

# PHOTOCHEMISTRY OF RHODOPSIN AND ISORHODOPSIN INVESTIGATED ON A PICOSECOND TIME SCALE

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**ABSTRACT** Bovine rhodopsin and isorhodopsin were excited with a single 530-nm, 7-ps light pulse emitted by a mode-locked Nd<sup>3+</sup> glass laser at room temperature. Within 3 ps of excitation, absorbance changes due to formation of bathorhodopsin were observed. The difference spectra generated during and 100 ps after pulse excitation are presented. The data show that bathorhodopsin formation is completed within 3 ps for both the primary pigments and suggest that a single common bathorhodopsin is photochemically formed from both primary pigments. Our findings provide additional support for the *cis-trans* isomerization model of the primary event in vision. Additional absorption transients that were observed near 670 and 460 nm are discussed.

## INTRODUCTION

The visual pigment in disk membranes of vertebrate rod cells, called rhodopsin, consists of an 11-*cis* retinal chromophore covalently linked through a protonated Schiff base to a small protein called opsin (Wald, 1968). When rhodopsin absorbs a photon, a consecutive series of thermal intermediates is formed ending in the release of all *trans* retinal and free opsin (for reviews see Honig and Ebrey, 1974; Ebrey and Honig, 1975). The first thermal intermediate, bathorhodopsin, has been shown to be generated from rhodopsin in less than 6 ps at room temperature (Busch et al., 1972; Green et al., 1977) and thermally decays in about  $10^{-7}$  s (Busch et al., 1972; Rosenfeld et al., 1972).

The opsin binding site also accommodates a 9-*cis* retinal. This pigment, isorhodopsin, has the same thermal intermediates as rhodopsin. It is possible to establish a photoequilibrium between rhodopsin, isorhodopsin, and bathorhodopsin at low temperatures (Yoshizawa and Wald, 1963; Aton et al., 1978) and at room temperature (Goldschmidt et al., 1976). The fact that bathorhodopsin is the common intermediate between two different *cis* isomers in this photoequilibrium strongly suggests that the primary photochemical event in the visual process involves a *cis-trans* isomerization (Hubbard and Kropf, 1958; Rosenfeld et al., 1977). Alternatively, proton translation has been proposed to describe bathorhodopsin formation (van der Meer et al., 1976; Peters et al., 1977).

We recently reported that the photochemical formation of bathorhodopsin from isorhodopsin and rhodopsin occurs in about 3 ps at room temperature (Green et al., 1977). In the present work, we investigate the bathorhodopsin difference spectra that are generated at room

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temperature on a picosecond time scale from both rhodopsin and isorhodopsin. Our principal goal in comparing the photochemistry of rhodopsin and isorhodopsin is to extract information on the primary visual event. Considering the various models of the visual process that have been proposed, we analyze whether bathorhodopsin formed initially from isorhodopsin is the same chemical species as that formed initially from rhodopsin. We also ask whether bathorhodopsin formed immediately after pulsed excitation of either primary pigment relaxes to a species with modified absorption characteristics at later times. Our picosecond results with both rhodopsin and isorhodopsin are consistent with describing the initial photochemical event in the visual process as a *cis-trans* isomerization.

In view of the 3-ps risetime estimated for bathorhodopsin from rhodopsin and isorhodopsin (Green et al., 1977), we have studied the difference spectra generated within our resolution time. Our data suggest that there may be a precursor that absorbs to the red of the bathorhodopsin absorption band. In addition, an apparent absorption in the blue region of the spectrum is observed. We also compare our results to those obtained by Sundstrom et al. (1977), who studied aspects of the rhodopsin-to-bathorhodopsin picosecond absorption kinetics.

## MATERIALS AND METHODS

All procedures were carried out under dim red light or in the dark, at 4°C or in an ice bath, unless noted otherwise. Rod outer segment membrane fragments were isolated from dark-adapted bovine retinae (George A. Hormel & Co., Austin, Minn.) essentially as described previously (Hong and Hubbell, 1973). Solubilized rhodopsin was obtained by extracting the membrane fragments in 1% Ammonyx-LO (also known as lauryldimethylamine oxide) and 100 mM phosphate buffer (pH 6.6). Isorhodopsin was prepared photochemically by taking rhodopsin samples to liquid nitrogen temperature, irradiating by using the 528.2-nm krypton laser light, and warming. Samples prepared this way have been shown to contain better than 95% isorhodopsin (Oseroff and Callender, 1974; Eyring and Mathies, 1979), and resonance Raman measurements on these samples and samples of opsin regenerated with 9-*cis* retinal are virtually identical (Mathies et al., 1976). Sample concentrations were about  $1 \text{ A cm}^{-1}$  at the absorption peaks near 500 nm. The absorbance ratios  $A_{400} : A_{500}$  and  $A_{280} : A_{500}$  were about 0.3 and 3, respectively. The absorbance ratio  $A_{530} : A_{500}$  was about 0.7 for rhodopsin and 0.5 for isorhodopsin.

All measurements were obtained at room temperature with the samples held in 1-cm cuvettes and carefully shielded from spurious light. The samples were stirred between successive laser shots, and mixed with a reservoir (about 30 ml) after about six shots. From standard UV-visible absorption spectroscopy, we estimated that 10% average sample degradation was accumulated during pulse excitation (about 100 shots) of rhodopsin and about 4% for isorhodopsin.

A schematic representation of the picosecond apparatus is shown in Fig. 1. A single 1.06- $\mu\text{m}$  pulse with a full width at half maximum of 9 ps was extracted from the output train of a mode-locked  $\text{Nd}^{3+}$  glass oscillator and amplified. The apparatus was as described previously (Green et al., 1977), except that the laser triggered spark gap was replaced by an Xonics electronic pulse generator (Xonics Inc., Santa Monica, Calif.). The second harmonic at 530 nm with a full width at half maximum of 7 ps was generated in a KDP (Potassium Dihydrogen Phosphate) crystal. The 530-nm pulse was separated from the fundamental with specially coated mirrors after using appropriate filters directed by additional mirrors to a delay prism. The pump beam was focused into the sample to initiate sample excitation. The delay prism was adjusted so that laser-induced absorbance changes were measured either before, during, or after sample excitation. The zero time was located by using a 1-cm  $\text{CS}_2$  Kerr optical shutter (Duguay and Hansen, 1969). A resolution time of about 4 ps was determined from the  $\text{CS}_2$  shutter prompt response curve. The fundamental at 1.06  $\mu\text{m}$  was filtered, then passed through a 15-cm cell containing  $\text{CCl}_4$  to produce a super broad band light continuum of about 9-ps duration throughout the visible region of the spectrum (Alfano and Shapiro, 1970*a,b,c*; 1971; 1974). Residual light at the

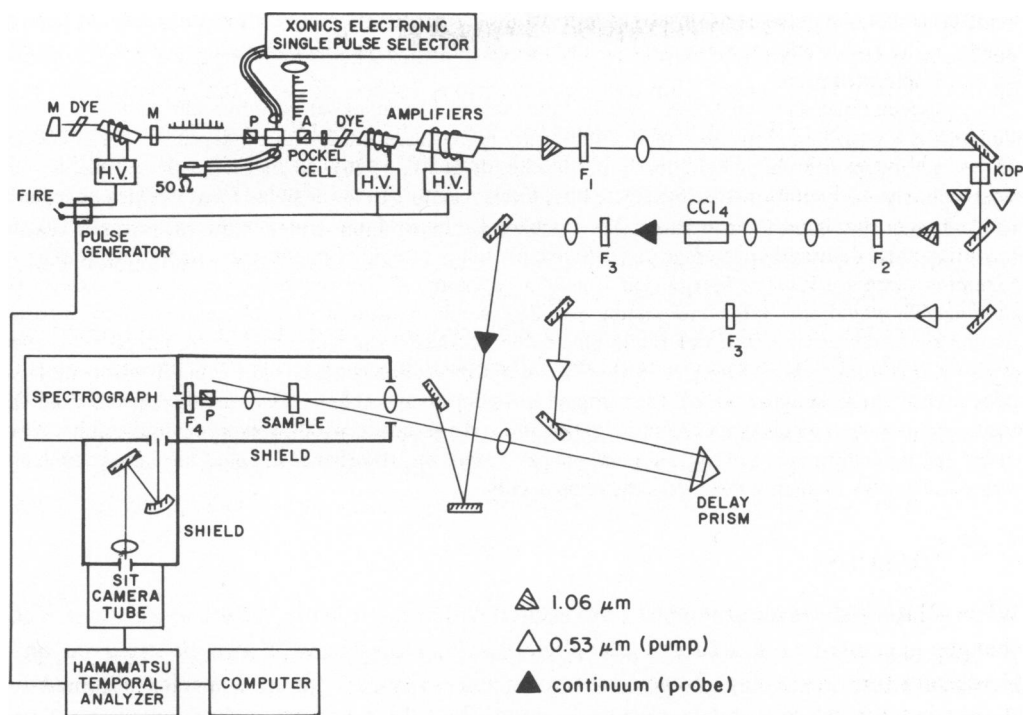


FIGURE 1 Schematic arrangement of the experimental system. The components include: an  $\text{Nd}^{3+}$  glass oscillator with cavity mirror  $M$  and saturable dye absorber; and Xonics electronic high voltage pulse generator with two crossed polarizers and a Pockel cell; a half-wave plate and "clean-up" saturable dye absorber; two  $\text{Nd}^{3+}$  glass amplifiers (gain  $\times 20$ ); filter  $F_1$  to eliminate flash lamp; a Lasermetric second harmonic generator (Lasermetrics, Inc., Teaneck, N.J.); specially coated mirrors to separate 1.06  $\mu\text{m}$  from 530nm; (in the 1.06- $\mu\text{m}$  path) filter  $F_2$  to eliminate 530 nm, a collimating lens system, a 15-cm  $\text{CCl}_4$  cell to produce the continuum; (in the continuum path) a filter  $F_3$  to eliminate 1.06  $\mu\text{m}$ , directing mirrors, a focusing lens on the sample, an imaging lens on the spectrograph, filter  $F_4$  to eliminate scattered light and to adjust the intensity of the input light; (after the spectrograph), a mirror, a directing mirror, a focusing lens onto a Hamamatsu model 145 temporal analyzer SIT video camera; (in the 530-nm path) filter  $F_3$  to eliminate 1.06  $\mu\text{m}$ , directing mirror, a delay prism, a focusing lens on the sample. The full width at half maximum for the 1.06- $\mu\text{m}$ , continuum, and 530-nm pulses are 9 ps, 9 ps, and 7 ps, respectively.

fundamental was removed by the appropriate filters. Mirrors and a focusing lens directed the continuum into a  $\frac{1}{2}$ -m spectrograph onto the Silicon Intensified Target (SIT) video camera of a Hamamatsu model 145 temporal analyzer detector system for data processing (Hamamatsu Corp., Middlesex, N.J.). Care was taken to minimize the effects of video camera lag. The system was calibrated for linearity. The camera and sample sites were carefully shielded from scattered light.

The picosecond apparatus described in Fig.1 produces pump and probe pulses that are polarized perpendicularly. In this case an additional Polaroid polarizer (Polaroid Corp., Cambridge, Mass.) minimized the amount of scattered pump light entering the spectrograph. For most of the measurements, Polaroid polarizers were placed in both the 530-nm and continuum light paths so that the pump and probe pulses were polarized in the same direction.

The energies of the 530-nm excitation pulse, about 0.6 mJ, and the probe pulse, about 0.2  $\mu\text{J}$ , were measured at the sample site with a Laser Precision Corporation (Yorkville, N.Y.) energy meter. The beam sizes were about 0.1  $\text{cm}^2$  for the pump and about 1  $\text{cm}^2$  for the probe. Two windows on the temporal analyzer were used to detect the continuum such that both the region of the sample where the pump and probe pulses overlapped and the unexcited sample region were monitored simultaneously for a

single laser shot. Absorbance changes during or after sample excitation were calculated from the ratio of counts in the two windows, normalized to the ratio obtained when the continuum probed the sample before sample excitation.

In picosecond measurements, care must be taken to avoid nonlinear effects. In addition, for the visual pigments it is possible to introduce photoequilibrium effects associated with rhodopsin  $\rightleftharpoons$  bathorhodopsin  $\rightleftharpoons$  isorhodopsin at high light levels (Goldschmidt et al., 1976). To minimize the problems, our measurements were performed at moderate light levels resulting in lower signal strength with, therefore, a reduction of the signal-to-noise ratios. We calculate for most of our experiments that a single 530-nm excitation pulse delivered an average of about one photon per visual pigment molecule in the pump path. Assuming there is a Poisson distribution of incident photons, 37% of the molecules would encounter no photon, 37% would encounter one photon, and 26% would encounter multiple photons. If we further assume that only ground states of rhodopsin, bathorhodopsin, and isorhodopsin absorb photons, then using the quantum yields (Hurley et al., 1977) and the absorption constants at the pump wavelength we calculate that at most about 30% of the sample for rhodopsin and about 10% for isorhodopsin within the excitation path are bleached per excitation pulse, in good agreement with the experimental results. Also, within the twofold range of excitation pulse energies used, the absorbance changes measured are linear with pulse energy, indicating that no saturation occurs.

## RESULTS

When solubilized rod outer segments are excited with a single 7-ps, 530-nm pulse, absorbance changes generated as a result of the photobleaching of rhodopsin can be observed on a picosecond time scale. Fig. 2 shows the room temperature difference spectra obtained for rhodopsin when the absorbance changes are measured during pump excitation (Fig. 2 *a*) and 100 ps after pump excitation (Fig. 2 *b*). The salient features of both curves are essentially identical. The difference spectra show a bleaching of the rhodopsin ground-state absorption band with a maximum- $\Delta A$  near 485 nm, the development of a strong absorption band in the red with a maximum  $\Delta A$  near 570 nm, and two isobestic points near 460 and 510 nm. In addition, the difference spectrum obtained at zero time (Fig. 2 *a*) may indicate a small absorption band near 670 nm that appears to be absent in the difference spectrum obtained 100 ps after excitation (Fig. 2 *b*).

For both difference spectra the 570-nm band and the isobestic point near 510 nm most likely reflect the formation of bathorhodopsin, the only intermediate in the rhodopsin photobleaching sequence known to absorb strongly to the red of the rhodopsin absorption band. The similarity of both difference spectra suggests that the rhodopsin-to-bathorhodopsin photoconversion is completed in less than our resolution time. In this region of the spectrum there is close agreement between our difference spectra and that generated from low temperature steady-state absorption measurements of rhodopsin in *Ammonyx-LO* (Applebury et al., 1974) and in digitonin (Yoshizawa and Wald, 1963; Pratt et al., 1964). The low temperature isobestic point is located about 5 nm to the red of the room temperature isobestic point shown in Fig. 2. This may be expected because the rhodopsin absorption band shifts slightly to the red upon cooling. The 570-nm band in our difference spectra also resembles that observed in the picosecond room temperature measurements previously reported by Sundstrom et al. (1977); however, for reasons that are not clear, their results show an isobestic point 15 nm red-shifted from ours.

Analysis of the blue region of the difference spectra shown in Fig. 2 is somewhat complicated. In the rhodopsin-to-bathorhodopsin photoconversion, the rhodopsin ground-state

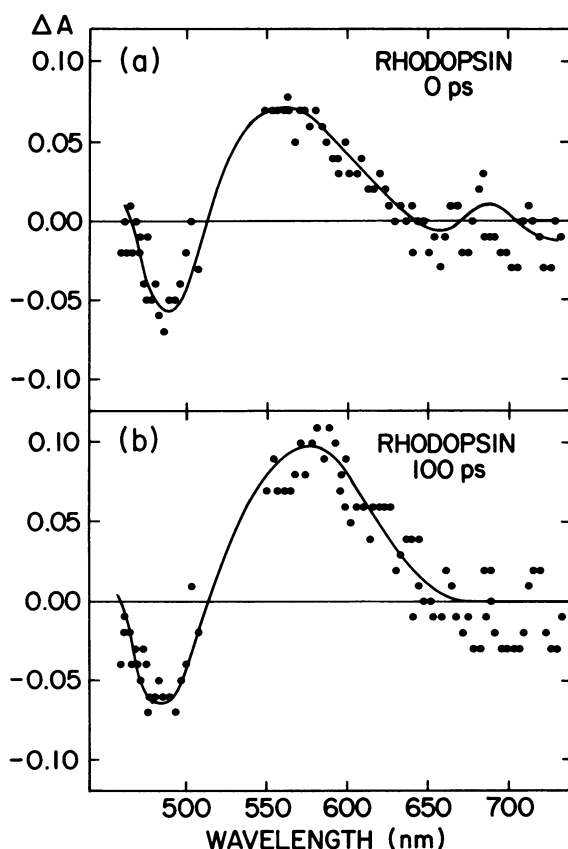


FIGURE 2 Laser-induced difference spectra for the formation of bathorhodopsin in detergent-solubilized bovine rhodopsin. Sample concentration was about  $0.9 \text{ A cm}^{-1}$ . Each data point shown is the average of about six laser shots. Typical data reproducibility is about  $\pm 0.02$ . (a) Absorbance changes measured during laser excitation (zero time). (b) Absorbance changes measured 100 ps after laser excitation. The yield of bathorhodopsin is about 20%.

absorption band is depleted, producing the bleaching near 485 nm. Low temperature steady-state absorption measurements (Yoshizawa and Wald, 1963; Pratt et al., 1964; Applebury et al., 1974) and the picosecond absorption measurements of Sundstrom et al. (1977) and Peters et al. (1977) suggest that this bleaching should extend from about 510 to 400 nm. However, Figs. 2 *a* and *b* show an isobestic point near 460 nm, indicating that additional positive absorbance changes are generated in this region of the spectrum. For convenience, we shall refer to this positive absorption change near 460 nm as the “blue transition.” To consider whether the “blue transition” is a property of the primary pigments, the blue region of the difference spectrum was measured for a photobleached rhodopsin sample. Within our data reproducibility, we could obtain no laser-induced absorbance changes for the bleached photoproduct. We shall consider possible sources of the “blue transition” in the Discussion.

When the pigment isorhodopsin absorbs a photon, the same consecutive series of thermal intermediates generated as a result of the photobleaching of rhodopsin can be observed. To

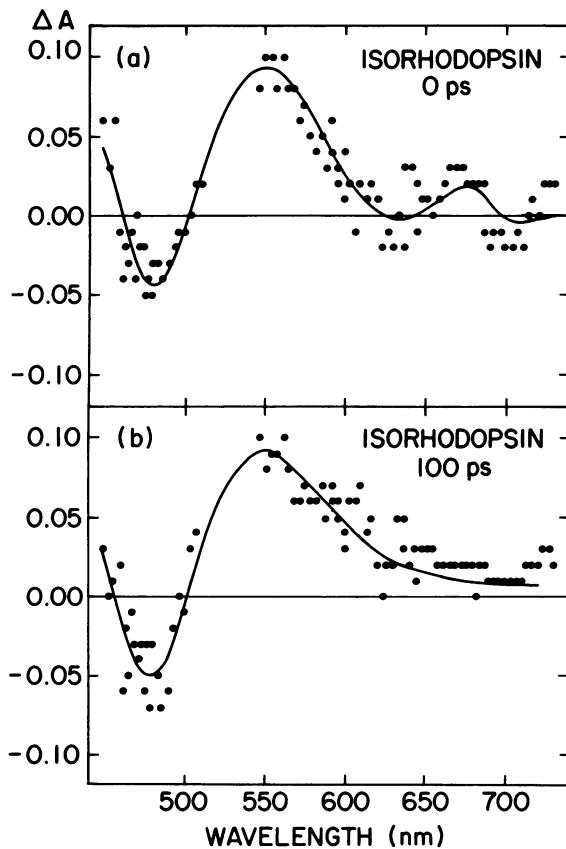


FIGURE 3 Laser-induced difference spectra for the formation of bathorhodopsin in detergent-solubilized bovine isorhodopsin. Sample concentration was about  $1 \text{ A cm}^{-1}$ . Each data point shown is the average of about six laser shots. Typical data reproducibility is about  $\pm 0.02$ . (a) Absorbance changes measured during laser excitation (zero time). (b) Absorbance changes measured 100 ps after laser excitation. The yield of bathorhodopsin is about 10%.

consider similarities in the primary photochemistry of both pigments, we examined the photobleaching of isorhodopsin on a picosecond time scale. Fig. 3 shows the room temperature difference spectra obtained with picosecond pulse excitation of isorhodopsin. Absorbance changes are measured during pump excitation in Fig. 3 *a* and 100 ps after pump excitation in Fig. 3 *b*. As with rhodopsin, the salient features of both curves are essentially identical. The difference spectra show a bleaching of the isorhodopsin ground-state absorption band with a maximum  $-\Delta A$  near 480 nm, the development of a strong absorption band in the red, with a maximum  $\Delta A$  near 550 nm, and two isobestic points near 460 and 500 nm. In addition, the zero time difference spectrum (Fig. 3 *a*) may show a small absorption band near 670 nm which is not apparent in the difference spectrum obtained 100 ps after excitation (Fig. 3 *b*). The similarity of both difference spectra and the close agreement between Fig. 3 and the difference spectrum that can be generated for the isorhodopsin-to-bathorhodopsin photoconversion from low temperature steady-state absorption measurements (Yoshizawa and Horiuchi, 1973) suggest that the photochemical formation of bathorhodopsin from isorhodopsin at

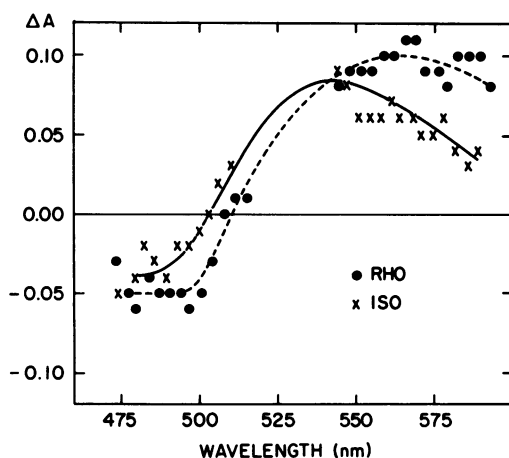


FIGURE 4 Isobestic regions of the laser-induced difference spectra measured at zero time for the formation of bathorhodopsin in detergent-solubilized bovine rhodopsin and isorhodopsin. For these measurements the pump and probe pulses are polarized perpendicularly. Sample concentrations were about  $1 \text{ A cm}^{-1}$ . Each data point shown is the average of about six laser shots. Typical data reproducibility is about  $\pm 0.02$ .

room temperature is completed in less than our resolution time. As with rhodopsin, the only feature of the isorhodopsin difference spectrum that is not in accord with the low temperature studies occurs in the blue region of the spectrum. The isobestic point near 460 nm indicates that the blue transition is partially masking the depletion of the isorhodopsin ground-state absorption band. That the blue transition is observed similarly for both rhodopsin and isorhodopsin suggests that we are not observing photoequilibrium effects associated with rhodopsin  $\rightleftharpoons$  bathorhodopsin  $\rightleftharpoons$  isorhodopsin photo-interconversions (Goldschmidt et al., 1976).

Perhaps the most precise way to compare bathorhodopsin formation in the 11-*cis* and 9-*cis* primary pigments is to reexamine the isobestic region near the bathorhodopsin absorption band. If bathorhodopsin generated from rhodopsin is the same chemical species as bathorhodopsin generated from isorhodopsin, then this region of the difference spectra should be similar for both primary pigments, except that the isobestic points should be shifted by about 5 nm, reflecting the  $\lambda_{\text{max}}$  difference in the room temperature rhodopsin and isorhodopsin ground-state absorption bands. Fig. 4 shows that the expected shift in the isobestic points is observed.

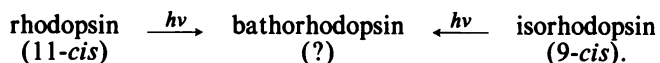
## DISCUSSION

There has been much recent interest in the underlying mechanism concerning the primary event in vision, namely bathorhodopsin formation. The rhodopsin-bathorhodopsin reaction has been thought to involve a photochemical 11-*cis* to all-*trans* isomerization (Yoshizawa and Wald, 1963; Rosenfeld et al., 1977). However, other models not involving *cis-trans* isomerization have been proposed recently (van der Meer et al., 1976; Peters et al., 1977).

The central argument for the *cis-trans* isomerization model as the primary event in vision is that a single common intermediate, namely bathorhodopsin, is formed photochemically from

an 11-*cis* chromophore (rhodopsin) and a 9-*cis* chromophore (isorhodopsin) and that these three pigments are photochemically interconvertible. Then, the bathorhodopsin chromophore being the common photoproduct of two different *cis* isomers is most naturally thought of as having a *trans* conformation (Yoshizawa and Wald, 1963; Rosenfeld et al., 1977). The evidence for a single bathorhodopsin intermediate is quite strong. Low temperature (77°K) photostationary-state absorption experiments can be easily understood in terms of a three component (rhodopsin, isorhodopsin, and bathorhodopsin) analysis (Yoshizawa and Wald, 1963). This picture is supported by the fact that rhodopsin and isorhodopsin are interconvertible by light through bathorhodopsin at 4 K (resonance Raman experiments; Aton et al., 1978), 77 K (resonance Raman experiments; Oseroff and Callender, 1974), and at room temperature (absorption experiments; Goldschmidt et al., 1976; resonance Raman measurements; Eyring and Mathies, 1979).

In addition, we have augmented these arguments by showing that *cis-trans* isomerization does take place in a picosecond time scale even for molecules as large as retinal (Green et al., 1977). In this work, we measured the formation time of bathorhodopsin from both isorhodopsin and rhodopsin. We and others showed that the room temperature bathorhodopsin formation time from rhodopsin is less than 3 ps (Bush et al., 1973; Peters et al., 1977; Green et al., 1977) and, additionally, that the bathorhodopsin formation time from isorhodopsin was also 3 ps at room temperature. In our interpretation of our results, the photochemical reactions were assumed to be (Yoshizawa and Wald, 1963):



Assuming there is a single bathorhodopsin, it could have a chromophore conformation of 11-*cis*, 9-*cis*, or some intermediate structure ("transoid"). Under any one of these three conformations, one or the other of the two photoreactions must involve a geometric isomerization.

Our present findings provide additional strong support for the *cis-trans* model of the primary event in vision for a variety of reasons. First, we have shown here that the same bathorhodopsin absorption results (within our signal-to-noise) are obtained for both the 11-*cis* (rhodopsin) and the 9-*cis* (isorhodopsin) primary pigments on a picosecond time scale. The bathorhodopsin absorption band (near 570 nm) is visible in the zero time difference spectra of both primary pigments (Figs. 2 *a* and 3 *a*) and appears essentially unchanged in the difference spectra measured 100 ps after pump excitation (Figs. 2 *b* and 3 *b*). These results strongly suggest that the rhodopsin-bathorhodopsin and isorhodopsin-bathorhodopsin transitions are completed within our 3-ps resolution time and that these photoconversions have essentially the same kinetic behavior. Second, our results are at room temperature and so remove any concern that the photoconversions observed at 77°K may be different from those closer to physiological temperatures. Third, the results augment the argument by Green et al. (1977), showing that photoisomerization does take place in the picosecond time scale in one or both of the photoconversions from the primary pigments to bathorhodopsin. In the experiment of Green et al. (1977) only one absorption point indicating bathorhodopsin formation was measured; here the entire visible spectrum is determined.

It seems difficult to rationalize all these results with any model not involving *cis-trans*



isomerization. The only model not involving *cis-trans* isomerization but which attempted to include isorhodopsin into its specific scheme is that of van der Meer et al. (1976). In this case, bathorhodopsin was proposed to be a mixture of two spectroscopically indistinguishable species in thermal equilibrium, one having an 11-*cis* retinal and the other a 9-*cis* retinal. However, this has been shown to be incorrect (Aton et al., 1978) because isorhodopsin has been shown to be formed from rhodopsin at 5.5°K, a temperature too low to permit the establishment of thermal equilibrium between the species suggested by van der Meer et al. (1976). Peters et al. (1977) have suggested a proton translocation as the essential step in the rhodopsin-to-bathorhodopsin transformation. However, they did not incorporate isorhodopsin into their model, and it seems clear that this must be done in view of our results.

The possibility of a transiently observed band, red-shifted from the absorption maximum of bathorhodopsin, is of special interest in light of two recent reports. Aton et al. (1978) have proposed a model for the visual process that includes a bathorhodopsin ground-state precursor. Briefly, bathorhodopsin is formed by a *cis-trans* isomerization during which the Schiff base proton accompanies the nitrogen (because of increased electron density at the nitrogen in the chromophore excited state), but the chromophore is temporarily without a counter-ion in a ground-state configuration. A protonated Schiff base without a counter-ion would result in a more red-shifted chromophore. Subsequent fast protein relaxations to reform in some way a Schiff base counter-ion would then blue shift this absorption spectrum and result in the final bathorhodopsin spectrum. The details of this model will appear elsewhere (Honig et al., 1979). In addition, a red-shifted band is observed in picosecond studies of bacteriorhodopsin (Applebury et al., 1978). In this case it has been shown that there exists a precursor state to *K* (the batho product of this system) formation that exhibits a red-shifted absorption band to the absorption band of *K*.

Our data suggest that a small absorption band is observed (with low signal-to-noise) near 670 nm in the zero time difference spectra obtained for both primary pigments (Figs. 2 *a* and 3 *a*). It seems most likely to represent a precursor of bathorhodopsin with a lifetime at or less than our resolution time. That it may be a precursor follows from the fact that it is absent in the difference spectra obtained at latter times (Figs. 2 *b* and 3 *b*). This interpretation is further supported by the fact (Rosenfeld et al., 1977) that the quantum yields for the forward and backward reactions in the rhodopsin-bathorhodopsin photoreaction sum to one. As has been pointed out by Rosenfeld et al. (1977), that the quantum yields sum to one implies that only two photochemical pathways are available when rhodopsin absorbs a photon, namely bathorhodopsin formation and rhodopsin reformation. The picosecond absorption kinetics of rhodopsin measured at liquid helium temperatures (Peters et al., 1977) suggest there are no ground-state precursors generated during rhodopsin reformation. The 670-nm band would then represent a ground-state precursor to bathorhodopsin or the common excited state precursor to both pigments. If the 670-nm band is a bathorhodopsin precursor, then at later times there should be additional absorption strength in the bathorhodopsin spectra compared to earlier times. While there is some evidence for this in the rhodopsin spectra (Fig. 2 *a* compared to 2 *b*) the opposite is apparent in the isorhodopsin spectra (Fig. 3 *a* compared to 3 *b*). Further work on the existence and kinetic behavior of this 670-nm band is clearly required, since our signal-to-noise is not sufficient to come to clear conclusions.

We finally consider the source of the "blue transition" that limits the extent of bleaching

observed for rhodopsin and isorhodopsin. Perhaps the most attractive explanation would be that we are observing formation of hypsorhodopsin (Yoshizawa and Horiuchi, 1973). Recently Shichida et al. (1978) have observed the formation of hypsorhodopsin from squid rhodopsin at room temperature using 347-nm picosecond pulse excitation. Their room temperature and liquid nitrogen temperature results clearly show that hypsorhodopsin is a precursor of bathorhodopsin in the squid rhodopsin photobleaching sequence. This could not be the case for our results, since the blue transition does not appear to precede bathorhodopsin formation, but rather to parallel it, persisting for at least 100 ps. In addition, Bensasson et al. (1977) have generated a difference spectrum in nanosecond flash photolysis of cattle rhodopsin with 530-nm light at room temperature, which shows an initial transient absorption at 420 nm with an isobestic point near 460 nm. They report a lifetime for the 420-nm transient similar to that observed for bathorhodopsin. It thus appears that if we assign the blue transition to hypsorhodopsin, then the sequential formation of hypsorhodopsin and bathorhodopsin is peculiar to the squid rhodopsin system and not a general feature of the visual process. While ascribing the blue transition to hypsorhodopsin seems unlikely from a kinetic standpoint, other explanations, such as beta band absorption in bathorhodopsin, or excitation by energy transfer of a forbidden transition in the protein moiety, appear less acceptable. Further work is required to clarify the source of blue transition and to determine why the blue transition is not apparent in the picosecond results of Sundstrom et al. (1977). We are currently performing additional experiments toward this end.

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