIG. 2. The normalized Raman scattering intensity S' is plotted as a function of the photoalteration parameter F, as calculated by numerical integration of Eq. [12].

When the extent of photoisomerization is low ($F \ll 1$), Eq. [12] can be simplified by expanding the exponential and evaluating the resulting integrals to give

$$S' = 1 - F/2 \quad (\text{for } F \ll 1) \qquad [13]$$

When $F \ll 1$, F is simply the fraction of molecules isomerized in one transit through the beam. At a higher extent of photoisomerization, F can be interpreted as the number of absorptions that an equivalent nonphotolabile molecule would experience while passing through the beam. Note that when F is high, the normalized Raman scattering does not drop as rapidly as might be expected from the composition of the sample after it has traversed the beam.

It is important to experimentally ascertain the value of F since it is the key parameter in the rapid-flow studies. When the extent of photolysis is low, F can be determined by measuring B, the fraction of the sample in the *entire* jet stream that is bleached. Since the effective length l of a Gaussian laser beam is $\sqrt{\pi}\omega$, the fraction of molecules in the jet stream that passes through the laser beam is $2\omega/(\sqrt{\pi}a)$, which gives

$$F = B(\sqrt{\pi}a)/(2\omega) \qquad [14]$$

MATERIALS AND METHODS

Raman Spectrometer and Flow System. Raman spectra were obtained with a Spex 1401 double monochromator and a model 1419 sample illuminator. A cooled RCA 31034 photomultiplier, operated in the photon-counting mode, was interfaced to a Hewlett-Packard 2114B computer through an SSR 1105 photon counting system. The 600 nm laser beam was produced by pumping a Spectra-Physics jet stream dye laser (model 375) with an argon ion laser (model 171). The beam was passed through an interference filter and focused on the jet stream with a 150 mm focal length lens. The monochromator was operated with a slit width of 6.6 cm^{-1}. The spectra were digitized in 2 cm^{-1} increments with a nominal scan rate of 1 cm^{-1}/sec. All spectra are an average of four to six scans that have been smoothed with a three point sliding average. The background has been removed by subtracting the average of an equivalent number of scans taken after the sample was bleached. All spectra have been calibrated with the 983 cm^{-1} $SO_4^=$ line and are accurate to ± 2 cm^{-1}.

The jet stream sampling system was selected instead of existing techniques for minimizing sample decomposition (12, 13) because of our requirements for high-flow velocities and well-defined sample recirculation. The jet stream was formed by inserting a small variable speed pump (Micro-Pump model 12-41-303-758) into a recirculating system with a 200 ml reservoir cooled in an ice bath. The solution was forced out of a Teflon syringe needle having an internal diameter of 0.44 mm and the laser beam was focused on the free jet stream about 1 mm from the tip of the nozzle. The mean velocity was determined by measuring the bulk flow rate, which was about 1 ml/sec. The observed temperature of the sample in the jet stream was about 12°C. We calculate that the temperature of the illuminated sample cannot increase by more than 0.01°C during its passage through the laser beam.

An important parameter in these rapid flow studies is ω, the beam radius at which the intensity is I_0/e^2. This quantity was determined by translating a magnified image (8:1) of the focused beam across the 10 μm entrance slits of the monochromator while monitoring the intensity of the 983 cm^{-1} $SO_4^=$ line from a sample. The beam radius determined in this way was 20 ± 2 μm after deconvoluting for the slit size.

Sample Preparation. Rod outer segments were isolated from bovine retinas (Hormel, Austin, Minn.), and sonicated disc membranes were prepared by the procedures of Waggoner and Stryer (14). Rhodopsin was purified by chromatography on hydroxylapatite by the method of Applebury *et al.* (15), except that a two-step gradient was used instead of a continuous phosphate gradient (M. Yeager, to be published). Isorhodopsin was prepared by bleaching rod outer segments in 67 mM sodium phosphate at pH 6.5, 1 mM 2-mercaptoethanol, and 10 mM hydroxylamine. The pellet was washed twice with this buffer minus the hydroxylamine. Isorhodopsin was then formed by the addition of a 3-fold excess of 9-*cis*-retinal (Sigma). Isorhodopsin was purified in the same way as rhodopsin. Ammonyx LO was a gift of the Onyx Chemical Co. (Jersey City, N.J.).

Absorption spectra of the solutions of rhodopsin and of isorhodopsin were taken before and after each flow experiment to determine the extent of bleaching caused by the laser beam. The fraction of the entire stream that was bleached per pass, B, was calculated from the observed reduction in the absorbance at 500 nm, the bulk flow rate, and the duration of the Raman scan. This experimentally determined value of B could then be compared with the one predicted by Eq. [14] on the basis of the calculated value of F. In addition, a separate experiment was performed to measure B as a function of F for Ammonyx-solubilized rhodopsin flowed once through the laser beam. These experimental values of B agreed within experimental error with those calculated from the predicted values of F where F ranged from 0.05 to 0.7.

RESULTS AND DISCUSSION

Evaluation of extent of photoisomerization in the scattering volume

Using Eqs. [10] and [13], we calculated that it is feasible to obtain the resonance Raman spectrum of essentially unmodified rhodopsin. For a laser power of 25 mW at 600 nm ($P = 7.54 \times 10^{16}$ photons/sec), $\omega = 20$ μm, $v = 350$ cm/sec, $\epsilon = 470$ cm^{-1} M^{-1}, and $\varphi = 0.67$, the calculated photoalteration parameter F is 0.07, and the normalized Raman scattering intensity is 0.96. This means that nearly all of the Raman scattering under these experimental conditions should come from unmodified rhodopsin. This prediction was tested by measuring the extent of bleaching of the entire sample at the end of the flow experiment. The observed value of B for rhodopsin in Ammonyx LO detergent solution was 0.005 ± 0.002, which agrees well with the value of 0.007 ± 0.003 calculated from Eq. [14] for $F = 0.07$ and $a = 0.22$ mm. For isorhodopsin in Ammonyx LO detergent solution, there also was good agreement between the observed and predicted values of B, which were 0.0012 ± 0.0006 and 0.0015 ± 0.0006, respectively (for experimental conditions, see Fig. 4). We conclude that the resonance Raman spectra shown below are in fact those of essentially unmodified rhodopsin (or isorhodopsin).

Resonance Raman spectra of rhodopsin

The jet-stream resonance Raman spectra of rhodopsin in Ammonyx LO detergent solution and in sonicated disc

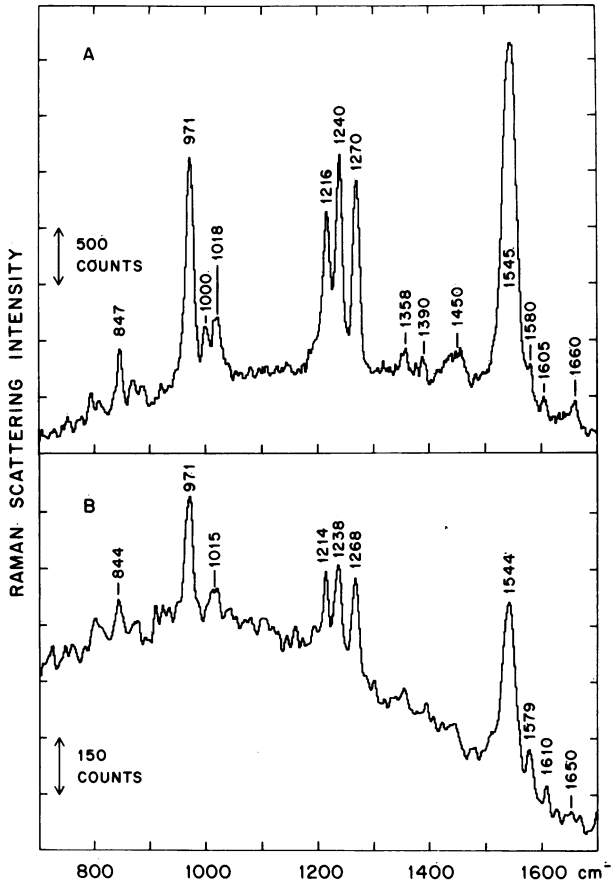

FIG. 3. (A) Resonance Raman spectrum of purified rhodopsin (1.8×10^{-5} M) in 60 mM sodium phosphate at pH 7.0, 0.4% Ammonyx LO, 1 mM dithiothreitol, and 10 mM hydroxylamine. The laser power at 600 nm was 25 mW and the flow velocity was 350 cm/sec. The calculated value of F was 0.07, which predicts that $B = 0.007 \pm 0.003$. The observed value of B was 0.005 ± 0.002. (B) Resonance Raman spectrum of sonicated retinal disc membranes (0.9×10^{-5} M rhodopsin) in 50 mM sodium phosphate at pH 6.5, 1 mM dithiothreitol, and 10 mM hydroxylamine. The laser power at 600 nm was 20 mW and the flow velocity was 660 cm/sec. The calculated value of F was 0.03.

membranes are shown in Fig. 3. The spectra are nearly identical apart from the lower signal-to-noise ratio of the membrane spectrum, which masks the weaker bands. It is evident that the Ammonyx LO detergent does not appreciably alter the conformation of the bound retinal in rhodopsin, though it markedly increases the rates of some of the dark steps after photoisomerization (15). The studies of Rimai *et al.* (16) and of Warshel and Karplus (17) provide a basis for tentatively assigning the major bands in these spectra. *The line at 1660 cm^{-1} strongly suggests that 11-cis-retinal is joined to opsin by a protonated Schiff base, as proposed on the basis of some previous resonance Raman studies (8, 9).* The ethylenic C=C stretching vibrations gives the intense band at 1545 cm^{-1}. The three lines at 1216, 1240, and 1270 cm^{-1} are probably due to C—C stretching and C—C—H bending vibrations. The lines at 1000 and 1018 cm^{-1} come from C—CH_3 stretching vibrations. The 971 cm^{-1} line is mostly due to C—H bending (16); it may also contain a contribution from C—CH_3 stretching (17).

The jet stream spectra of rhodopsin shown in Fig. 3 bear little resemblance to the spectrum reported by Lewis, Fager, and Abrahamson (8) for a digitonin solution of bovine rhodopsin excited with a 30 mW beam at 582.5 nm. Since their

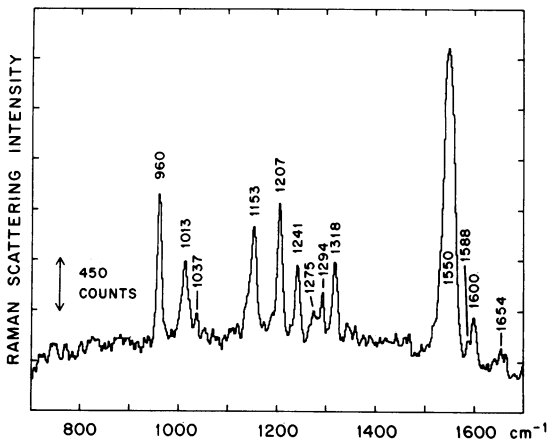

FIG. 4. Resonance Raman spectrum of purified isorhodopsin (1.3×10^{-5} M) in 43 mM sodium phosphate at pH 7.0, 0.3% Ammonyx LO, 1 mM dithiothreitol, and 10 mM hydroxylamine. The laser power at 600 nm was 61 mW and the flow velocity was 660 cm/sec. The calculated value of F was 0.015, which predicts that $B = 0.0015 \pm 0.0006$. The observed value of B was 0.0012 ± 0.0006.

sample was stationary, it seems likely that most of the rhodopsin molecules in their scattering volume were modified by light.

Resonance Raman spectrum of isorhodopsin

The jet-stream resonance Raman spectrum of isorhodopsin (Fig. 4) is considerably more complex than that of rhodopsin. The C—C stretching and C—C—H bending region contains four prominent lines at 1153, 1207, 1241, and 1318 cm^{-1}, and two weaker ones at about 1275 and 1294 cm^{-1}. The C—H bending vibration appears at 960 cm^{-1}, and there is a broad C—CH_3 stretching band at 1013 cm^{-1}. The ethylenic line is at 1550 cm^{-1}, and a line characteristic of a protonated Schiff base is at 1654 cm^{-1}. In comparing Figs. 3 and 4, *it is evident that the spectra of rhodopsin and isorhodopsin are strikingly different, showing that resonance Raman spectroscopy is a powerful technique for exploring in detail the conformation of retinal in rhodopsin and its photolytic intermediates.*

The jet-stream spectrum of isorhodopsin (Fig. 4) is nearly identical to the one reported by Oseroff and Callender (9) for isorhodopsin produced by illuminating retinal disc membranes at 77 K. The identity of these spectra is significant for two reasons. First, it demonstrates that *isorhodopsin produced by two isomerizations—that is, by the pathway of rhodopsin → prelumirhodopsin → isorhodopsin—is the same species as isorhodopsin formed by the addition of 9-cis-retinal to opsin.* Second, it shows that low temperature (77 K) or the presence of Ammonyx LO has little effect on the conformation of retinal in isorhodopsin.

PROSPECTS

The rapid-flow technique described here makes it feasible to control the extent of interaction between light and *any* photolabile molecule. We have used this technique to obtain the first resonance Raman spectra of unmodified rhodopsin, and also of unmodified isorhodopsin at 12°C. The striking differences between the spectra of rhodopsin and isorhodopsin show that these spectra are rich in information about the precise structure of the retinal chromophore. Rapid-flow resonance Raman spectroscopy is likely to be a choice technique for elucidating in atomic detail the sequence of con-

formational changes in retinal after the absorption of a photon. To achieve this goal, it will be necessary to obtain the resonance Raman spectrum of each photolytic intermediate. The spectrum of prelumirhodopsin can now be calculated from the published spectrum (9) of a photostationary steady state of rhodopsin, isorhodopsin, and prelumirhodopsin. The resonance Raman spectra of lumirhodopsin, metarhodopsin I, and metarhodopsin II should be observable by our rapid-flow technique. Then, the lines in the spectra of rhodopsin and these photolytic intermediates must be assigned. Previous experimental work (16) and recent rapid flow studies (A. Doukas, R. Callender, T. Yudd, R. Crouch, and K. Nakanishi, to be published) on various isomers of retinal and their Schiff bases are important in this regard, as are theoretical studies (17). Spectra of isotopically substituted retinals and of retinal analogs can also be expected to facilitate the interpretation of the spectra of rhodopsin and its photolytic intermediates (18, 19).

The idea of controlling the degree of photoisomerization by moving the sample evolved through discussions between A.R.O. and Dr. Robert Callender at City College of New York. We are indebted to Mr. Mark Yeager for the rhodopsin purification procedure and to Mr. Jerry Johnson for imaginative technical assistance in constructing the Raman spectrometer. This work was supported by grants from the National Eye Institute (EY-01070) and the National Institute of General Medical Sciences (GM-16708). R.M. is a Helen Hay Whitney Fellow.

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