BATHOPRODUCTS OF RHODOPSIN, ISORHODOPSIN I, AND ISORHODOPSIN II

BORYEU MAO and THOMAS G. EBREY, *Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801*
ROSALIE CROUCH, *Departments of Biochemistry and Ophthalmology, Medical University of South Carolina, Charleston, South Carolina 29403 U.S.A.*

ABSTRACT Bathorhodopsins were prepared by partially (10–15%) photoconverting bovine rhodopsin (11-*cis* chromophore) or isorhodopsin I (9-*cis* chromophore) at 77°K; care was taken to avoid establishing photostationary states. The absorption spectra calculated for the bathorhodopsins derived from the two parent pigments are identical in their λ_{max}'s, bandwidths, and extinction coefficients. This result provides further support for the hypothesis that bathorhodopsin is a common intermediate between an 11-*cis* pigment (rhodopsin) and a 9-*cis* one (isorhodopsin I) and thus probably has an all-*trans* chromophore. This in turn is strong evidence for the *cis-trans* isomerization model of the primary event in vision. The spectrum of the bathoproduct of isorhodopsin II (9,13-di*cis* chromophore) is different from the other pigments' bathoproducts.

INTRODUCTION

The bleaching of the visual pigment rhodopsin begins with an initial absorption of light followed by a series of absorption changes that characterize the intermediates of bleaching. These absorption changes have been studied at temperatures as low as 4°K and on time scales as fast as several picoseconds after photon absorption (Yoshizawa and Wald, 1963; Yoshizawa, 1972; Peters et al., 1977; Green et al., 1977). The first photoproduct observed in photostationary state mixtures at 77°K is bathorhodopsin (also called prelumirhodopsin) whose absorption maximum is red-shifted from rhodopsin (Yoshizawa and Wald, 1963). Bathorhodopsin is also seen in photostationary state mixtures at 77°K; with appropriate exciting wavelengths, such a mixture can be converted into one that contains predominantly isorhodopsin I, whose absorption maximum is at 485 nm, blue-shifted by 15 nm from rhodopsin (Yoshizawa and Wald, 1963; Oseroff and Callender, 1974). The chromophore of rhodopsin is 11-*cis* retinal whereas that of isorhodopsin I is 9-*cis* retinal.

Because bathorhodopsin can be formed from both the 11-*cis* pigment and the 9-*cis* pigment, it has been argued that bathorhodopsin contains all-*trans* retinal as its chromophore so that the interconversions at 77°K follow the scheme (Yoshizawa and Wald, 1963; Rosenfeld et al., 1977; Hurley et al., 1977):

$$\text{Rhodopsin (Rh)} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{Bathorhodopsin (Batho)} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{Isorhodopsin I (Iso-I)}$$

Scheme 1

As the above observations were based on measurements on photostationary state mixtures, the

following type of conversion scheme could not be excluded:

$$\text{Rh} \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{Batho}' \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{Batho}'' \underset{h\nu}{\overset{h\nu}{\rightleftharpoons}} \text{Iso-I}$$

Scheme 2

Batho' and Batho" in this scheme would be two different species and presumably have different absorption spectra. The assignment of all-*trans* to the conformation of the chromophore of either Batho' or Batho", or both, would be more difficult, although clearly isomerization still has to take place at 77°K on going from rhodopsin to isorhodopsin I. Under Scheme 2, what was previously thought to be the spectrum of bathorhodopsin would then be a composite spectrum of a mixture of Batho' and Batho".

For Scheme 1 to be valid, it is necessary (but not sufficient) that Batho' and Batho" in Scheme 2 be spectrally identical. Experimentally, this can be achieved, starting from either rhodopsin or isorhodopsin, by converting only small fractions of the parent pigments into their respective bathoproducts (without forming photostationary states), and then comparing their absorption spectra. In addition, the spectrum of bathorhodopsin derived from photostationary state mixtures can be compared with the individually measured spectra of the bathoproducts.

Using the reported spectrum of bathorhodopsin (Yoshizawa and Wald, 1968) and the values for quantum efficiencies of pigment conversion (Hurley et al., 1977) it can be estimated that converting ~20% of either Rh or Iso-I into its bathoproduct will give <1% of the third pigment in Scheme 1. At lower levels of conversion, this fraction is reduced even further. Typically a 15% conversion was used in the experiment to be described. Estimates of the third component can be reevaluated after spectra of Batho' and Batho" are obtained.

For the bathoproduct of Iso-I, isorhodopsin is obtained by either regenerating opsin with 9-*cis* retinal (Iso-I_r) or irradiating rhodopsin at 77°K with 580 nm light (Iso-$I_{h\nu}$). (580 nm light drives rhodopsin or a mixture containing bathorhodopsin and rhodopsin to almost pure isorhodopsin [Yoshizawa and Wald, 1963; Maeda et al., 1978].) The photoregenerated isorhodopsin I, presumably having a minimally perturbed protein conformation, is found to have a bathoproduct very similar to that of rhodopsin. The bathoproduct of the isorhodopsin I regenerated from 9-*cis* retinal has an absorption spectrum of similar shape and position but with a slightly higher extinction.

Besides rhodopsin and isorhodopsin I, there is a third stable pigment, isorhodopsin II (Iso-II), whose chromophore is 9,13–di*cis* retinal (Crouch et al., 1975). It is photobleachable at room temperature, and the final photo product is also all-*trans* retinal. The spectrum of the "bathoproduct" of Iso-II appears to be quite different from the spectrum of bathorhodopsin derived from rhodopsin or isorhodopsin I.

MATERIALS AND METHODS

Preparations of Rhodopsin and Regenerated Isorhodopsins

Bovine rod outer segment (ROS) membranes are purified according to Ebrey (1971) and rhodopsin solubilized with 2% digitonin in 67 mM phosphate buffer, pH 7.0. Iso-I_r or Iso-II are prepared from regenerating opsin contained in purified ROS membranes with either 9-*cis* (Iso-I_r) or 9,13-di*cis* retinal (Iso-II) (Ebrey et al., 1975) and then solubilizing the membranes as above. The purity of both isomers are checked by high-performance liquid chromatography (HPLC) before regeneration (Crouch et al., 1975).

The solubilized pigments are mixed with two volumes of glycerol, which allows formation of optical glasses at 77°K. The samples also contain 0.2 M NH_2OH, which combines with the bleaching product all-*trans* retinal to form retinal oxime.

Preparation of Iso-$I_{h\nu}$ at 77°K

Rhodopsin is cooled to 77°K in a 2-mm path length cuvette and then illuminated for 3 h with 580 nm light (combination of a 400-W projector lamp and a 580-nm interference filter, band pass 20 nm) until no further absorption change is observed. The absorption spectra at 77°K before and after the illumination are shown in Fig. 1 *a*.

Fig. 1 *b* shows the absorption spectrum of rhodopsin before cooling and that of the pigment formed with irradiation of 77°K and subsequently warmed to room temperature. This spectrum, λ_{max} at 485 nm, indicates that the photoconverted pigment is isorhodopsin. A similar conversion procedure has been used for rhodopsin in cattle ROS membrane (Oseroff and Callender, 1974; Maeda et al., 1978). The resonance Raman spectrum of the photoconverted pigment is very similar if not identical to that of Iso-I_r, chemically regenerated from 9-*cis* retinal (Mathies et al., 1976). Also the chromophore of the photoconverted pigment, when extracted and analyzed on HPLC, is found to be the 9-*cis* isomer of retinal (Maeda et al., 1978). Direct confirmation by a simple HPLC experiment could not be performed with our samples, due to the detergent isomerizing the extracted retinals (Crouch et al., 1975). However, the absence of a significant amount of rhodopsin in our photoregenerated isorhodopsin samples was also suggested by our finding that the ratio of the extinction coefficient of isorhodopsin I to that of rhodopsin in Fig. 1 *b* is ~1.06, very close to the ratio of the published values of the isorhodopsin and rhodopsin extinction coefficients, 43,000/40,600 (Wald and Brown, 1953, and Hubbard, 1956). The amount of bathorhodopsin in the 77°K sample in Fig. 1 is also very small, as measured by the negligible absorption change at 365 nm due to retinal oxime in Fig. 1 *b*.

The isorhodopsin I so formed at 77°K with 580 nm illumination can be used immediately for conversion to bathorhodopsin (Iso-$I_{h\nu}$), or it can be warmed to room temperature and then recooled to 77°K before conversion (Iso-$I_{h\nu/\Delta}$). A possible difference between Iso-$I_{h\nu}$ and Iso-$I_{h\nu/\Delta}$ at 77°K is that

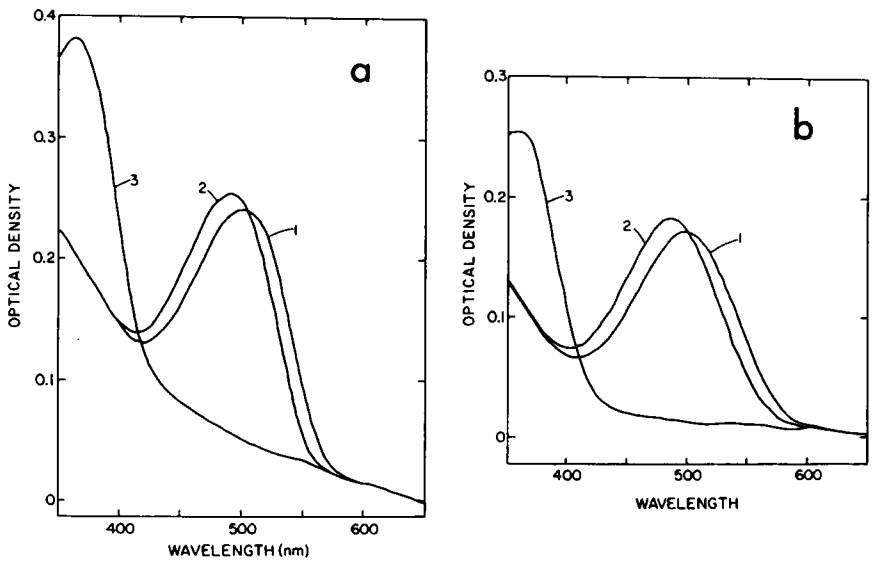

FIGURE 1 Photoconversion of isorhodopsin I from detergent-solubilized rhodopsin. (*a*) Spectra of rhodopsin (curve 1) and isorhodopsin I (curve 2) at 77°K. The conversion to isorhodopsin was achieved by 580 nm irradiation for 3 hr. (*b*) Spectra of rhodopsin (curve 1) before the cooling and irradiation, and isorhodopsin I (curve 2) at room temperature. Curve 3 in either *a* or *b* is the totally bleached sample.

Iso-$I_{h\nu/\Delta}$ could adjust its conformation to the 9-*cis* chromophore, whereas Iso-$I_{h\nu}$ should have a protein environment that is close to, if not identical to, that of rhodopsin from which Iso-$I_{h\nu}$ was made photochemically at 77°K.

Absorption Measurements at 77° K

A 2-mm path length quartz cuvette containing the sample is attached to the holder at the end of a cold finger. This is then placed in a dewar with optical windows, which is gradually evacuated while liquid nitrogen is slowly poured into the cold finger. The sample cracking causes an increase in light scattering affecting absorption measurements. The effect is reduced by placing a piece of ground (opal) glass behind the cuvette and another in the reference beam of the spectrophotometer (Varian Instrument, Monrovia, Calif.; Cary model 118C).

After cooling to the liquid nitrogen temperature, the sample is allowed to fully equilibrate for 1 h before the experiment. Little or no drift in the base line is observed during measurements as measured by the absorbance at 700 nm. For ΔOD^i required below, the spectra are measured on expanded absorbance scales.

The optical dewar is securely fixed to the bottom of the spectrophotometer sample chamber. For the conversion to the bathorhodopsin, the sample is irradiated in situ with the light (480-nm interference filter) passing through a hole cut on the side of the spectrophotometer and reflected onto the sample by a removable mirror. This is necessary for making the accurate measurements needed below.

Calculation of the Absorption Spectra of Bathorhodopsin

The following equation summarizes the method of calculating the spectrum of bathorhodopsin and the experimental quantities needed:

$$\epsilon^i_{B(p)} = \epsilon_p + \frac{\Delta OD^i_{B\text{-}p}}{f^i} \cdot g^i, \tag{1}$$

where p = pigment (i.e., Rh, Iso-I_r, Iso-$I_{h\nu}$, Iso-$I_{h\nu/\Delta}$, or Iso-II); $\epsilon^i_{B(p)}$ is the calculated spectrum of Batho from p at 77° K in the ith experiment for p, i = 1–5 or 7; ϵ_p is the extinction spectrum of the parent pigment p at 77°K; $\Delta OD^i_{B\text{-}p}$ is the difference optical density spectrum between Batho and its parent pigment p of 77°K in the ith experiment; f^i is the fraction of p that is converted to Batho in the ith experiment; g^i is a scaling factor in the ith experiment so that the calculated spectra of Bathos are comparable on an absolute scale of extinction coefficients.

A set of spectra of the pigments, ϵ_p, are first determined at 77°K. The corresponding set of spectra at room temperature, ϵ^{RT}_p, are also recorded. They were normalized to identical pigment concentrations, with (*a*) the ratio of $\epsilon_{\text{Iso-I}_{h\nu}}(\lambda_{max})$ to $\epsilon_{Rh}(\lambda_{max})$ taken from Fig. 2 *a*, (ϵ^{RT}_{Rh}[500 nm] = 40,600), (*b*) $\epsilon_{\text{Iso-I}_{h\nu/\Delta}}(\lambda_{max})$ equal to $\epsilon_{\text{Iso-I}_{h\nu}}(\lambda_{max})$ from experimental observations, and (*c*) $\epsilon_{\text{Iso-I}_r}(\lambda_{max})$ and $\epsilon_{\text{Iso-II}}(\lambda_{max})$ equal to $\epsilon_{\text{Iso-I}_{h\nu}}(\lambda_{max})$ by assumption. In a single experiment for any given pigment, four spectra are recorded in the following sequence: No. 1 (at room temperature) and No. 2 (at 77°K) before the irradiation with 480 nm light at 77°K for ~15% conversion, then No. 3 (at 77°K) and No. 4 (at room temperature) after the irradiation. The completely bleached sample gives base lines for these spectra between 440 and 630 nm. The difference between No. 3 and No. 2 gives $\Delta OD^i_{B\text{-}p}$, whereas the ratio of No. 4 to No. 1 gives f^i. The ratio of ϵ^{RT}_p to No. 1 gives g^i, which scales $\Delta OD^i_{B\text{-}p}$ to the concentration of ϵ_p. With the scaling, $\epsilon^i_{B(p)}$ from all the experiments for all the parent pigments are of identical concentrations and hence are directly comparable.

RESULTS

Spectra of Bathorhodopsin Derived from Rh, Iso-I_r, Iso-$I_{h\nu}$, and Iso-$I_{h\nu/\Delta}$

The primary concern of this report is to obtain spectra of bathorhodopsin from various parent pigments without establishing photostationary state mixtures. Specifically, we wish to obtain mixtures containing just the parent pigment and its bathoproduct, without significant amounts of other components. In general, a low-level (10–20%) conversion of a pigment to its

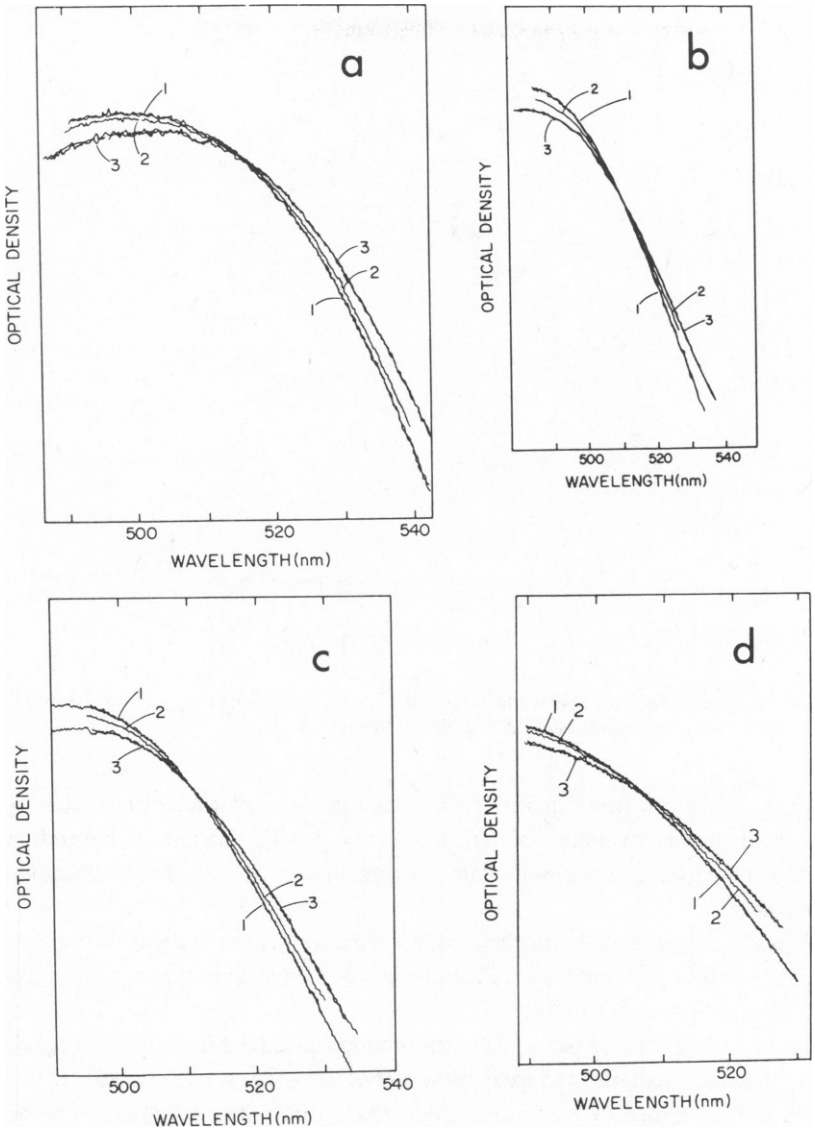

FIGURE 2 Observations of the approximate isosbestic point during initial conversions of four parent pigments into bathoproducts with 480 nm light. Spectra are recorded in regions of interests on expanded scales for clarity. Little or no drift in the base line occurs during the conversion, as measured by an unchanged absorbance at 700 nm. The full scale of optical density shown is ~0.1. (*a*) Rh; curve 3 represents ~13% conversion. (*b*) Iso-I_r; curve 3 represents ~9% conversion. (*c*) Iso-I_{bs}; curve 3 represents ~15% conversion. (*d*) Iso-II; curve 3 represents ~17% conversion.

bathoproduct will meet this condition. An independent verification of the presence of just two species after irradiation is the observation of an isosbestic point during the initial photoconversion, which would ensure that only the parent pigment and the bathoproduct are involved. When the concentration of a third component starts to increase significantly and the mixture starts to approach the photostationary state, the crossover point will progressively deviate

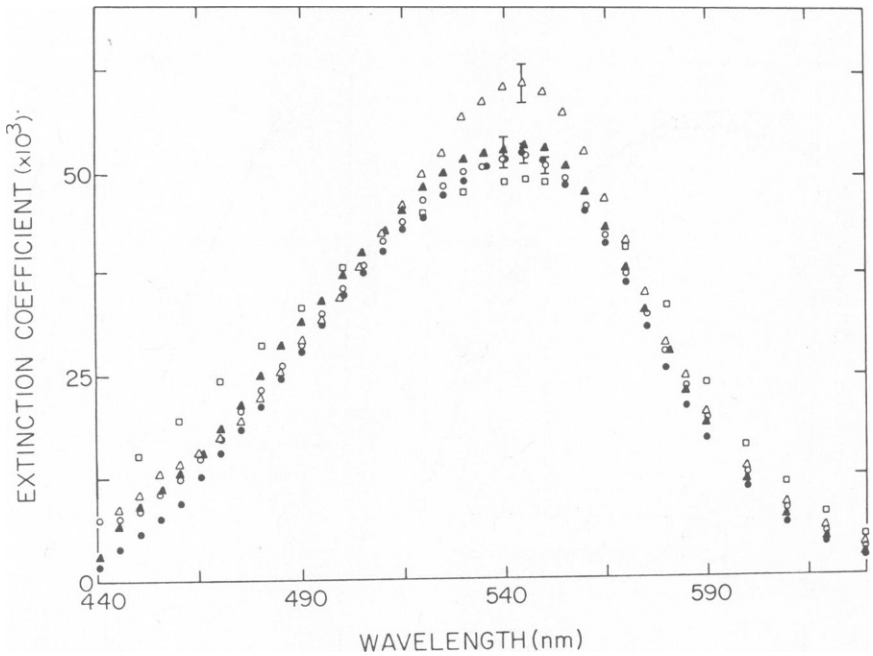

FIGURE 3 Calculated spectra of bathorhodopsin from Rh (○), Iso-I_r (△), Iso-$I_{h\nu/\Delta}$ (●), and Iso-$I_{h\nu}$ (▲). The spectrum from Yoshizawa and Wald (1963) is also included (□).

from the approximate isosbestic point of the initial conversion. Our observation of isosbestic points in typical photoconversions of Rh, Iso-I_r, and Iso-$I_{h\nu}$ to their bathoproducts (Fig. 2) confirms the expectation of the low-percent conversion experiment, that bathorhodopsin is the only photoproduct.

Fig. 3 shows the spectra of bathorhodopsins calculated from five to seven experiments for each of the four parent pigments. Table I summarizes various experimental quantities that are of interest, for all of the experiments.

From Fig. 3, it is clear that spectra of bathorhodopsin derived from Rh, Iso-$I_{h\nu}$, and Iso-$I_{h\nu/\Delta}$ are similar in their λ_{max}, shape, and extinction coefficients. They are also similar to the spectra of bathorhodopsin obtained by Yoshizawa and Wald (1963). The spectrum of bathorhodopsin derived from Iso-I_r, although similar in shape and λ_{max}, seems to have a slightly higher extinction coefficient than the other pigments.

Spectrum of "Bathorhodopsin" Derived from Iso-II

For comparison with the bathorhodopsins of Rh and Iso-I, Iso-II was photoconverted to a mixture of ~15% bathoproduct. Up to this level of photoconversion however, the successive crossovers do not appear to be isosbestic (Fig. 2 *d*). The average spectrum of the "bathorhodopsin" of Iso-II calculated as above is shown in Fig. 4. Compared to the bathorhodopsins of the other pigments, the absorption maximum is blue-shifted, the bandwidth is broader, and the extinction coefficient is lower. Preliminary data indicate that the photochemical behavior of Iso-II at 77°K may be different from those of Rh and Iso-I and will require further experiments.

TABLE I

CONVERSION TO BATHOPRODUCTS OF FIVE DIFFERENT PARENT PIGMENTS

Expt. no.	f	λ_{max}^{Batho}	λ_x	$\epsilon_{B(p)}$
Rh (λ_{max}^{Batho} = 542 ± 1 nm)		*nm*	*nm*	
1	0.20	541 ± 1	514	54,100
2	0.13	542 ± 1	516	51,200
3	0.12	541 ± 1	515	52,300
4	0.18	542 ± 1	515	51,800
5	0.13	543 ± 1	512	53,100
Average				52,500 ± 500
Iso-I_{hv} (λ_{max}^{Batho} = 542 ± 1 nm)				
1	0.13	541 ± 1	509	53,500
2	0.16	542 ± 1	512	51,100
3	0.09	546 ± 2	510	54,900
4	0.11	542 ± 1	508	53,500
5	0.08	550 ± 4	508	60,200
6	0.12	545 ± 2	510	57,800
7	0.16	538 ± 2	509	44,100
Average				53,600 ± 2,000
Iso-$I_{hv/\Delta}$ (λ_{max}^{Batho} = 544 ± 1 nm)				
1	0.20	544 ± 1	512	52,200
2	0.13	544 ± 2	512	55,500
3	0.12	542 ± 1	510	52,400
4	0.08	542 ± 1	510	53,700
5	0.10	542 ± 1	512	49,900
Average				52,700 ± 900
Iso-I_r (λ_{max}^{Batho} = 543 ± 1 nm)				
1	0.09	541 ± 1	505	64,200
2	0.07	543 ± 1	510	57,800
3	0.07	547 ± 2	508	73,600
4	0.11	542 ± 1	507	52,300
5	0.10	544 ± 1	506	62,900
6	0.10	542 ± 1	505	65,000
7	0.14	542 ± 1	506	54,800
Average				61,500 ± 2,700
Iso-II (λ_{max}^{Batho} = 532 ± 2 nm)				
1	0.17	533 ± 1	(505)	46,900
2	0.17	531 ± 1	(505)	48,200
3	0.13	530 ± 1	(507)	45,800
4	0.10	530 ± 1	(508)	52,000
5	0.15	534 ± 1	(508)	47,800
Average				48,100 ± 1,000

f is the fraction of the parent pigment that is converted into Batho. λ_{max}, λ_x are the wavelengths of maximum absorption and crossover, respectively, in the calculated spectrum of Batho for individual experiments. λ_{max}^{Batho} is the wavelength of maximum absorption in the averaged spectrum of the bathoproduct. $\epsilon_{B(p)}$ is the calculated molar extinction coefficient of the bathoproduct assuming ϵ_{Rh} = 40,600 and $\epsilon_{Iso\text{-}I_{hv}}$ = 43,000.

DISCUSSION

In calculating the spectrum of bathorhodopsin, Yoshizawa and Wald (1963) had used photostationary state measurements of 77°K. From the observation that isorhodopsin I can be photoregenerated from rhodopsin at that temperature, it was then concluded that isorhodop-

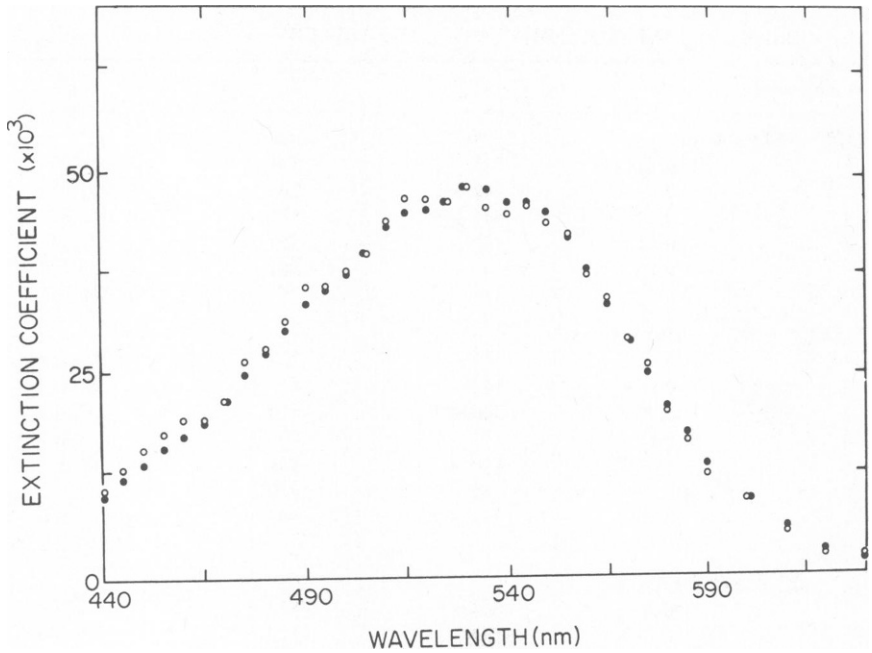

FIGURE 4 The calculated spectrum of the "bathoproduct" of isorhodopsin II. Results of a typical experiment (○) and the average of five experiments (●) are plotted.

sin I is also photoconverted into the same Batho species. This conclusion is important to the argument that the chromophore of bathorhodopsin is an all-*trans* retinal, and that the *cis-trans* isomerization, proposed first by Kropf and Hubbard (1958), and generalized to the photoreactions at 77°K by Yoshizawa and Wald (1963), is the primary photochemical reaction of visual pigments (Rosenfeld et al., 1977; Hurley et al., 1977). In experiments reported here, bathorhodopsins are generated from rhodopsin and isorhodopsin I without establishing photostationary states. The results, shown in Fig. 3 and Table I, show that at 77°K, the bathoproduct from isorhodopsin I is spectrally identical to the bathorhodopsin from rhodopsin. The agreement among the extinction coefficients, λ_{max}, and the bandwidths strongly support the notions that the two bathorhodopsins are in fact the same species. Scheme 1 described in the Introduction is therefore an appropriate description of the photochemical interconversions of the pigments at 77°K. As also shown in Fig. 3, the spectra of bathorhodopsin in low-level conversions are spectrally identical to that in photostationary states reported by Yoshizawa and Wald (1963), as expected from Scheme 1.

Recently kinetic measurements of bathorhodopsin generated from rhodopsin and isorhodopsin (photoconverted from rhodopsin at 77°K) with picosecond excitation at room temperature have been reported (Monger et al., 1979). Difference spectra between rhodopsin and bathorhodopsin and between isorhodopsin and bathorhodopsin were measured 100 ps after excitation at room temperature. The crossovers Monger et al. (1979) found for the Rh/Batho and the Iso-I/Batho difference spectra are near 510 and 500 nm, respectively. These values are blue-shifted slightly from the corresponding values we report in Table I, as expected from the temperature dependence of the absorption spectra of the pigments. However, both sets of

crossover points are in disagreement with the recently reported values (at room temperature) of ~525 nm for Rh/Batho (Peters et al., 1977) and ~530 nm for Iso-I/Batho (Applebury et al., 1979). Moreover, since Iso-I is blue-shifted with respect to rhodopsin, if both parent pigments had the same Batho, the crossover between Iso-I and Batho should also be blue-shifted. The red shift of the Iso-I/Batho crossover with respect to the Rh/Batho crossover reported by Applebury et al. (1979) would require that the bathoproduct of Iso-I be spectrally different from that from Rh. This result is in contrast to the 77°K results reported here and the room temperature and picosecond results by Monger et al. (1979), that the bathorhodopsins are spectrally identical. At present we have no explanation for this discrepancy.

Although the bathoproducts of Rh, Iso-$I_{h\nu}$, and Iso-$I_{h\nu/\Delta}$ are spectrally identical, the bathoproduct of Iso-I_r has a higher maximum extinction. As $\epsilon_{\text{Iso-}I_{h\nu}} = \epsilon_{\text{Iso-}I_{h\nu/\Delta}}$, and $\epsilon_{B(\text{Iso-}I_{h\nu/\Delta})} = \epsilon_{B(\text{Iso-}I_{h\nu})}$, this difference is not likely to be due to the dependence of ϵ_B on a particular frozen protein conformation. Other factors such as the bleaching and regeneration, and the exposure of the Iso-I_r containing ROS membrane to hexane (to remove excess 9-*cis* retinal) before solubilization, could affect the value of $\epsilon_{\text{Iso-}I_r}$ (which the value of $\epsilon_{B\,[\text{Iso-}I_r]}$ depends on), or $\epsilon_{B(\text{Iso-}I_r)}$, or both. The shape and the position of $\epsilon_{B(\text{Iso-}I_r)}$, however, indicate that the principal interactions between the chromophore and the protein in this pigment are similar to those in bathorhodopsin produced from Rh or Iso-$I_{h\nu}$.

Finally, it is intriguing that the bathoproduct of Iso-II is so different from the bathoproducts of the other parent pigments. Since the final product of bleaching at room temperature for Iso-II is all-*trans* retinal (Crouch et al., 1975), the primary photoproduct might be expected to have an all-*trans* chromophore. If it does, the spectral differences between the two bathoproducts suggest that there are other conformational differences in the bathoproducts' structures.

We would like to thank Professor Motoyuki Tsuda for many helpful discussions.

This work was supported by National Institute of Health grants EY01323 and EY00025.

Received for publication 7 August 1979 and in revised form 11 October 1979.

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