tein exists in two conformations T and R, of which only R, which comprises only a small fraction of the free protein, binds the hapten. For this system the relaxation time for the association is $1/\tau = K_R (R_0 + H) + k_{-R}$, and since $R_0 \ll H$ always, $1/\tau \cong k_R H + k_{-R}$. The broken lines in the figure represent the best fit to this mechanism, with $K_R = 1.9 \times 10^6\ M^{-1}\ s^{-1}$, $k_{-R} = 45\ s^{-1}$, and $K_0 = 0.14$. An additional relaxation time representing the monomolecular $R_0 \rightleftharpoons T_0$ equilibrium is also expected. We suspect that this time is very slow and lies outside the time range of the temperature-jump method. This mechanism is not, however, in accord with the static measurements, since it predicts a hyperbolic saturation curve. We then proceeded to analyze the kinetic data using the MWC mechanism. For an allosteric dimer this model is redundant, i.e., many different K_T, K_R, and L sets may conform with a particular binding curve, as far as they all arise from the same pair of Adair constants. For the titrations of DNPL these were found to be $K_1 = 3.5 \times 10^3$ and $K_2 = 1.4 \times 10^4\ M^{-1}$. A fit to the nonexclusive MWC model using the formulas of Kirschner et al. (4) and assuming that only an intermediate (R association) relaxation time is observed, gave a good agreement (full curve in the figure) the best fit parameters being $k_R = 1.9 \times 10^6\ M^{-1}\ s^{-1}$, $k_{-R} = 52\ s^{-1}$, $K_T = 1.9 \times 10^3\ M^{-1}$, $K_R = 3.7 \times 10^4$, and $L = 36$. These represent $K_1 = 2.8 \times 10^3$, $K_2 = 1.5 \times 10^4\ M^{-1}$, in good agreement with the titrations. The value of L obtained is intermediate between those found when exclusive MWC, or when equality of L for both haptens, was assumed. An analysis of relaxation amplitudes gave only a moderate agreement with the MWC model, implying that the actual mechanism may be somewhat more complicated. However, the very good fit of the relaxation time data suggests that the MWC model is a good first-approximation description. Relaxation measurements with NBDA are now underway. A complex spectrum, containing at least two relaxation times, is observed, which serves as a preliminary indication for the complexity of the binding mechanism.

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THE STRUCTURE OF THE RETINYLIDENE CHROMOPHORE IN BATHORHODOPSIN

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ABSTRACT Resonance Raman data on bathorhodopsin (bovine and squid) at 95, 77, and 4°K support a mechanism of excitation proposed by Lewis in which both a

protein conformational transition and chromophore structural alteration to a "dicisoid" configuration are required to generate the bathorhodopsin species observed in steady-state photostationary mixtures. However, these results also suggest that the molecular structure with a red-shifted chromophore absorption detected at room temperatures in 1 ps using picosecond absorption spectroscopy may not necessarily have the same chromophore conformation as the steady-state bathorhodopsin species.

Recently, I proposed a mechanism of excitation in visual transduction which accounts for all the presently available photophysical and photochemical data on rhodopsin (1). In this mechanism, the primary action of light is to cause significant electron redistribution in the retinylidene (retinal) chromophore, which induces a protein conformational rearrangement (such as proton translocation in the opsin matrix) and subsequent structural alteration in the chromophore isoprenoid chain. The original description of this visual excitation mechanism (1) proposed a distorted retinal structure in bathorhodopsin that could be generated from either rhodopsin (containing the chromophore in an initial 11-*cis* isomeric configuration) or isorhodopsin (containing the chromophore in an initial 9-*cis* isomeric configuration) by a similar out-of-plane torsional motion. Furthermore, although our proposed bathorhodopsin chromophore structure is not consistent with mechanisms that predict a photochemical 11-*cis* to all-*trans* isomerization (2), the structure we propose can readily relax to an all-*trans* conformation by metarhodopsin I and is consistent with experiments that demonstrate the bathorhodopsin retinal structure is common to both rhodopsin and isorhodopsin (3, 4). In this note we demonstrate that our proposed bathorhodopsin chromophore structure is also consistent with the resonance Raman data on bovine and squid visual pigments.

Resonance Raman spectra of bovine and squid photostationary mixtures obtained with 514.5 nm illumination at 77 and 95°K, respectively, are displayed in Fig. 1. When our high-resolution bovine spectra (Fig. 1 B) are compared to the squid spectra (Fig. 1 A), detailed similarities are revealed that are not apparent from comparing our squid results to the earlier low-resolution experiments of Oseroff and Callender (5). The various peak assignments shown in Fig. 1 are made on the basis of dual-beam and variable temperature-experiments to be described in more detail elsewhere.[1] These assignments are in general agreement with our earlier results on squid visual pigments (6), except for the reassignment in this paper of the 1,147 cm^{-1} peak to isorhodopsin. Our earlier conclusion on the assignment of this band was made on the basis of observations by Oseroff and Callender (5) and Mathies et al. (7). that bovine isorhodopsin contained only one vibrational mode in this region. However, our higher resolution bovine data show two vibrational modes at 1,143 and 1,153 cm^{-1} even when the photostationary mixture is 96% isorhodopsin. Therefore, even though a pure squid isorhodopsin spectrum cannot be obtained at low temperatures, we are confident

[1]Sulkes, M., A. Lewis, and M. A. Marcus. 1978. Resonance Raman spectroscopy of squid and bovine visual pigments; the primary photochemistry in visual transduction. Submitted for publication.

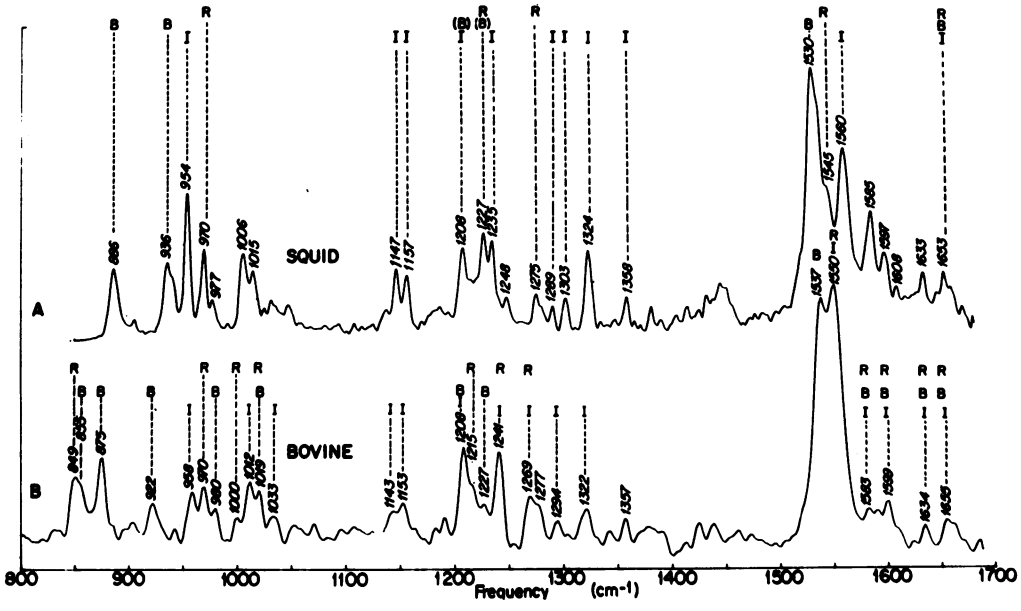


FIGURE 1 Photostationary state resonance Raman spectra of (A) Squid retinular membrane fragments prepared by the method of Hubbard and St. George (17) and (B) bovine rod outer segments prepared by the method of Applebury et al. (18). These spectra were recorded at 77°K (A) and 95°K (B), with a 514.5-nm laser beam, and are virtually identical to spectra recorded at 4°K. The intensity of the C—H bond in squid isorhodopsin indicates that this species makes a large contribution to the photostationary state mixture at 514.5 nm illumination and this obscures certain rhodopsin bands (such as the 1,215 cm^{-1} mode) in squid rhodopsin, which can be detected in the bovine spectra. It is important to note that peaks are assigned to either rhodopsin (R), isorhodopsin (I), or bathorhodopsin (B), with tentative assignments in brackets, and that squid spectra of these species exhibit no bands between 800 cm^{-1} and the peak at 886 cm^{-1} seen in A. In addition, there is a shoulder at 1,277 cm^{-1} in bovine spectra and a peak at 1,248 cm^{-1} in squid spectra, which may be associated with bathorhodopsin.

that both the frequencies in this spectral region (see Fig. 1 A) arise mainly from squid isorhodopsin.

Based on these assignments, it is obvious that the isorhodopsin, rhodopsin, and bathorhodopsin bands exhibit little overlap. In addition, there are no bathorhodopsin vibrational modes in the 1,170–1,200 cm^{-1} region, where metarhodopsin exhibits strong scattering modeled well by all-*trans* protonated Schiff base resonance Raman spectra (6). These data indicate that the configuration of the chromophore in bathorhodopsin is not all-*trans*, and although it is common to rhodopsin and isorhodopsin (3, 4), it is structurally distinct from either the 11-*cis* or 9-*cis* isomeric configurations. I have proposed (1) a structure that meets all of the above criteria and can relax to an all *trans* conformation by metarhodopsin I; it is shown in Fig. 2. Notice that the C_9 and C_{13} methyl groups in this structure are in very similar structural configurations and that the 11-*cis* 12-s-*trans*/ 11-*cis* 12-s-*cis* C_{13} methyl-hydrogen interactions are absent in this "dicisoid" configuration. In these terms one can understand the absence

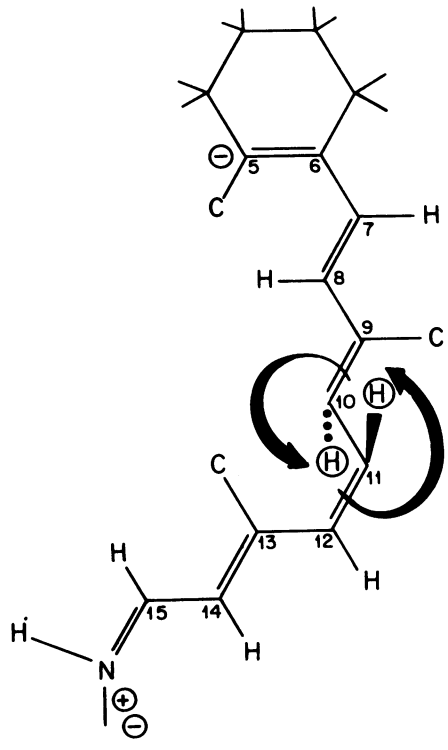
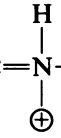


FIGURE 2 A proposed structure for the retinylidene chromophore in bathorhodopsin. The Hs at positions 10 and 11 are depicted out of the plane of the paper. This structure is generated by out-of-plane torsional motions from either rhodopsin or isorhodopsin by rotating as a unit the $C_9—C_{10}$ and $C_{11}—C_{12}$ ground-state double bonds, which are single bonds in the excited state. The exact out-of-plane rotation is not known.

in bathorhodopsin of the 1,000 and 1,019 cm^{-1} splitting in the $C—CH_3$ vibrational mode in rhodopsin (associated with the 11-*cis* 12-s-*trans* and 12-s-*cis* C_{13} methyl-hydrogen interactions [8]) coalesces to one band at ~1,019 cm^{-1}. Furthermore, the distortions around double bonds in this structure may be the cause of the lowered C=C stretching frequency in bathorhodopsin in Fig. 1[2] and may also account for the appearance only in bathorhodopsin of an intense out-of-plane torsional mode between 860 and 900 cm^{-1}. It is interesting in this regard that, even though squid and bovine pigment spectra exhibit detailed similarities in other spectral regions (see Fig. 1 A), bovine pigments (rhodopsin and bathorhodopsin) have an additional mode at ~850 cm^{-1} not detected in squid photostationary state spectra. The calculations of Warshel

[2]This is suggested by the observation that bacteriorhodopsin with a 570 nm absorption and squid bathorhodopsin with a 545 nm absorption both have C=C stretching frequencies at 1,530 cm^{-1} (see ref. 9 and Fig. 1 A). This may indicate that in the highly strained bathorhodopsin chromophore structure the C=C stretching frequency is not linearly correlated to the chromophore absorption maximum, as some workers have suggested (10.)

and Karplus (11) indicate that this could occur as a result of ring constraints and without structural alterations in the isoprenoid chain configuration.

Finally, the  stretching frequency at 1,655 cm^{-1} (see Fig. 1) is identical in rhodopsin, bathorhodopsin, and isorhodopsin. Since this is the only exchangeable proton on the chromophore, deuterium isotope effects on the time evolution of bathorhodopsin must result from a proton movement in the protein, as I recently proposed (1), and must be unrelated to changes in the state of protonation of the Schiff base (12). Thus, these results suggest that bathorhodopsin is produced by structural transitions in both the protein and chromophore, in support of the excitation mechanism and energy surface I proposed (1).

In conclusion, it should be noted that there are now three possible explanations for the red shift in bathorhodopsin. This red shift could be the result of: (*a*) protein structural alterations; (*b*) chromophore structural alterations; or (*c*) chromophore and protein structural alterations. However, it is known that widely differing (9- and 11-*cis*) chromophore structures in similar protein conformations have nearly identical absorption maxima (3, 4). This can be explained by the suggestion (6, 13, 14) that the color of visual pigments is controlled by the charged environment of the protein, which causes chromophore excited-state stabilization. Thus, it is quite possible that proton transfer in the protein changes the protein charge environment, which results in the bathorhodopsin red-shifted absorption. If this is so, subsequent chromophore structural alteration may not detectably alter the bathorhodopsin visible absorption and in these terms picosecond absorption spectroscopy (4, 12, 15) may be monitoring only the protein structural alteration and not the chromophore structural changes detected, even at 4°K in steady-state resonance Raman spectra. Kinetic resonance Raman measurements (16) with mode-locked lasers should be capable of testing this hypothesis.

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CONTRACTILE DEACTIVATION BY RAPID, MICROWAVE-INDUCED TEMPERATURE JUMPS

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Activation of muscle is controlled by Ca^{++} (released by changes in membrane potential), myofilament overlap, and muscle load. Understanding has been hindered by inability in many cases to achieve rapid changes in potential or intracellular concentration of agents. Biochemical approaches are hindered by the loss of the physiological ordered lattice and by loss of the coupling to external load.

A microwave temperature-jump system has been developed for the study of rapid-heating relaxation of single frog striated muscle cells, using a modified magnetron conditioner (4J50, 9.4 GHz, 200 kW peak power, 0.002 duty cycle) (Fig. 1). The isolated muscle cell is bathed in saline contained in an acrylic chamber (150 μl volume) passing through a waveguide (RG51) one quarter wavelength from the shorted end. Slits are provided to allow continuous measurement of sarcomere spacing by means of the diffraction of a HeNe laser beam, and isometric force is recorded using an AME silicon transducer element. Temperature increase was linear with duration of the heating train 0.2°K/ms, and cooling of the ambient temperature had a half-time exceeding 10 s. No cellular damage is apparent even with scores of T-jumps over some hours, if excessive heating (T <28°C) is avoided. Heating for 5–10 ms preceding a twitch altered the twitch in the same fashion as altered steady T; heating during the rising phase caused an increase in the rate of tension development, followed by the more rapid relaxation