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Interpretation of the Resonance Raman Spectrum of Bathorhodopsin Based on Visual Pigment Analogues†

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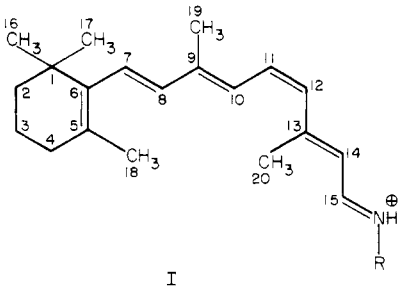
ABSTRACT: Resonance Raman spectra of visual pigment analogues have been used to evaluate various models for the structure of the retinal chromophore in bathorhodopsin. Deuteration or removal of the 18-methyl on the β-ionyl ring or of the 19-methyl on the polyene chain demonstrates that the three intense low wavenumber bands of bathorhodopsin at 853, 875, and 920 cm^{-1} are not due to exomethylene or ring modes. Rather, assignment of these lines to out-of-plane vinyl hydrogen motions on the chain best accounts for the experimental data. Our calculations show that the intensity of these vibrations can be explained by twists of 10–30° about chain single bonds. The 1100–1400-cm^{-1} Raman fingerprint indicates that the configuration of the double bonds is trans. This suggests that the structure of the bathorhodopsin chromophore is twisted all-trans.

Black and white vision in vertebrates is triggered by light-induced changes in the rod cell photoreceptor pigment, rhodopsin. This pigment consists of an 11-*cis*-retinal chromophore (I) bound covalently to a lysine residue of the apo-



I

protein opsin by a protonated Schiff base (PSB) linkage.[1] Absorption of a photon by the 11-cis chromophore raises the pigment to a high free energy form which decays thermally via a series of distinct, metastable intermediates to a bleached mixture of *all-trans*-retinal and opsin as depicted in Figure 1 (Yoshizawa & Wald, 1963; Hubbard & Kropf, 1958). The chromophore binding site of opsin can also accommodate the 9-cis isomer of retinal to form isorhodopsin, which bleaches by the same pathway. Thus, the net effect of light absorption on the visual pigment chromophores is to isomerize them from an 11-cis or 9-cis configuration to an all-trans configuration.[2] It is not known precisely when in the bleaching process this isomerization is first achieved, although resonance Raman (RR) evidence indicates that by the time metarhodopsin I is formed the chromophore configuration is relaxed all-trans (Doukas et al., 1978a).

The fact that bathorhodopsin is the common photoproduct of both 11-*cis*-rhodopsin and 9-*cis*-isorhodopsin, together with the observed thermal decay of bathorhodopsin to *all-trans*-retinal and opsin, led naturally to the proposal that bathorhodopsin's chromophore configuration is distorted trans (Yoshizawa & Wald, 1963). Recent workers have coined the term "transoid" (Honig et al., 1979) to describe this trans configuration with an unspecified conformation. However, other possible batho structures involving out-of-plane deformations of chain carbons may be accessible to both 11-cis and 9-cis chromophores (Warshel & Deakyne, 1978; Lewis, 1978). Given the paucity of experimental data, a detailed evaluation of even these simplified models has not yet been possible.

Considerable evidence indicates that the formation of bathorhodopsin is, in fact, quite complex. The observed fast rise time of bathorhodopsin (~6 ps; Busch et al., 1972) seems intuitively to be too rapid for the movement of bulky groups involved in isomerization, although this assumption has been challenged (Warshel, 1976; Weiss & Warshel, 1979; Birge & Hubbard, 1980). Furthermore, picosecond absorption studies on rhodopsin equilibrated with D_2O at low temperature have demonstrated a kinetic isotope effect on the formation time of bathorhodopsin (Peters et al., 1977). This indicates that the primary photochemical event involves proton translocation. Reported incorporation of modest but persistent amounts of deuterium into the retinal chromophore of rhodopsin bleached in D_2O tends to support this view (Fransen

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[1] Abbreviations used: RR, resonance Raman; PSB, protonated Schiff base; 18-CD_3, 18-trideuterioretinal; 19-CD_3, 19-trideuterioretinal; HOOP, hydrogen out-of-plane.

[2] We define *configuration* to indicate the cis or trans structure of the double bonds and use *conformation* to refer to the set of rotation angles about single bonds in a given configuration.

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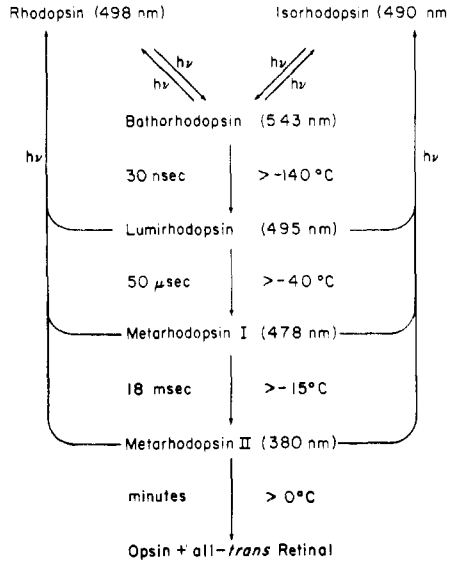


FIGURE 1: Bleaching sequence for rhodopsin (11-*cis*-retinal and opsin) and isorhodopsin (9-*cis*-retinal and opsin). The absorption maxima, characteristic decay times, and temperatures at which transitions are blocked are indicated.

et al., 1976). However, care must be taken in constructing a model for "the primary photochemical event" from these data. There is no reason why the actual potential surface trajectory followed in bathorhodopsin formation cannot involve configurational and conformational changes in the chromophore and protein, as well as changes in proton coordinates.

Early characterization of the visual pigments and their discrete photolysis intermediates was done almost exclusively by UV–visible absorption spectroscopy, a technique which has provided little *detailed* information about the configurations of the chromophores in these molecules. Although the stable pigments could be denatured and their chromophores extracted, this yielded only a knowledge of the configurations to which these retinals would relax in the extraction process (Hubbard & Kropf, 1958). The only technique which has provided abundant information on the chromophore configuration in situ has been RR spectroscopy (Mathies, 1979; Callender & Honig, 1977; Sulkes et al., 1978; Terner et al., 1979). The success of this method in the past has depended upon the existence of a good correlation between the 1100–1400-cm^{-1} fingerprint regions of the Raman spectra of the pigments and those of particular geometrical isomers of retinal PSB's in solution. This comparison has shown that rhodopsin, isorhodopsin (Mathies et al., 1977), and the metarhodopsins (Doukas et al., 1978a) contain chromophores with substantially the same structures as the 11-*cis*-, 9-*cis*-, and *all-trans*-retinal PSB's, respectively. Thus, there appears to be no large distortion associated with the incorporation of retinal into the binding sites of these pigments. However, the assignment of the chromophore configuration in bathorhodopsin by the fingerprint comparison method has been blocked pending an experimentally verified explanation of three anomalous lines which appear at 853, 875, and 920 cm^{-1} in the RR spectrum (Oseroff & Callender, 1974). No lines of comparable intensity are observed in this region of the spectra of rhodopsin, isorhodopsin, or the metarhodopsins, nor do they appear in the Raman spectra of the free chromophores (Mathies, 1979).

A number of models have been advanced to account for these low wavenumber bathorhodopsin vibrations. One proposal attributes these bands to vibrations of an exomethylene group at the C_5 position in a retroretinal structure (van der Meer et al., 1976). While this exomethylene hypothesis provides a natural explanation of the observed kinetic isotope effect on the rise time of bathorhodopsin, it has been seriously questioned by previous absorption (Kropf, 1976) and RR (Eyring & Mathies, 1979; Aton et al., 1980) experiments. Another suggested model explains the low wavenumber modes of bathorhodopsin as out-of-plane vibrations about weakened 9 and 11 double bonds (Lewis, 1978). Theoretical calculations (Warshel, 1977) have indicated that distortions of the ionyl ring or hydrogen out-of-plane (HOOP) motions of a strained all-trans polyene chain may also account for these lines. Evidently, the assignment of these low wavenumber bands will provide detailed conformational information about the bathorhodopsin chromophore.

Traditionally, the vibrational spectrum of a molecule is assigned with the help of isotopically substituted derivatives. A close correlation between the observed frequency shifts and intensity changes in the derivative spectra and those calculated for the normal modes provides a necessary check on the initial assignment. This method is ideal for the investigation of the low wavenumber lines of bathorhodopsin since it introduces no ambiguities due to changes in the chromophore's electronic structure or protein–chromophore interaction. Furthermore, with such an isomorphous modification, the photostationary steady-state compositions and resonance enhancement factors of the analogue pigments are identical with those of the native pigments. These advantages would not necessarily accrue, for example, in RR studies of chemically modified pigments.

Recent workers have assigned many of the Raman bands of retinal on the basis of chemical modifications (Cookingham et al., 1978). These studies have also been extended to encompass isotopically (Lewis et al., 1978) and chemically modified retinal chromophores incorporated into the purple membrane protein bacteriorhodopsin (Marcus et al., 1977). Limited RR studies of this type have also been used to assign the Schiff base vibrations of rhodopsin, isorhodopsin (Oseroff & Callender, 1974), and bathorhodopsin (Eyring & Mathies, 1979). We have previously obtained the RR spectrum of bathorhodopsin (Eyring & Mathies, 1979) by using a dual-beam pump-probe technique (Oseroff & Callender, 1974). In order to interpret our bathorhodopsin data, we present here the RR spectra of isomorphous visual pigment analogues regenerated with isotopically modified retinals. Our 18- and 19-trideuterio analogue Raman studies demonstrate that chain vinyl HOOP motions enhanced by a twisted conformation about chain single bonds can best account for bathorhodopsin's low wavenumber lines, while the similarity of the fingerprint region of the Raman spectrum to that of the all-trans PSB of retinal indicates that the configuration of the double bonds in bathorhodopsin is all-trans. This is the first experimental study to address the important question of bathorhodopsin's configuration and conformation in such detail.

Materials and Methods

Deuterated retinals were prepared by first synthesizing *trans*-β-ionone deuterated on the 5-methyl group (J. Lugtenburg, M. R. Fransen, and I. Palings, unpublished work) or on the 9-methyl group (Johansen & Liaaen-Jensen, 1974). Isomeric vitamin A acetate mixtures were prepared according to Pommer's method (Pommer, 1977). After conversion by $LiAlH_4$ reduction and MnO_2 oxidation to the retinals, the pure isomers were isolated by preparative high-performance liquid chromatography (LC) with a Porasil column (hexane–ether, 9:1). Liquid chromatographic analysis of the isolated isomers showed only one peak with the appropriate R_f value. The isomeric purity was 98% or better. The isomers showed the expected electronic absorption spectra. The ^1H NMR spec-

trum of 18-trideuterio-11-*cis*-retinal was identical with that of unmodified 11-*cis*-retinal except that no peak at δ 1.63, characteristic of the 5-methyl group, was observed. Similarly, peaks at δ 1.94 and 1.96 were eliminated in spectra of the 19-trideuterio-11-cis and 9-cis isomers, respectively. Mass spectral analysis showed that the deuterium incorporation was 98% or better.

Frozen bovine retinas were purchased from American Stores (Lincoln, NE), and rod outer segments were isolated by a sucrose flotation method (Hong & Hubbell, 1973). Analogue pigments were regenerated with the appropriate retinals after bleaching the rhodopsin in 100 mM phosphate buffer (pH 7) containing 10 mM 2-mercaptoethanol and 10 mM hydroxylamine. Demethylretinals were obtained by $LiAlH_4$ reduction of the corresponding demethylretinoic acids followed by MnO_2 oxidation. A mixture of *cis*-retinals obtained by isomerization in methanol was separated by thin-layer chromatography and added directly to opsin. LC analysis of the 5-demethyl mixture showed that the predominant isomer was 13-cis, with 20% 9-cis and 11-cis present. The 9-demethyl pigment regenerated rapidly with ~63% yield based on opsin. The observed absorption maximum (464 nm) was strongly blue shifted from that of the native pigment (Blatz et al., 1969; Kropf et al., 1973). The 5-demethyl pigment (λ_{max} = 485 nm) regenerated slowly (10% in 8 h) at room temperature (Kropf, 1976). In order to control for the possibility that enzymatic isomerization of unmodified *trans*-retinal present in the membrane suspension might permit autoregeneration of native pigment, we subjected a parallel aliquot of membranes to the identical procedure except that no exogenous demethylretinal was added. No regeneration of opsin was observed, indicating that all regenerated pigment contained a demethyl chromophore.

The regenerated pigments were solubilized in Ammonyx-LO (Onyx Chemical Co., Jersey City, NJ) and purified by hydroxylapatite chromatography (Applebury et al., 1974). Room temperature rapid-flow RR spectra were obtained with 25 mL of sample (10 mM hydroxylamine and 1.5% Ammonyx-LO) having an absorbance of 0.8/cm at 500 nm. These spectra were excited with the 488-nm line of a Spectra-Physics 165 argon laser and detected with a dry ice cooled intensified vidicon detector (PAR 1205A and 1205D) coupled to a mismatched subtractive dispersion double monochromator (Mathies & Yu, 1978). The laser power (200 μW), flow rate (400 cm/s), and beam waist (10 μm) were chosen to make the effects of photolysis on the spectra negligible (photoalteration parameter $F < 0.1$) (Mathies et al., 1976; Callender et al., 1976). The monochromator was adjusted to display the region 700–1500 cm^{-1} with 8-cm^{-1} resolution. Frequencies are accurate to ± 3 cm^{-1}. Rapid-flow spectra of the all-trans PSB of retinal were obtained in methanol solution (457.9-nm excitation) with the vidicon spectrometer following the methods of Mathies et al. (1977).

In the low-temperature studies the purified pigments were concentrated by vacuum dialysis (Schleicher & Schuell) and frozen onto a copper cold finger at 77 K (Eyring & Mathies, 1979). Spectra were obtained by using a standard photon-counting Raman system (Mathies et al., 1976). The monochromator was calibrated with the Rayleigh line, and slit widths were 5 to 6 cm^{-1}; wavenumber assignments are accurate to ± 2 cm^{-1}. Pooled data from identical runs were smoothed with a three-point sliding average, and quartic polynomial fluorescence backgrounds were subtracted with a PDP 8/e minicomputer. The laser beam at 585 nm was produced by pumping a Coherent 590 dye laser with the all-lines output of a Spectra-Physics 165 argon laser. The beam at 568 nm was generated by a Spectra-Physics 171-01 krypton laser.

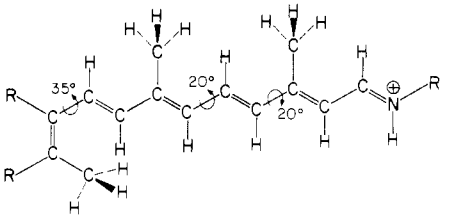


FIGURE 2: The pseudoretinal chromophore modeled in the calculations. The $C_5C_6C_7C_8$ (retinal numbering system) dihedral angle was fixed at 35° for all calculations. The $C_9C_{10}C_{11}C_{12}$ and $C_{11}C_{12}C_{13}C_{14}$ angles were constrained to 20° in the twisted ground state. The R group has the mass and potential parameters of a saturated carbon atom.

Low-temperature RR spectra were obtained by a dual-beam pump-probe technique (Oseroff & Callender, 1974). This method permits the unambiguous identification of bathorhodopsin vibrations in spectra of the photostationary steady-state mixture of rhodopsin, isorhodopsin, and bathorhodopsin formed below −140 °C (Figure 1). The steady-state compositions of these pigments were determined as described previously (Eyring & Mathies, 1979). Under comparable conditions the deuterated analogues gave identical compositions. The possibility of exchange of hydrogen and deuterium in the course of the experiments was tested by exposure of the deuterated pigments to prolonged irradiation at 488 nm and 77 K and subsequent mass spectral analysis of the extracted retinal. No exchange occurred within our experimental error of ±5%.

Normal mode and Raman intensity calculations were performed on models of the retinal chromophore by using a modified version of Warshel's QCFF-π program (Warshel, 1977; Warshel & Dauber, 1977). This method uses a PPP π-electron field coupled with an empirical σ force field to calculate optimal geometries and normal modes independently in the ground and $\pi \rightarrow \pi^*$ excited states. The resulting equilibrium geometries and Cartesian normal mode vectors allow the determination of relative resonance Raman intensities according to the Albrecht *A* term formalism (Tang & Albrecht, 1970). Raman excitation profiles of retinal (Doukas et al., 1978b; Cookingham, 1978) and of β-carotene (Inagaki et al., 1974; Sufrâ et al., 1977) have indicated that the observed intensities of the major spectral lines can be satisfactorily described by a single Albrecht *A* term centered near the origin of the strongly allowed transition.

Because of computer space and time limitations, only the conjugated portions of the retinylidene chain and ring were modeled. The molecule used for calculations was 3,7-dimethyl-2,4,6,8,10-dodecapentaenal ("pseudoretinal", Figure 2) PSB with the addition of two carbon masses at C_5 and C_6 (retinal numbering system) to simulate the ring coupling. The "ring"–chain dihedral angle was fixed at 35° from *s*-cis, in agreement with the observed angle in 11-*cis*-retinal crystals (Gilardi et al., 1972). Our justifications for these simplifications will be discussed below.

Calculations of the twisted retinal PSB were performed by constraining the $C_7C_8C_9C_{10}$ and $C_{11}C_{12}C_{13}C_{14}$ dihedral angles to 20° in the ground state, while allowing the excited states to relax to a planar geometry. The force field parameters of Warshel & Karplus (1972) for C—CH_3 stretching, C=C torsion, and C—C—CH_3 bending were slightly modified to give a better fit to the spectral analyses of butadiene and isoprene (Sverdlov et al., 1974). Details of the modifications will be provided elsewhere (B. Curry and R. Mathies, unpublished work).

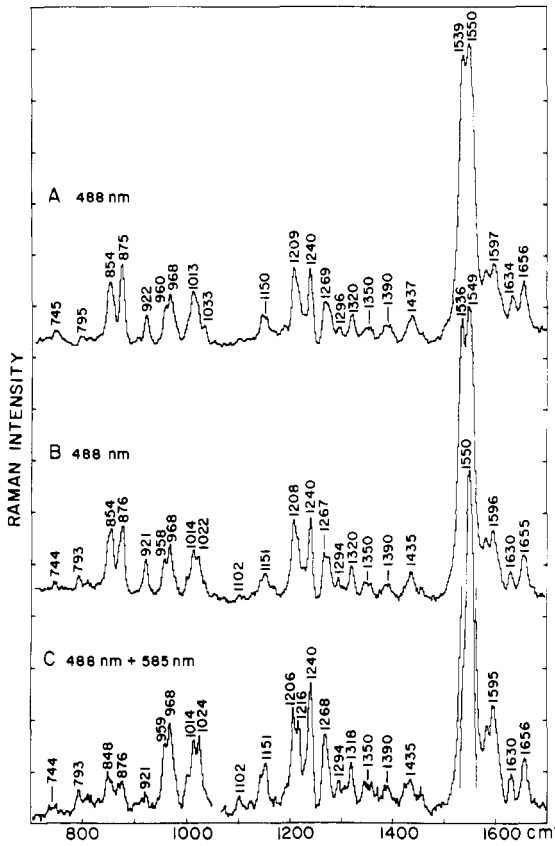


FIGURE 3: Resonance Raman spectra of steady-state mixtures of unmodified pigments (A) and 18-CD_3 pigments (B and C) at 77 K, probed with 488-nm light. In (C) the sample is simultaneously irradiated with a pump beam at 585 nm with a pump/probe power ratio of 4.

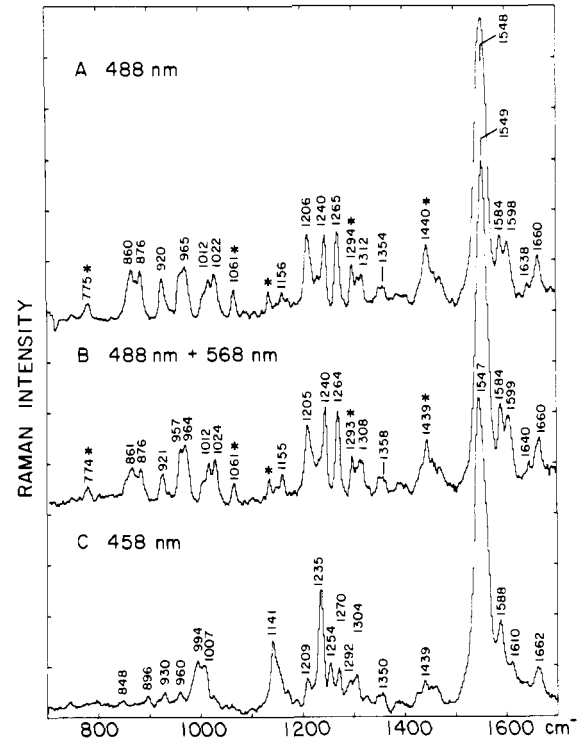


FIGURE 4: (A) Resonance Raman spectra of a steady-state mixture of 5-demethyl pigments at 77 K, probed with 488-nm light. In (B) the sample is simultaneously irradiated with a pump beam at 568 nm with a pump/probe power ratio of 3. Asterisks denote lines which have components due to Ammonyx-LO. (C) Resonance Raman spectrum of a steady-state mixture of 9-demethyl pigments at 77 K, probed with 458-nm light.

Results

Figure 3A presents the resonance Raman spectrum of a steady-state mixture of unmodified rhodopsin, isorhodopsin, and bathorhodopsin at 77 K taken with 488-nm light. Figure 3B shows the steady-state spectrum obtained under the same conditions with 18-CD_3 visual pigments. Within our experimental error the frequencies and intensities in spectra A and B are identical. Addition of a yellow pump beam at 585 nm (Figure 3C), which is preferentially absorbed by bathorhodopsin (Figure 1), reduces the bathorhodopsin concentration in the steady state from 55 to 23%. Because there is a commensurate intensity drop in the lines at 854, 876, and 921 cm^{-1}, they can be attributed to bathorhodopsin (Oseroff & Callender, 1974).

The analogous RR spectra for the 5-demethyl steady state are shown in Figure 4. Each band in the 5-demethyl spectrum (Figure 4A) is within a few wavenumbers of a corresponding band in the native pigment spectrum (Figure 3A). The only significant difference is that the bathorhodopsin ethylenic line is not resolved from scattering of rhodopsin and isorhodopsin near 1550 cm^{-1} in the 5-demethyl spectrum. Lines marked with an asterisk have components due to Ammonyx-LO. Because lines at 860, 876, and 920 cm^{-1} have their intensities reduced by half with the addition of a 568-nm pump beam (Figure 4B), they can be assigned to 5-demethylbathorhodopsin. The obvious similarity of the frequencies and intensities of these lines to those observed in native bathorhodopsin demonstrates not only that 5-demethylbathorhodopsin can be formed but also that its chromophore conformation is virtually identical with that of native bathorhodopsin. A spectrum of the 9-demethyl steady-state mixture at 77 K is given in Figure 4C. No strong lines are observed below 994 cm^{-1} with 457.9-nm irradiation. However, upon warming in the dark ~50% of the 9-demethyl pigments bleach. Evidently, a bathorhodopsin-like species is formed which does not have intense low wavenumber lines.

In Figure 5A we repeat the unmodified steady-state spectrum to facilitate comparison. Figure 5B shows the resonance Raman spectrum of the 19-CD_3 steady state taken under the same conditions. Above 1200 cm^{-1} the spectra appear very similar. Between 700 and 1200 cm^{-1}, however, a number of significant differences are observed. The most dramatic change is the intensity drop in the band at ~1010 cm^{-1}. New bands at 831, 887, and 914 cm^{-1} are observed. To determine the bathorhodopsin contribution, we added a yellow pump beam at 568 nm (Figure 5C). The bathorhodopsin concentration dropped from 55 to 14%, with a consequent intensity drop in the lines at 831, 855 (shoulder, sh), 864, 887, 892 (sh), and 927 cm^{-1}. Raman bands with nearly constant intensity (~913 cm^{-1}) or with significant residual intensity (838 and 864 cm^{-1}) should have components due to rhodopsin and/or isorhodopsin.

The unambiguous assignment of observed bands to particular molecules in the steady state requires a decomposition of the spectrum into contributions from pure components. Figure 6 compares rapid-flow spectra of 19-CD_3 rhodopsin (B) and isorhodopsin (D) with their unmodified counterparts (A and C, respectively). The 19-CD_3 modification causes the band in rhodopsin at 1018 cm^{-1} to lose most of its intensity while a new band appears at 911 cm^{-1}. Bands at 792, 843, and 867 cm^{-1} in rhodopsin appear to correspond with bands at 787, 840, and 862 cm^{-1} in 19-CD_3 rhodopsin. In 19-CD_3 isorhodopsin a line appears at 1063 cm^{-1}. Also, the 1011-cm^{-1} line loses intensity while scattering increases at 908 cm^{-1}. Lines

Table I: Calculated Chain Vibrations of *all-trans*-Retinal PSB between 600 and 1400 cm^{-1} [a]

19-CH_3 relaxed		19-CH_3 twisted [b]		19-CD_3 twisted [b]		normal mode description [c]
1412	(0.8)	1412	(0.2)	1428	(0.4)	CCH bend
1373	(2)	1372	(0.4)	1373	(0.4)	CCH bend
1339	(1)	1338	(0.7)	1342	(0.1)	CCH bend
1326	(12)	1326	(1)	1327	(0.5)	CCH bend + C–C str
1230	(44)	1230	(24)	1230	(21)	C–C str + CCH bend
1211	(17)	1212	(12)	1209	(36)	C–C str
1181	(6)	1180	(2)	1174	(0.3)	C–C str
1043	(<0.01)	1045	(<0.01)	1043	(<0.01)	$C_{20}H_3$ rock
1038	(<0.01)	1038	(0.3)	874	(0.02)	$C_{19}H_3$ rock
1035	(0.1)	1033	(0.1)	852	(0.2)	$C_{19}H_3$ rock
1024	(6)	1024	(2)	1026	(0.2)	$C_{20}H_3$ rock
934	(<0.01)	932	(<0.01)	926	(<0.01)	C–CH_3 str
906	(0.04)	905	(0.03)	878	(<0.01)	C–CH_3 str
988	(<0.01)	988	(<0.01)	988	(<0.01)	C_{15}=N A_u HOOP
974	(<0.01)	975	(<0.01)	976	(<0.01)	C_{11}=C_{12} A_u HOOP
970	(1)	970	(0.08)	970	(<0.01)	C_7=C_8 A_u HOOP
896	(<0.01)	898	(0.05)	898	(0.1)	C_{14} HOOP
866	(0.002)	868	(2)	843	(2)	C_{10} HOOP
819	(0.002)	821	(0.003)	816	(0.002)	C_7=C_8 B_g HOOP
817	(<0.001)	817	(4)	810	(4)	C_{11}=C_{12} B_g HOOP
786	(<0.01)	789	(<0.01)	789	(<0.01)	C_{15}=N B_g HOOP
685	(0.1)	686	(0.6)	671	(0.001)	CCC bend

[a] Calculations were performed on pseudoretinal PSB (Figure 2) as described in the text. Raman intensities were calculated by summing vibronic contributions, assuming 2000 cm^{-1} from exact resonance with the lowest $\pi \rightarrow \pi^*$ state and a phenomenological line width (Γ) of 250 cm^{-1}. The calculated intensities are qualitatively insensitive to the values of these parameters. Calculated Raman intensities are given in parentheses as a percentage of intensity calculated for the most intense C=C stretching vibration. All vibrations between 600 and 1400 cm^{-1} which have calculated relative intensities of >0.1%, as well as those discussed explicitly in the text, are included. [b] The twists were applied in the ground state as discussed in the caption to Figure 2. [c] The major motion contributing to the calculated normal coordinate is listed. HOOP vibrations of hydrogens trans to double bonds are described in the C_{2h} local point group as discussed in the text.

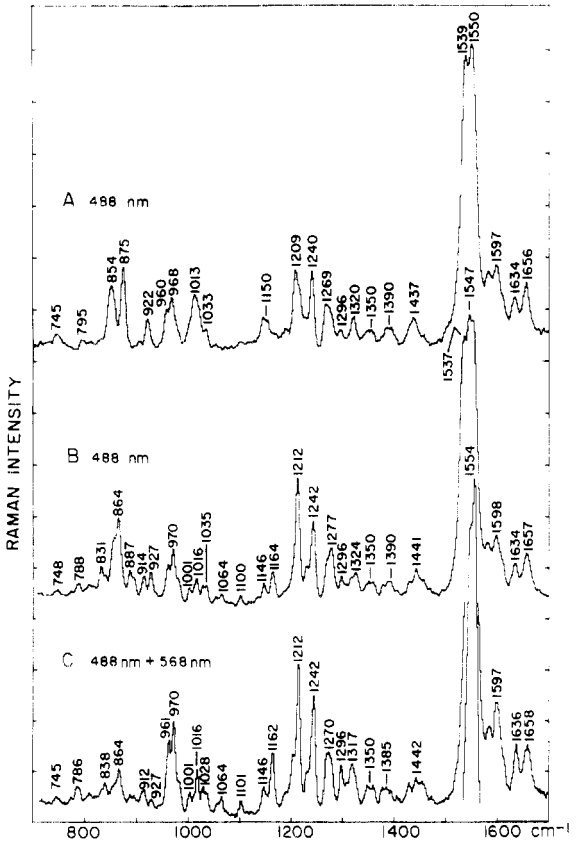


FIGURE 5: Resonance Raman spectra of a steady-state mixture of unmodified pigments (A) and 19-CD_3 pigments (B and C) at 77 K, probed with 488-nm light. In (C) the sample is simultaneously irradiated with a pump beam at 568 nm with a pump/probe power ratio of 10.

at 793 and 830 cm^{-1} in isorhodopsin appear to correspond with lines at 782 and 834 cm^{-1} in 19-CD_3 isorhodopsin.

The assignment of low wavenumber lines to 19-CD_3 bathorhodopsin in Figure 5B can be made by careful comparison of Figures 5 and 6. Lines at 831, 855 (sh), 864, 887, 894 (sh), and 927 cm^{-1} can be attributed to 19-CD_3 bathorhodopsin because they are not observed in the spectra of 19-CD_3 rhodopsin or isorhodopsin or because they decrease in relative intensity when the yellow pump beam is added. The contribution of bathorhodopsin can be made more obvious by using probe wavelengths that selectively enhance bathorhodopsin scattering (Eyring & Mathies, 1979; Oseroff & Callender, 1974). Figure 7C shows a spectrum of the 19-CD_3 steady state taken with a 585-nm probe beam and a 488-nm pump. Under these conditions the bathorhodopsin scattering dominates the spectrum. These data confirm the previous identification of 19-CD_3 bathorhodopsin lines. In addition, the intensities in Figure 7C demonstrate that 19-CD_3 bathorhodopsin has lines at 1210, 1274, and 1533 cm^{-1}. These same results can be obtained by computer subtraction of pure 19-CD_3 rhodopsin and isorhodopsin from the blue probe photostationary steady-state mixture following the methods of Eyring & Mathies (1979). For purposes of comparison, spectra of the all-trans PSB of retinal and unmodified bathorhodopsin (Eyring & Mathies, 1979) are given in parts A and B of Figure 7, respectively.

The results of our QCFF-π calculations are presented in Table I. The first column lists the calculated frequencies and relative Raman intensities of relaxed *all-trans*-pseudoretinal PSB (Figure 2). The second and third columns compare the calculated results for the 19-CH_3 and 19-CD_3 PSB's which have been artificially twisted by 20° about the C_{10}–C_{11} and C_{12}–C_{13} single bonds. Twists around single rather than double bonds were modeled because they are energetically more reasonable. For example, the twisted PSB is calculated to have a ground state energy only 2.1 kcal/mol above that of the untwisted all-trans isomer. A relaxed planar excited state equilibrium geometry was used for all three calculations. Note

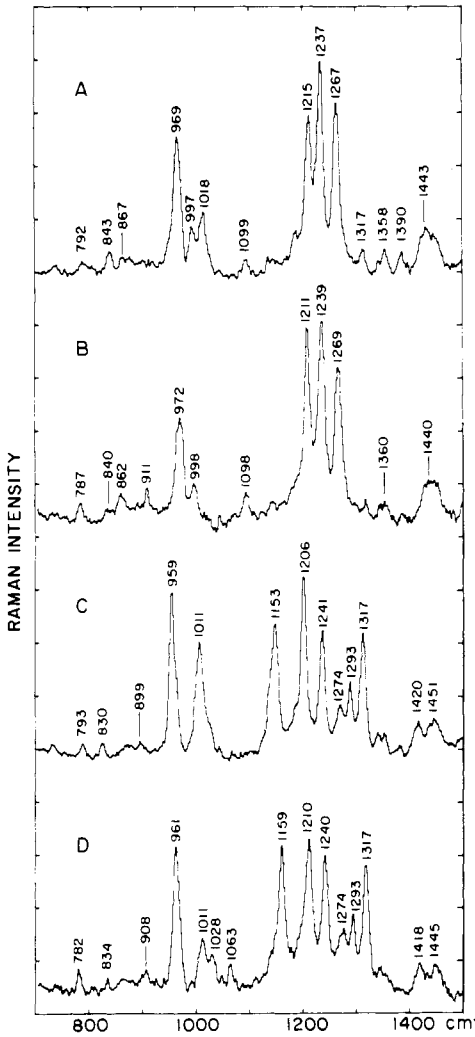


FIGURE 6: Room temperature rapid-flow RR spectra (488 nm) of pure rhodopsin (A), isorhodopsin (C), and their respective 19-CD_3 analogues (B and D).

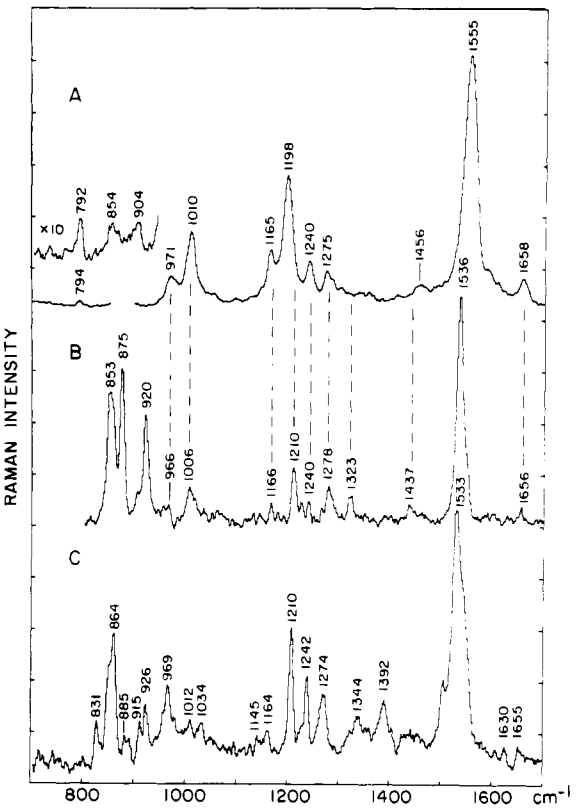


FIGURE 7: (A) Resonance Raman spectrum of the all-trans PSB of retinal (488 nm) from Mathies et al. (1977). The inset presents an expanded all-trans PSB spectrum (457.9 nm) in methanol. (B) Spectrum of unmodified bathorhodopsin (585 nm) from Eyring & Mathies (1979). (C) 77 K steady-state mixture of 19-CD_3 pigments with 585-nm probe and 488-nm pump. The pump/probe power ratio is 0.6.

that deuteration of the 19-methyl group shifts the 19-methyl rocks into near degeneracy with the chain vinyl hydrogen HOOP modes. Twists of only 20° about chain single bonds enhance the C_{11}=C_{12} (B_g) and C_{10} HOOP modes by more than 3 orders of magnitude. More elaborate twists in either the ground or the excited state would lead to further enhancement of other chain HOOP vibrations. This HOOP mode enhancement occurs with negligible changes in the frequencies of the 1100–1400-cm^{-1} fingerprint lines.

Discussion

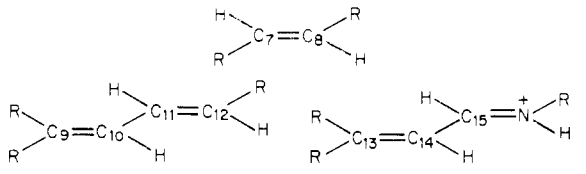
The interpretation of the Raman spectrum of bathorhodopsin is an important unsolved problem in vision photochemistry. The most striking feature of the bathorhodopsin spectrum is the presence of three anomalous low wavenumber lines at 853, 876, and 920 cm^{-1} (Oseroff & Callender, 1974; Eyring & Mathies, 1979). Possible origins for these lines include vibrations of a chemically modified chromophore (van der Meer et al., 1976), distortions of the β-ionyl ring (Warshel, 1977), and HOOP vibrations of vinyl hydrogens on the polyene chain (Warshel, 1977; Lewis, 1978). Our data on pigment analogues can be used to evaluate these proposals.

Several workers have suggested that the bathorhodopsin low wavenumber lines are due to vibrations of a chromophore which is not a geometric isomer of retinal. The observation of a sevenfold deuterium isotope effect on the bathorhodopsin formation time at low temperature has led to a group of such proposed structures. These include as a common feature proton transfer to the chromophore, with consequent single- and double-bond reversal (Peters et al., 1977). Thus, for example, the Schiff base group C=NH^+ in rhodopsin becomes C—N—H in bathorhodopsin, so that large differences in Schiff base vibrational frequencies and deuterium shifts would be expected in the Raman spectra of these pigments. No such differences are in fact observed (Eyring & Mathies, 1979). This is strong evidence against all such retrostructure models. Furthermore, the 5-demethyl spectra of Figure 4, which are nearly identical with those of the unmodified steady state, demonstrate that the 18-methyl group is not involved in the primary photochemistry. This confirms the earlier observation (Kropf, 1976) that a 5-demethyl species with an absorption spectrum and thermal stability analogous to those of native bathorhodopsin can be formed from 5-demethylrhodopsin at low temperature. Moreover, the absence of any frequency shifts of the low wavenumber lines of 18-CD_3 bathorhodopsin (Figure 3) demonstrates that these lines are not due to vibrations of an exomethylene group between C_5 and C_{18} (Fransen et al., 1976). Finally, the <20-cm^{-1} shifts of the bathorhodopsin low wavenumber lines caused by deuteration of the 19-methyl group (Figure 5) preclude the alternative possibility that these lines could be due to deformations of an exomethylene group involving a double bond between C_9 and C_{19}. The characteristic out-of-plane hydrogen vibrations of an exomethylene group in the 800–1000-cm^{-1} region (Fransen et al., 1976) should shift by ~200 cm^{-1} upon deuteration. These data, together with the more general arguments of Honig et al. (1979) and Rosenfeld et al. (1977), render all *proposed* chemically modified structures for the bathorhodopsin chromophore improbable. Note that our data do not preclude the possibility that proton translocation may occur

without chemical modification of the bathorhodopsin chromophore. Several authors have suggested such models (Lewis, 1978; Warshel, 1978; Honig et al., 1979).

The remaining possibility is that the low wavenumber lines are due to vibrations of a chromophore which is a geometric isomer of retinal but is conformationally distorted by interactions with opsin (Warshel, 1977; Lewis, 1978). Previous calculations on strained retinals (Warshel, 1977) and group frequency considerations (Potts & Nyquist, 1959) indicate that lines in the 820–950-cm^{-1} region can arise either from deformations of the ionyl ring or from vinyl hydrogen wags (HOOP vibrations).[3] First-order vibronic theory predicts that chain vibrations should be more intense than ring modes in the RR spectrum, since they are more strongly coupled to the resonant $\pi \rightarrow \pi^*$ transition. This coupling occurs when the nuclear distortion in the resonant excited state corresponds closely with the vibrational nuclear displacements (Tang & Albrecht, 1970). A variational open-shell all valence electron INDO calculation (Birge, 1973) has been used to confirm the intuitive idea that the intense $^1A_g \rightarrow {}^1B_u$ transition in retinal is localized on the conjugated atoms (L. M. Hubbard and R. R. Birge, personal communication). The prediction that the saturated portion of the ring contributes little to the Raman spectrum has been experimentally confirmed for the free aldehydes by the Raman analogue data of Cookingham et al. (1978) which show no significant spectral changes for the 5-demethyl-, 3,4-dehydro-, or trimethylthienylretinals. The minor shifts observed in the 5-demethyl pigment spectra (Figure 4) support this conclusion, since removal of this methyl group would be expected to perturb dramatically most of the ring deformation modes and especially those involving the $C_5{=}C_6$ ring double bond. The 19-CD_3 substitution, on the other hand, which is a comparatively small perturbation, produces significant shifts in the 800–950-cm^{-1} bathorhodopsin lines (Figure 5). This indicates that the low wavenumber modes are coupled to the $C_{19}H_3$ motion and must therefore involve the polyene chain. In addition, large changes are observed in steady-state spectra of the 9-demethyl pigments (Figure 4C). Thus, because minor chain modifications perturb the low wavenumber lines while major ring alterations have little effect, we conclude that these bathorhodopsin vibrations are localized on the polyene chain. Specifically, we now show that these lines can best be assigned to HOOP vibrations of chain vinyl hydrogens.

Evidence To Support the HOOP Assignment: (1) Number and Frequencies of the Low Wavenumber Lines Are Consistent with Those Expected for HOOP Vibrations. Our QCFF-π calculations predict that for unsubstituted polyenes the HOOP motions are delocalized among the chain hydrogens. The presence of methyl groups on the retinal chain, however, decouples these motions. Thus, the calculated vibrations are largely localized in one of the three resulting fragments:



[3] Previous authors have described such modes as "torsions". We prefer to reserve the term torsion to describe motions which, by twisting about chain bonds, distort the carbon backbone out of the plane. The distinction in kinetic motion between the torsion and the wag is detailed by Wilson et al. (1955). Because of the small hydrogen mass, the HOOP wag does not mix greatly with the C=C torsion, which appears at a lower frequency.

To a first approximation, these motions can be considered as vibrations of 1,2-disubstituted or 1,1,2-trisubstituted ethylenes. Characteristic frequencies for such fragments have been thoroughly discussed (Potts & Nyquist, 1959; Sverdlov et al., 1974), and eight HOOP vibrations of planar *all-trans*-retinal PSB are predicted: two IR-active modes between 960 and 980 cm^{-1} of $A_u(C_{2h})$ symmetry about $C_{11}{=}C_{12}$ and $C_7{=}C_8$, two weakly IR-active modes between 800 and 890 cm^{-1} due primarily to C_{10} and C_{14} hydrogen wags, two IR-inactive vibrations between 750 and 860 cm^{-1} of B_g symmetry about $C_{11}{=}C_{12}$ and $C_7{=}C_8$, and two modes involving the C_{15} hydrogen and the Schiff base proton. Our calculations predict that the coupled Schiff base and C_{15} protons will give rise to two bands near 790 and 990 cm^{-1}, albeit with significant uncertainty because no model compound assignments are presently available. These group frequency considerations are consistent with our calculations (Table I) and with reported IR spectra of the free retinaldehydes (Cookingham et al., 1978). Thus, at least four HOOP vibrations are expected in the 800–930-cm^{-1} range. On frequency considerations alone, therefore, the three lines observed for bathorhodopsin at 853, 875, and 920 cm^{-1} could all be consistently assigned to HOOP wags. The large intensities of these lines, however, seem inconsistent with the weak scattering observed in this frequency region for polyenes (Popov & Kogan, 1964), retinal PSB's (Mathies et al., 1977), and the unphotolyzed pigments (Figure 6). Therefore, a reasonable explanation for the anomalous Raman intensities of the HOOP modes of bathorhodopsin is required.

(2) HOOP Modes Can Be Enhanced by Chain Distortion. Vibronic theory (Tang & Albrecht, 1970) predicts that the intensity of a resonance Raman transition depends upon products of vibrational overlap integrals (Franck-Condon factors) between the initial (and final) vibrational states and excited vibronic levels. These products of Franck-Condon factors will be large for a particular vibration if electronic excitation results in a displacement of the equilibrium position of that normal coordinate. Thus, displacement of the chain hydrogens out of the molecular plane in either the ground state or the excited electronic state would be expected to enhance the local HOOP modes. For example, crystalline *trans*-stilbene, which has a planar geometry, has an extremely weak Raman line corresponding to the IR-active ethylenic HOOP (A_u) vibration at 963 cm^{-1} (Edelson & Bree, 1976). In solution, however, *trans*-stilbene is twisted $\sim 10°$ about the central double bond and $\sim 20°$ about each C–C_6H_5 single bond, displacing the ethylenic hydrogens out of the original molecular plane. A strong Raman line appears at 963 cm^{-1} in the spectrum of *trans*-stilbene in solution, while the rest of the spectrum is virtually unchanged from that of the crystal. This example demonstrates that ground state torsional perturbations can produce large relative displacements of the hydrogen wagging coordinate between the ground and resonant excited states, resulting in large enhancements of the HOOP modes.

Resonance enhancement of the HOOP modes of retinals could be achieved equally well by twists about single or double bonds in the ground or excited state. We chose to model this effect by twisting the ground state, which seems more compatible with the fact that bathorhodopsin is a transient, high energy intermediate. We calculated the *A* term intensities for modes of *all-trans*-retinal PSB twisted by 20° about the 10–11 and 12–13 single bonds (Figure 2). The results, shown in Table I, indicate that significant HOOP mode intensity can be introduced. Note also that the calculated frequencies of

fingerprint lines change only slightly upon twisting. The particular twists modeled were arbitrarily chosen, and there is no evidence to suggest that they represent the actual conformation of bathorhodopsin. However, the calculations do demonstrate that strong resonance enhancement of the HOOP vibrations can be achieved by a simple conformational distortion of the retinal chain.

(3) Isotopically Induced Shifts Observed in 19-CD_3 Bathorhodopsin (Figure 5) Support Our HOOP Assignment for the Low Wavenumber Lines. Based on relative intensities and proximity of frequencies, we have correlated the 853-, 875-, and 920-cm^{-1} bands of unmodified bathorhodopsin (Figure 7B) with the 855- (sh), 864-, and 926-cm^{-1} bands of 19-CD_3 bathorhodopsin, respectively (Figure 7C). The lines at 831 and 887 cm^{-1} (Figure 5B) are new bathorhodopsin lines, while scattering at ~915 cm^{-1} is largely due to 19-CD_3 rhodopsin (Figure 6B). It appears that this more complicated vibrational spectrum arises because deuteration shifts methyl vibrational modes into the 800–950-cm^{-1} region. In order to account for these effects, we must analyze the methyl group vibrations in greater detail.

Only three modes directly involving methyl group hydrogen motion would be expected to appear in the 700–1200-cm^{-1} range: two CCH_3 rocking modes[4] and a methyl stretching mode. For isoprene and pentadiene the two methyl rocks appear between 1000 and 1040 cm^{-1}, while the stretches are observed between 920 and 1020 cm^{-1} (Sverdlov et al., 1974). Gill et al. (1971) tentatively assigned the intense Raman bands appearing near 1010 cm^{-1} in the retinal isomers to the C_9–CH_3 and C_{13}–CH_3 stretches. Comparison of crystal and solution RR spectra of retinal isomers led recent workers (Callender et al., 1976) to the same conclusion. Further RR studies of C_9- and C_{13}-butyl-substituted retinals showed that the 1017-cm^{-1} line of 11-*cis*-retinal and part of the 1010-cm^{-1} intensity in the 9-cis, 13-cis, and all-trans isomers are indeed associated with motion of the $C_{19}H_3$ group (Cookingham et al., 1978; Cookingham & Lewis, 1978), and these lines were also attributed to stretching vibrations. Our observation of the disappearance of the intense 1018-cm^{-1} line of 19-CD_3 rhodopsin (Figure 6B) and of the major component of the 1011-cm^{-1} line of 19-CD_3 isorhodopsin (Figure 6D) confirms that $C_{19}H_3$ motions are responsible for these bands in the pigments as well. However, the methyl stretch assignment cannot be made automatically, because two $C_9C_{19}H_3$ methyl rocking modes, with significant Raman intensity, are also expected in this frequency region. Our calculations on retinals (Table I) place the four methyl rocks from 1020 to 1050 cm^{-1} and the methyl stretches at 934 and 906 cm^{-1}. However, the semiempirical force field used here does not allow us to make a conclusive assignment based on the absolute frequency correspondence. It is clear that both methyl rocks and methyl stretches can consistently be assigned in the 1010-cm^{-1} region.

Because isotopic shifts are due primarily to mass effects, they are less sensitive than the absolute frequencies to the details of the force field. Upon 19-CD_3 substitution two methyl rocks are calculated to shift down into the 800–950-cm^{-1} region, bringing these vibrations into near degeneracy with the chain HOOP modes (Table I). Therefore, a small degree of mixing and slight frequency shifts are expected for the HOOP vibrations (e.g., 868 → 843 cm^{-1} and 817 → 810 cm^{-1}). The observed shifts of the low wavenumber lines of bathorhodopsin from 875 to 864 cm^{-1} and from 922 to 927 cm^{-1} (Figure 5B) are within the range predicted for this effect.

In summary, the number and frequencies of the bathorhodopsin low wavenumber lines are consistent with those expected for the HOOP vibrations. The anomalous intensities of these lines can be explained by small relative twists about single bonds. Also, the isotopically induced low wavenumber line shifts for 19-CD_3 bathorhodopsin are consistent with the HOOP assignment.

Assuming that this assignment is correct, it should be possible to determine the double-bond configuration of the bathorhodopsin chromophore based on its Raman fingerprint, since our calculations predict that the ground state twists of 20° about chain single bonds required to enhance the HOOP modes should not seriously perturb the fingerprint region. This result is experimentally observed in the stilbene example cited above (Edelson & Bree, 1976). In Figure 7, we compare the Raman spectra of bathorhodopsin (Eyring & Mathies, 1979) and the all-trans PSB of retinal. *The close correspondence in both frequency and relative intensity in the 1100–1400-cm^{-1} fingerprint region suggests that the configuration of the bathorhodopsin chromophore is trans. The intense low wavenumber lines of bathorhodopsin would then originate from the latent HOOP modes of the all-trans PSB between 850 and 950 cm^{-1} (Figure 7) which are resonantly enhanced by twists about chain single bonds.* While such correlations must be drawn with caution (Auerbach et al., 1979), this conclusion is consistent with previous proposals of a twisted all-trans ground state to explain many of the photochemical properties of the pigments (Rosenfeld et al., 1977; Honig et al., 1979). Potential surface calculations have shown that certain assumed protein charge distributions could stabilize such a twisted state (Warshel, 1978). Further work with analogue pigments deuterated at vinyl chain positions should permit the assignment of particular bathorhodopsin low wavenumber lines to specific HOOP vibrations. This should define more precisely the degree and location of the chain twists in the bathorhodopsin chromophore.

[4] The two methyl rocks are linear combinations of CCH valence bends, in which the central carbon is the methyl carbon. Qualitatively, all the methyl hydrogens move together without changing their C–H bond lengths or HCH bond angles. There are two independent rocking vibrations for each methyl group, one parallel and one perpendicular to the plane of the polyene chain. For standard methyl geometry, the isotopic frequency ratio for CH_3 and CD_3 rocks is 0.765, very close to the value of 0.707 which would result if the mode involved purely hydrogen motion.

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