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*Topical Review*

# Synthetic Retinals as Probes for the Binding Site and Photoreactions in Rhodopsins

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## Introduction

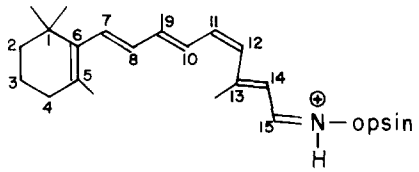
Rhodopsins are membrane-protein pigments responsible for a variety of photobiological functions, such as visual transduction (visual rhodopsins—Rh), photosynthesis (bacteriorhodopsin—bR and halorhodopsin), photoaxis (sensory rhodopsins) and photoisomerization (retinochrome). Independently of their specific function, all rhodopsins share the same basic chromophore system: A retinyl polyene, 11-*cis* in Rh and all-*trans* in bR, bound to the opsin via a protonated Schiff base linkage with a lysine $\varepsilon$-amino group. Light absorption induces a sequence of cyclic spectroscopic transformations, reflecting changes in the structures of both polyene and opsin, which induce the specific biological activity. In the past decades rhodopsins have been the focus of intensive investigations aiming at understanding, on a molecular level, the mechanisms by which they absorb, convert, store and subsequently utilize solar radiation. Substantial progress has been achieved due to the combined application of fast laser excitation methods and of spectroscopic techniques such as resonance-Raman, FTIR and NMR. Recently, the genes of several rhodopsins have been cloned and in some cases expressed, providing new powerful tools based on the production of pigments with selective modifications in their amino acid sequence.

General aspects of rhodopsins have been considered in several review papers (Ebrey & Honig, 1975; Ottolenghi, 1980; Birge 1981; Uhl & Abrahamson, 1981; Packer, 1982; Stoeckenius & Bogomolni, 1982; Dencher, 1983; Kobayashi, 1987). The purpose of the present topical review is to analyze the structure and function of rhodopsins with special emphasis on a relatively novel and powerful technique: The replacement of the native retinal chromophore by a suitably tailored synthetic analog, yielding artificial pigments in which intrinsic properties of the polyene (e.g., electronic structure, conformation, bond rotation and isomerization capability), as well as specific (steric and electrostatic) polyene-protein interactions may be selectively modified. (The reader is referred to several reviews on artificial rhodopsins: Balogh-Nair & Nakanishi, 1982; Crouch, 1986; Derguini & Nakanishi, 1986; Sheves et al., 1987; Sheves & Ottolenghi, 1989).

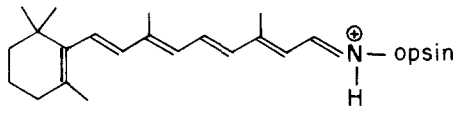
Our discussion will be focussed, in a comparative way, on the two basic and most extensively investigated systems: Visual rhodopsins and bacteriorhodopsin. In the first system the absorption of a light quantum induces a conformational change in the protein, producing an activated form of the pigment which causes the exchange of GTP for GDP in a G-protein. The letter in turn activates a cyclic GMP phosphodiesterase (Fung & Stryer, 1980; Fung et al., 1981). This leads to changes in the electrical potential across the cell plasma membrane, which are transmitted to the brain through appropriate synaptic processes. On the other hand, the role of bacteriorhodopsin, the retinal pigment of the halophilic microorganism *Halobacterium halobium*, is to convert light energy into a proton gradient across the membrane which is subsequently used via a chemiosmotic mechanism to synthesize ATP (for a review, *see* Stoeckenius & Bogomolni, 1982).

In spite of their basically different biological roles, Rh and bR share numerous common properties, not only in relation to their basic structure (seven $\alpha$-helical segments vertically spanning the membrane), but especially in respect to their spectroscopic and photophysical behavior. In the following sections we shall consider the spectra of the pigments which control the light-absorption process, serving also as important indicators of the molecular changes occurring in the subsequent photo-

**Key Words** rhodopsin · bacteriorhodopsin · synthetic retinals · visual photocycle · visual pigments.




11-cis (Rh)




all-trans (bR)

reactions. These will be discussed with special emphasis on the primary photophysical events, for which dramatic insights have been recently obtained due to the application of picosecond ($10^{-12}$ sec) and femtosecond ($10^{-15}$ sec) laser pulses. We note that the use of artificial rhodopsins serves as a powerful indicator of the intrinsic properties of the retinyl moiety and of its interactions with the close protein environment. Other methods, primarily FTIR spectroscopy, are being applied for monitoring light-induced changes in protein regions which are outside the retinal binding site.

## The Absorption Spectra

### Basic Factors Affecting the Spectra of Rhodopsins

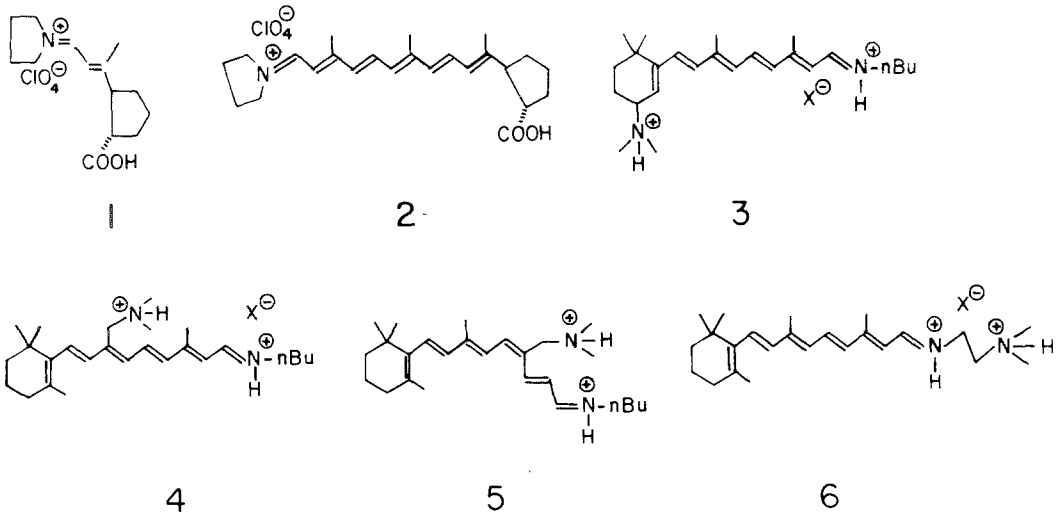
It is now well established that in all rhodopsins the retinyl chromophore is bound to the ε-amino terminal of a lysine residue of the apoprotein opsin via a protonated Schiff base linkage (Callender & Honig, 1977; Mathies, 1979; Bagley et al., 1982; Rothschild & Marrero, 1982). In itself, such a linkage cannot account for the observation that the absorption maxima of the various rhodopsins are characterized by a wide range of wavelengths (440–620 nm) despite the fact that they consist of a similar chromophore. In all cases a red shift is observed in respect to the spectrum of a model retinal protonated Schiff base ($RSBH^+$) in a methanol solution, which absorbs at 440 nm. The shift, which is due to very specific interactions between the retinyl chromophore and its protein binding site, was defined as the "opsin shift" (Nakanishi et al., 1980). The origin of the opsin shift (OS), i.e., the mechanism through which the protein regulates the absorption maxima of rhodopsins was studied extensively, giving rise to several molecular models. The early approaches outlined below have failed to provide quantitative spectroscopic models, but succeeded in establishing the basic factors which control the spectra of the pigments.

In 1958, Hubbard and Kropf suggested that the red shift in visual pigments is due to electrostatic interactions between the chromophore and the protein. This suggestion was later supported by theoretical calculations (Mantione & Pulman, 1971), indicating that a negative protein residue can influence π-electron delocalization along the polyene chromophore. This interaction can alter the relative stability of the ground and excited states of the chromophore, including shifts in the absorption maximum of the pigment.

Akhtar Jallo and Johnson (1968) proposed a charge transfer complex between an unprotonated Schiff base and an electron withdrawing group on the protein, causing a significant bathochromic shift. However, theoretical calculations (Komatsu & Suzuki, 1976) indicated that this model cannot satisfactorily explain the strong shifts found in visual pigments. Furthermore, it was shown by Raman spectroscopy (Lewis et al., 1974; Callender & Honig, 1977; Eyring & Mathies, 1979) that the chromophore is bound to the protein via a protonated Schiff base linkage, in variance with the unprotonated Schiff based invoked by Akhtar's model. Irving and Leermakers (1970) attributed the opsin shift to stabilization of the excited state by polyene interactions with polarizable aromatic amino acids. However, their model confronts serious difficulties in explaining opsin shifts as large as 5000–6000 $cm^{-1}$.

Electrostatic interactions in the vicinity of the Schiff base linkage were first suggested by Blatz and coworkers (1970, 1972) who studied the influence of the size of the counter-ion on the absorption spectra of model $RSBH^+$ in nonpolar solvents. Larger anions were found to induce larger red shifts. The experimental results were corroborated by theoretical calculations predicting a shift in the absorption, from 460 to 540 nm, due to an increased distance between the two ionic centers (the positively charged nitrogen and its counter-anion) from 3.5 to 10.5 Å. According to this approach, an effective way of achieving long-wavelength absorbing pigments is to design a protein in which the counter-anion is removed from the protonated Schiff base.

The role of electrostatic interactions between the chromophore and the opsin was also proposed on the basis of a negative protein charge located in the vicinity of the β-ionone moiety (Weisenfeld &




Abrahamson, 1968; Walech & Ingraham, 1973; Honig et al., 1976; Warshel, 1978). In this case a red shift, due to excited state stabilization, is induced as compared with the blue shift associated with the counterion of the positively charged nitrogen.

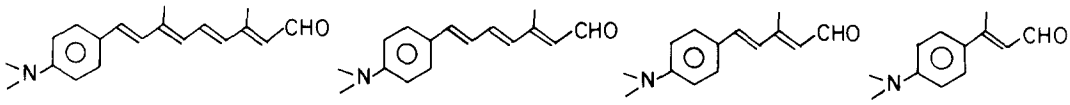
The capability of nonconjugated negative charges to influence the absorption of (protonated) polyene Schiff bases was directly demonstrated by examining chromophores **1** and **2** (Sheves & Nakanishi, 1983), which exhibited red shifts of 2500 and 300 $cm^{-1}$, respectively, upon conversion of the carboxyl groups to carboxylates. This demonstrated that a nonconjugated negative charge is capable of shifting the absorption maximum, and that its influence decreases upon increasing the polyene chain. However, the red shift observed for chromophore **2** was significantly smaller than the opsin shift observed for bR ($OS_{bR}$ = 5100 $cm^{-1}$).

Further insights into the influence of nonconjugated charges on the absorption maximum of $RSBH^+$ was obtained by studying a series of modified retinals bearing nonconjugated positive charges at various locations along the retinal skeleton (Sheves, Baasov & Friedman, 1983; Sheves & Baasov, 1984; Baasov & Sheves, 1986). It was found that in $CH_2Cl_2$ the absorption maximum ($\lambda_{max}$) of *n*-butyl amine (nBu) Schiff bases, is blue shifted due to the presence of the positive charge in the vicinity of $C_4$ (chromophore **3**) and $C_9$ (chromophore **4**), by 1150 and 1320 $cm^{-1}$, correspondingly. $\lambda_{max}$ is hardly affected by a charge close to $C_{12}$ (chromophore **5**), and it is red shifted by a charge in the vicinity of the Schiff base linkage (by 950 $cm^{-1}$, in chromophore **6**). It was concluded that the location of the external charge is crucial in determining the spectrum of the protonated Schiff base. It was also found that the magnitude of the above shifts largely depends on the degree of ion pairing between the nonconjugated positive charge and its counter-anion. Diminishing ion pairing, by increasing counter-anion solvation, causes a more effective interaction of the nonconjugated charge with the polyene, enhancing the spectral shift. The effect was observed (Baasov & Sheves, 1986) by applying fluorinated alcohols, which are strong anion solvators but only weakly interact with cations. For example, by using trifluoroethanol as a solvent, the absorption maximum of chromophore **2** was shifted by as much as 1800 $cm^{-1}$ relative to $RSBH^+$. We note, however, that the magnitude of the effect is smaller than that of the protein in bacteriorhodopsin ($OS_{bR}$ = 5100 $cm^{-1}$).

## The Opsin Shift in Bacteriorhodopsin

### *Schiff Base-Opsin Interactions*

The opsin shift of the light-adapted (all-*trans*) form of bR ($\lambda_{max}$ = 570 nm) is 5100 $cm^{-1}$. Important information relevant to the origin of this shift was obtained by studying the absorption spectra of artificial pigments based on synthetic retinal analogs, as well as from model $RSBH^+$ in solution. It was concluded that an important interaction affecting the absorption spectrum of bR originates from the protonated Schiff base linkage and its vicinity. This was shown by studying artificial pigments derived from analogs in which the basic polyene structure was perturbed by shortening the chain and by introducing aromatic rings (Sheves et al., 1985). Thus, the artificial pigments derived from the aromatic polyenes **7–10** exhibited a substantial drop in the value of $OS_{bR}$. However, in spite of the drastic per-

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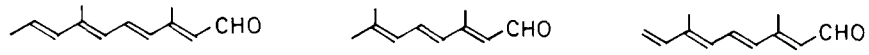



11 12 13

turbation of the polyene structure, a residual opsin shift of ca. 2500 $cm^{-1}$ was always observed. This applies even in the case of the artificial pigment derived from chromophore **10** bearing only one double bond. Opsin shifts of a similar magnitude were also found (Muradin-Szweykowska et al., 1984) in artificial bR pigments based on a series of short linear polyenes (**11–13**). More recently, the importance of Schiff base-protein interactions was further demonstrated by breaking the polyene chain using artificial pigments derived from a series of (5,6; 7, 8; 9,10) dihydroetinals (Lugtenburg et al., 1986; Spudich et al., 1986). A residual opsin shift of 3000–3500 $cm^{-1}$ was observed with the shortest polyene chain, which was assigned to the net contribution of Schiff base-protein interactions.

The above protein effect should be approached on the basis of the sensitivity of $RSBH^+$ spectra to environmental factors. As discussed above, Blatz and Mohler (1970) demonstrated that diminishing ion pairing between the positively charged nitrogen and its counterion induces a red shift in the spectrum. As demonstrated by studies with model compounds (Sheves & Baasov, 1984), this effect can contribute as much as 2500 $cm^{-1}$ to the red shift. It should be noted, however, that spectroscopic effects, analogous to those caused by ion pair separation, may also be associated with hydrogen bonding with protein dipoles (Warshel & Barboy, 1982; Warshel, Russell & Churg, 1984) or with residual water (Dupuis et al., 1980; Hildebrandt & Stockburger, 1984). For example, weak hydrogen bonds with the Schiff base nitrogen, but strong with the counteranion, may substantially contribute to the opsin shift in bR. This was demonstrated by studies with model compounds (Baasov & Sheves, 1986) using fluorinated alcohols as solvents. Such a description of the interactions prevailing in the vicinity of the Schiff base linkage in bR is attractive since it not only accounts for the opsin shift, but also for the high $pK_a$ value (13.3 ± 0.3) found for the protonated Schiff base of bR (Druckmann et al., 1982; Baasov & Sheves, 1986; Sheves, Friedman & Albeck, 1986*b*). The weakness of hydrogen bonding to the positively charged nitrogen in bR (compared to model $RSBH^+$ in methanol solutions) is strongly supported by $^{15}N$ NMR studies of a pigment with isotopically enriched lysines (Harbison, Herzfeld & Griffin, 1983).

A powerful indicator of the interactions between the Schiff base linkage and its environment is the C=N stretching frequency ($\nu_{C=N}$) of the protonated Schiff base, as measured by resonance-Raman and FTIR spectroscopy (Bagley et al., 1982; Siebert, Maentele & Kreutz, 1982; Rothschild, Cantore & Marrero, 1983; Mathies, Smith & Palings, 1987). Studies with model $RSBH^+$ in solution bearing nonconjugated positive charges along the polyene skeleton indicated that electrostatic perturbations in the vicinity of the ionone ring, or near $C_9$, affect both the absorption maximum and the C=C stretching frequency, but do not shift the C=N stretching mode (Baasov, Friedmann & Sheves, 1987) It is therefore evident that perturbations which are distant from the Schiff base cause only minor alterations in the $\pi$-electron delocalization along the C=N bond. A completely different situation prevails when the perturbation is in the vicinity of the C=N bond. Studies with model compounds (Baasov et al., 1987), theoretical calculations (Kakitani et al., 1983; Rodman Gilson et al., 1988) and an analysis of various intermediates in the bR photocycle (Rothschild et al., 1984) indicated that in this case a shift is induced in the absorption maximum of $RSBH^+$ which is correlated with a change in the C=N stretching frequency. The latter may be caused by two main effects: (i) alteration of hydrogen bonding to the N-H moiety; (ii) changes in charge delocalization along the C=N bond.

The first kind of perturbation is associated with

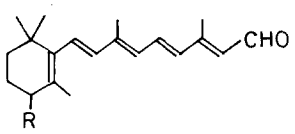
coupling between the C=N stretching and the N-H rock, which explains the high frequency of the C=N vibration (Aton et al., 1980; Kakitani et al., 1983; Baasov et al., 1987; Rodman Gilson et al., 1988) and implies that the C=N stretching will be strongly influenced by hydrogen bonding to the N-H moiety. Strong hydrogen bonding shifts the N-H bond vibration to higher energies (Nakanishi, 1962), resulting in effective coupling with the C=N mode, due to a close proximity of the energy levels. A similar explanation holds for changes in the isotope effect. Deuteration eliminates C=N/N-H coupling, due to a significant shift of the N-H frequency, which in turn affects the C=N stretching. The isotope effect will be smaller whenever coupling has already been diminished because of weak hydrogen bonding. Recently, an alternative explanation has been invoked to account for the relatively high frequency of the C=N stretching mode. It has been proposed that protonation increases the C=N force constant of the retinal Schiff base due to rehybridization of the C=N bond (Lopez Garriga et al., 1986*a–c*). In spite of the difference in approach, hydrogen bonding to the N-H bond is still considered as an important factor in determining the C=N stretching frequency. The second kind of perturbation due to nonconjugated charge in the vicinity of the Schiff base linkage involves charge redistribution along the C=N bond. Such interaction affects the absorption maximum as well as the C=N stretch, but does not change the deuterium isotope effect. This insensitivity indicates that C=N/N-H coupling is not affected by the nonconjugated charge.

In conclusion, accumulated evidence indicates that a change in the absorption maximum of $RSBH^+$, occurring without alteration of the C=N stretching frequency is indicative of a perturbation in the ring region or close to it along the $C_5$–$C_{10}$ section of the polyene chain. Alteration of both the absorption spectrum and the C=N stretching mode reflects perturbations in the vicinity of the C=N linkage. Thus, the C=N stretching mode provides valuable information on the interactions of the Schiff base moiety with its protein environment. In bR, the C=N mode shifts relative to $RSBH^+$ in a methanol solution by ~16 $cm^{-1}$ (1640 *vs.* 1656 $cm^{-1}$). This implies weaker hydrogen bonding of the $N^+$-H moiety to its counterion and/or to protein dipoles or residual water. This conclusion is directly supported by the observations of a deuterium isotope effect of ~17 $cm^{-1}$ on the C=N frequency of bR which is similar to that found in weakly H-bonded model systems (Baasov et al., 1987). Moreover, the value of $\lambda_{max}$ in model systems characterized by a $\nu_{C=N}$ value similar to that of bR (1640 $cm^{-1}$) shows a red shift of ca. 3000 $cm^{-1}$ in respect to methanol solutions, which is in keeping with the contribution of the Schiff base region to $OS_{bR}$ as estimated by the work with artificial pigments discussed above. These conclusions in respect to the absorption spectrum of bR will be discussed below with relation to the primary photoprocesses in rhodopsins.

### *Interactions in the Vicinity of Ring Moiety*

Apart from the Schiff base effects discussed above, protein-chromophore interactions in the vicinity of the ionophore ring play an important additional role in affecting the absorption maximum of bR. This was first suggested by the pioneering studies of Nakanishi et al. (1980) based on a series of dihydroretinals bearing a different reduced double bond. The chromophores were bound to bacterio-opsin and the absorption spectra of the resulting pigments were compared with those of the corresponding protonated Schiff bases in methanol. Analysis of the OS values of such artificial pigments led to the suggestion of an "external point charge model" based on the interaction with a negative protein charge residue in the vicinity of the $\beta$-ionone ring. e.g., $OS_{bR}$ of the 5,6-dihydro pigment (chromophore **22**) is substantially smaller (ca. 2400 $cm^{-1}$) than that of native bR (5100 $cm^{-1}$), indicating a significant contribution of the ring region to the opsin shift. It should be pointed out that this approach assumes that, in the absence of serious steric perturbations, the artificial chromophore adopts a geometry which is close to that of the native chromophore. Although no direct proof justifying this approximation is available, its feasibility is supported by studies with the series of retinal analogs with a perturbed ring region discussed above (Sheves et al., 1985; Muradin-Szweykowska et al., 1984). Independently of the way in which such perturbation is carried out, a similar decrease (of the order of 2000–2500 $cm^{-1}$) in $OS_{bR}$ is observed.

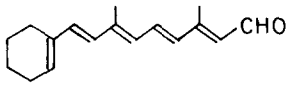
Additional information in this respect has emerged from studies with artificial pigments derived from synthetic retinal analogs. Retinals **14–16** (substituted at the $C_4$ position, Sheves et al., 1984*a*) **17** (lacking all ring substituents, Sheves et al., 1984*b*) and the linear chromophore **18** (Muradin-Szweykowska et al., 1984; Rao et al., 1985; Sheves et al., 1986*b*) are expected to sterically affect the chromophore-opsin interactions without altering the basic all-*trans* polyene system. Interestingly, the artificial pigments derived from $C_4$ substituted analogs (**14–15**) are unique in exhibiting two distinct absorption bands in the visible range: A main (blue) band at 465 nm, characterized by a small $OS_{bR}$, and a secondary band around 570 nm, reminiscent of the main band



14 R = $CH_3$

15 R = n-Bu

16 R = $N(CH_3)_2$



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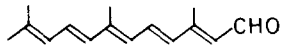

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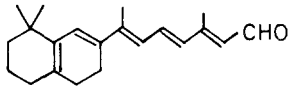

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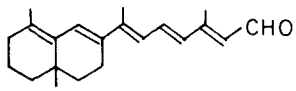

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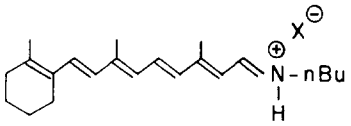
of bR. In the artificial pigment derived from the bulkier 4-dimethylamino derivate **16** only the blue (465 nm) band was observed. Therefore, large steric effects are induced by bulky substituents at the $C_4$ position, which drastically alter the conformation of the ring region and dramatically decrease the opsin shift. Accordingly, the two bands observed in the $C_4$-substituted pigments are attributed to two different ring conformations. One (the blue band) similar to that of a free $RSBH^+$ in solution. The second (the red band) similar to that characteristic of the native bR chromophore. Substantial drops in $OS_{bR}$ were also observed for an artificial pigment derived from 5-$CF_3$ retinal (Rao et al., 1986) for the linear chromophores (**11–13** and **18**) and for the aromatic polyenes **7–10.** In all cases the drop in $OS_{bR}$ was attributed to a release of steric constraints prevailing in the native system, resulting in a nonplanar ring-chain conformation similar to that of a free protonated retinal Schiff base in solution. Alternatively, alteration of the interaction of the chromophore with a protein charge in the vicinity of the ring (Nakanishi et al., 1980) was invoked.

These findings obviously call for a detailed molecular model of the polyene-opsin interactions at the ring region of the binding site. Specific, protein-induced, steric effects on the chromophore in the bR binding site were first suggested by Schreckenbach, Walckhopf and Oesterhelt (1978), who proposed a planar s-*cis* ring-chain conformation. A planar conformation was also suggested by Akhtar et al. (1982). More recently, Harbison et al. (1985) have assigned the $^{13}C$ NMR chemical shifts of all the polyenic carbon atoms of bR using solid state $^{13}C$ magic angle sample spinning. Comparison with the chloride salt of a protonated Schiff base in the solid state revealed pronounced differences in the chemical shifts of $C_5$, $C_7$ and $C_8$. A dramatic protein-induced perturbation of ca. 15 ppm of the isotropic $^{13}C$-chemical shift was detected in $C_5$. Studies with a retinal analog bearing a planar (s-*trans*) ring-chain conformation indicated a downfield of ca. 5 ppm in $C_5$. This observation confirmed the suggestion that the chromophore in bR is 6-s-*trans* (planar) in contrast to the 6-s-*cis* (twisted) conformation of $RSBH^+$ in solution. The conclusion was supported by the upfield shift observed at $C_8$, which was attributed to its nonbonded interaction with the gem-dimethyl groups in the s-*trans* conformation and by the $C_8$ longitudinal relaxation time.

The s-*trans* ring-planarization in the binding site was further supported by studies with retinal analogs bearing 6-s-*cis* (**19**) and 6-s-*trans* (**20**) locked conformations (Van der Steen et al., 1986). Both retinals form artificial pigments with $OS_{bR}$ = 3800 $cm^{-1}$. However, while the 6-s-*trans* analog easily forms a pigment exhibiting a 90% proton pump efficiency, the pigment derived from the 6-s-*cis* analog regenerated inefficiently to a mixture of pigments with only 20% proton pump efficiency.

The $^{13}C$ NMR data provided additional information, indicating that geometrical distortions cannot fully account for the perturbations in the vicinity of the ring and that additional (electrostatic) interactions should be considered. As discussed above, the $^{13}C_5$-labeled bR is further shifted downfield, in respect to 6-s-*trans* $RSBH^+$ derivatives, by ~10 ppm. Consequently, it was suggested (Harbison et al., 1985) that a negative charge is introduced by the protein adjacent to $C_5$ which contributes to $OS_{bR}$. The negative charge repels electrons from $C_5$, increasing the positive charge density, thus shifting the chemical shift downfield. The absence of large perturbations in the chemical shifts at the $C_6$ and $C_7$ positions suggests the presence of a positive counterion near $C_7$. Independent support for the presence of an ion pair in the vicinity of ring moiety of bR was obtained by recent studies (Lugtenburg et al., 1986; Spudich et al., 1986) of artificial bR pigments derived from the same series of dihydro retinal analogs first applied by Nakanishi et al. (1980). As mentioned above a "residual" $OS_{bR}$ of ca. 2000 $cm^{-1}$ is observed for the shortest (9,10-dihydro) pigment which is attributed to Schiff base-opsin interactions. However, the (revised) value of the opsin shift of the 7,8-dihydro pigment (ca. 3000 $cm^{-1}$) is

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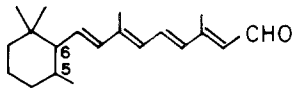


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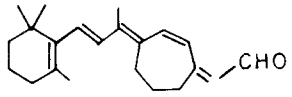


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larger than that (ca. 2400 $cm^{-1}$) of 5,6-dihydro (chromophore **22**), supporting the suggestion of an ion pair in the vicinity of carbons 5 and 7. (The polyene in 5,6-dihydro bR interacts with the positive end of the dipole. This induces in a blue shift in respect to 7,8-dihydro bR, which is less exposed to the dipolar charge).

Independent evidence supporting the planar ring-chain conformation and the interaction with a protein positive charge in the ring site in bR, was derived from $^{13}C$ NMR studies of model $RSBH^+$ systems (A. Albeck, M. Sheves and H. Gottlieb, *in preparation*). It was found that the $^{13}C$ NMR chemical shifts of the odd-numbered carbons are highly sensitive to charge delocalization along the retinyl polyene and that a linear correlation exists between such shifts and the absorption maximum. Especially relevant is the sensitivity of the chemical shifts to hydrogen bonding to the Schiff base: Weakening of the H-bond does not substantially affect the $^{13}C_5$ shift in a $RSBH^+$. However, a pronounced effect was observed in the case of chromophore **21.** The phenomenon was attributed to the planar s-*trans* ring-chain conformation adopted by this chromophore due to elimination of the interactions of the gem-dimethyl group with the chain hydrogens. An even higher resemblance between chromophore **21** and bR was achieved by weakening its Schiff base H-bonding, thus mimicking the situation in the protein. This shifted the absorption maximum of chromophore **21** to 550 nm and its $^{13}C_5$ chemical shift to 142 ppm, approaching the respective parameters in bR (570 nm and 144 ppm, respectively). Such studies clearly indicated that it is possible to closely mimic the absorption maximum of bR and its $^{13}C_5$ chemical shift by planarization of the ring-chain conformation and by an appropriate Schiff base environment, without requiring a nonconjugated negative charge in the vicinity of the ring. The above studies with model systems also indicated that the influence of a nonconjugated charge on the $^{13}C$ chemical shift depends on the charge arrangement around the double bond. A symmetric C=C/charge configuration causes only a minor change in the chemical shift but still substantially affects the absorption maximum of the chromophore. These observations are consistent with the postulated presence of a negative charge in the vicinity of the ionone ring of bR, which will shift the absorption maximum by ca. 500 $cm^{-1}$ without significantly affecting the $C_5$ chemical shift.

Further studies of model systems (Albeck et al., *in preparation*) analyzed the influence of a nonconjugated positive charge on the chemical shifts of the polyene carbons, supporting the presence of a positive charge in the vicinity of $C_7$–$C_9$. The conclusion was based on the observation that the $C_7$–$C_{12}$ chemical shifts of chromophore **21,** which absorbs at 550 nm, differ considerably (by 5–7 ppm) from the observed chemical shifts in bR. Relevant information is also derived from studies of chromophore **4** in which the presence of the positive charge affects the chemical shift of $C_9$ by 17 ppm. The magnitude of the analogous positive charge effect in bR is estimated as 5–7 ppm, implying that the protein charge site is further away from the polyene as compared to the model compound **4** (ca. 3 Å).

To summarize this discussion of the opsin shift in bR, we note the major conclusions which, in the absence of a detailed X-ray structure, provide important information on the polyene binding site: (i) compared to a model $RSBH^+$ in methanol, the Schiff-base moiety in bR is characterized by relatively weak hydrogen bonds. Such bonding (to the counteranion and/or to protein dipoles or residual water) contributes ca. 3000 $cm^{-1}$ to the opsin shift; (ii) the polyene is characterized by a planar strained s-*trans* ring-chain conformation which contributes ca. 1500 $cm^{-1}$ to the opsin shift; (iii) a small contribution of ca. 500 $cm^{-1}$ to $OS_{bR}$ is attributed to the effect of a protein dipole, consisting of a negative charge arranged symmetrically to bond $C_5$=$C_6$ and a positive charge located in the vicinity of $C_7$–$C_9$. This is schematically represented in Fig. 1. We note, however, that the presently available data cannot exactly locate the protein dipole with respect to the chromophore, so that the proposed arrangement should be treated as a qualitative approach.

## Acidic Forms of bR

Acidification of purple membrane suspensions leads to reversible spectroscopic changes associated with two acid modifications, absorbing at 605 nm ($bR_{605}$)

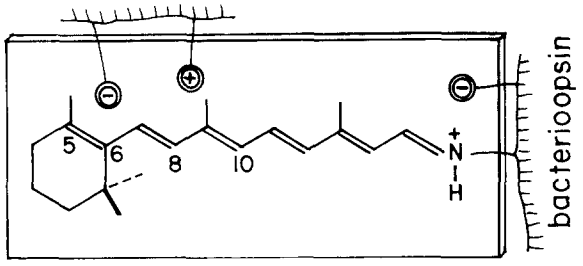



**Fig. 1.** Planar chromophore configuraiton in the binding site of bacteriorhodopsin showing the 6-s-*trans* ring-chain conformation. Location of the ring dipole charge (in the same or in other planes) is approximate and schematic

and 565 nm ($bR_{565}$). The corresponding transitions exhibit apparent $pK_a$ values of 2.9 and 0.5, respectively (Oesterhelt & Stoeckenius, 1971; Fischer & Oesterhelt, 1979). Later studies (Kimura, Ikegami & Stoeckenius, 1984; Chang et al., 1985*a*) have shown that the $bR_{570} \rightleftharpoons bR_{605}$ equilibrium is markedly affected by deionization of the membrane suspensions. Removal of bR-bound divalent cations causes an equilibrium shift to $bR_{605}$, even in neutral solution. Such phenomena have attracted considerable attention, mainly since they are considered to bear on polyene-opsin interactions in the binding site (*see* discussion above). They may also be related to the spectroscopic changes which characterize the photocycle of bR (*see below*). Several suggestions have been made to account for the above acid-induced spectral shifts. It was proposed (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Warshel & Ottolenghi, 1979; Smith & Mathies, 1985) that the formation of $bR_{605}$ is associated with protonation of the negative counterion of the Schiff base, or by a protein conformational change inducing an increased Schiff-base counterion separation (Fischer & Oesterhelt, 1979; Smith & Mathies, 1985; Szundi & Stoeckenius, 1987, 1988). The second transition, to $bR_{565}$, was attributed to protonation of a protein group in the vicinity of the $\beta$-ionone ring (Warshel & Ottolenghi, 1979) or to binding of an anion at the site responsible for the first transition (Fischer & Oesterhelt, 1979). More recently it has been suggested that low pH and/or high extrinsic ion concentration induce the formation of $bR_{565}$ by restoring a Schiff base environment which is similar to that of the native $bR_{570}$ chromophore (Smith & Mathies, 1985; Szundi & Stoeckenius, 1987, 1988).

Artificial bR pigments were recently applied in order to directly identify the specific regions in the retinal binding site where the above acid-induced changes in the protein-retinal intractions take place (Friedman et al., 1989). The spectroscopic effects of acid were investigated in a variety of artificial pigments, including retinals bearing bulky groups at $C_4$, short polyenes, and retinals in which the $\beta$-ionone ring was substituted by aromatic rings. The generation of an acid species, analogus to $bR_{605}$, was observed in all systems, including the extreme case of a pigment derived from chromophore **10** in which only one C=C bond is present. These results constitute direct evidence for the hypothesis that the generation of $bR_{605}$ is due to changes in polyene-opsin interactions in the vicinity of the Schiff base. However, they do not discriminate between various plausible specific effects, such as the direct titration of the counterion, (indirectly) increasing the Schiff base-counterion separation, or weakening of hydrogen bonding to protein dipoles or with residual water. All effects are expected to induce a red shift in the spectrum (Baasov & Sheves, 1986), to modify the C=N stretching (in keeping with the observations of Smith and Mathies (1985)) and, as discussed above, to shift downfield the $^{13}C_5$ NMR chemical shift (confirmed by De-Groot et al., 1988).

Analogous experiments were carried out in relation to the generation of $bR_{565}$. It was found that major changes in the ring structure of the retinal, such as substitution by aromatic rings, shortening of the polyene chain (including dihydro compounds), or addition of bulky substituents at the $C_4$ position, lead to elimination of the second transition. These observations are in keeping with mechanisms involving acid-induced changes in the interaction with the protein in the vicinity of the ring and/or with the release of the steric constraints responsible for the planar ring-chain conformation in $bR_{570}$ (and $bR_{605}$). Alternatively, if changes in the Schiff-base vicinity take place, it is implied that they are conditioned by an intact chromophore structure in the ring region.

## The Opsin Shift in Bovine Rhodopsin

The 498-nm absorption maximum of bovine rhodopsin represents an opsin shift of 2700 $cm^{-1}$ (Honig et al., 1979*a*), which is approximately half of that found in bR. Accumulated evidence indicates that neither Schiff base-protein nor ring-protein interactions, of the kind discussed above for bacteriorhodopsin, can account for the spectroscopic properties of the visual pigment.

Basic information on the interactions of the Schiff base linkage with its environment are obtained from the C=N vibration frequency. In variance with bR, the value observed in bovine rhodopsin (1660 $cm^{-1}$) indicates that the solvation (H-bonding) of the positively charged nitrogen is

similar (and possibly stronger) to that prevailing for $RSBH^+$ in methanol. This conclusion is confirmed by a large isotope effect, of ca. 30 $cm^{-1}$ (*see* discussion above). Since such a C=N environment should lead to an absorption maximum of ~440 nm (or even lower), one must conclude that the opsin shift in bovine rhodopsin ($\lambda_{max}$ = 498 nm) originates from interactions that do not affect the C=N stretching. A plausible suggestion would be the involvement of steric or electrostatic ring-opsin perturbations of the kind prevailing in bR, which are not supposed to affect the C=N frequency (Bassov & Sheves, 1986). However, there are several observations that rule out this mechanism. The first is associated with the early study of the opsin shifts of a series of dihydro bovine rhodopsins (Honig et al., 1979*a*). Thus, in variance with bR, the opsin shift was practically unaffected in the 5,6-dihydro pigment derived from the 11-*cis* isomer of chromophore **22**, in which the ring moiety is disconnected from the polyene sequence. The same applied to the 7,8-dihydro and 9,10-dihydro pigments. However, a remarkable increase in the opsin shift (5300 $cm^{-1}$) was observed with the 11,12-dihydro chromophore. It was concluded that electrostatic interactions between the polyene and a protein negative charge in the neighborhood of $C_{12}$–$C_{14}$ (*see* Fig. 2) account for the opsin shift in bovine rhodopsin. (The effect of the charge is essentially identical for the four pigments in which the polyene system extends from the charge towards the ring, sharply increasing for the 11,12-dihydro chromophore in which the charge is in a terminal position in respect to the (short) polyene chain).

The above conclusions are consistent with the spectrum of an artificial pigment derived from a bicyclic ring chromophore (Ito et al., 1985), which behaves similarly to the 5,6-dihydro pigment. Moreover, the solid state $^{13}C$ NMR studies (Smith et al., 1987*a*,*b*; Molevanger et al., 1987) reveal an (electrostatic) perturbation in the vicinity of the $C_{12}$–$C_{14}$. It also indicated that the retinal chromophore in the binding site adopts a s-*cis* twisted ring-chain conformation, similar to $RSBH^+$ solution. It is therefore possible to rule out any contribution to the opsin shift originating from special (protein-induced) ring-chain conformations. A schematic diagram representing these conclusions is shown in Fig. 2, which includes an external point charge along the polyene, in addition to the Schiff base counterion. We note, however, that a two-photon spectroscopy study of the binding site of vertebrate rhodopsin, using the locked 11-*cis* chromophore (**23**), indicates that the binding site is electrically neutral (Birge et al., 1985). This implies either that one of the negative charges should be replaced by a dipole or, alternatively, that there is only one negative (counterion) charge. In such a case it is evident that this charge cannot be intimately associated with the Schiff base linkage, but it should be located further along the polyene chain (Birge et al., 1988).

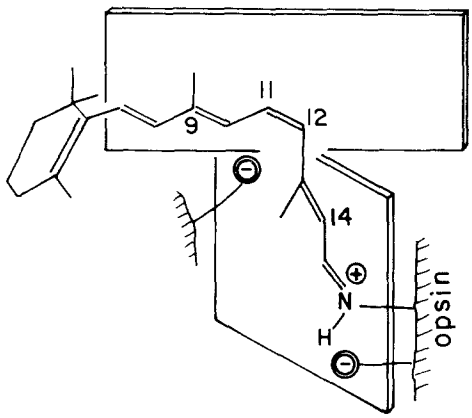



**Fig. 2.** Chromophore configuration in the binding site of bovine rhodopsin showing the nonplanar 6-s-*cis* ring-chain conformation. Location of negative protein charges is approximate and schematic

It is worthwhile noting that the above retinal-protein interactions characteristic of bovine rhodopsin are not a general feature of visual pigments. Thus, similarly to bovine (vertebrate) rhodopsin, a study of octopus (invertebrate) rhodopsin regenerated with synthetic retinals, attributes the opsin shift to the interaction with a protein negative charge near the middle of the polyene chain (Koutalos et al., 1989). On the other hand, a study of the chicken cone pigment, iodopsin, using a series of dihydrochromophores, is indicative of a binding site which is reminiscent of that of bR. Namely, a relatively large opsin shift (4950 $cm^{-1}$) is interpreted in terms of a weakened nitrogen-counterion interaction and of a protein dipole in the vicinity of the ring (Chen et al., 1989).

## The Photocycles

### Early Phenomena in the Photocycle

The most extensively applied approach for studying light-induced transformations in rhodopsins is to follow the optical absorption changes following excitation by a short laser pulse. Presently available techniques have been extended down to sub-picosecond time resolution (for a recent review, *see* Sharkov & Matveets, 1987), yielding the photocycle schemes for rhodopsin and light-adapted (all-*trans*) bacteriorhodopsin shown in Fig. 3. The initial phe-

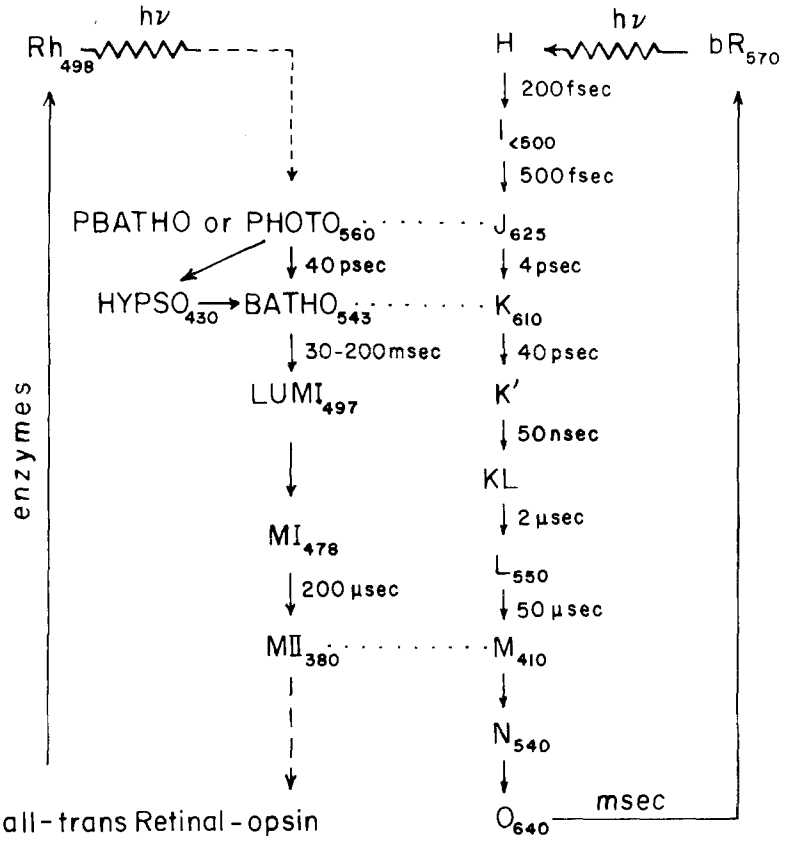



**Fig. 3.** The photocycles of (11-*cis*) rhodopsin (Rh) and (all-*trans*) bacteriorhodopsin (bR). Subscripts refer to wavelengths of maximum absorption. Time notations are approximate room temperature values. Horizontal dotted lines indicate analogous intermediates (*see* text)

nomena in the photocycles are focussed around the early phototransients: bathorhodopsin (BATHO) and K, respectively. These two intermediates are considered analogous, mainly since they are the first that can be stabilized at low temperatures and also for being spectrally red-shifted in respect to their parent pigments. Considerable attention has been devoted to fast phenomena associated with the evolution and the decay of both species, over time scales ranging from femtoseconds to microseconds. Early absorption experiments with picosecond resolution suggested that both BATHO (Peters, Applebury & Rentzepis, 1977) and K (Appelbury, Peters & Rentzepis, 1978), are preceded by further red-shifted precursors denoted (Dinur et al., 1980, 1981) as prebathorhodopsin (PBATHO) and J, respectively. An analysis (Dinur et al., 1980, 1981) of the early subpicosecond experiments of Ippen et al. (1978) indicated that in bacteriorhodopsin J is not the primary photointermediate, suggesting that it grows-in over a ~1 psec time scale, from a precursor denoted as I. Both I and J decay phases have been quantitatively examined by several groups, establishing lifetimes of 0.5 ± 0.1 psec for the former (Polland et al., 1984*a*,*b*, 1986; Sharkov et al., 1985; Nuss et al., 1986; Petrich, Breton & Martin, 1986; Mathies, et al., 1988) and 4 ± 1 psec for the latter (Polland et al., 1984*a*,*b*; 1986; Petrich et al., 1986). All data are consistent with the consecutive scheme of Fig. 3 in which I is a blue-shifted ($\lambda_{max} < 500$ nm) precursor of J, which in turn transforms to K. On the basis of this assumption an analysis was carried out (Polland et al., 1986) yielding a spectrum for J which is red-shifted by ~10 nm relative to K and by ~39 nm relative to bR. We note, however, that the presently available data cannot definitely rule out the alternative (parallel) scheme in which I is a precursor of both J and K. Namely, bR $\xrightarrow{h\nu}$ I followed by I $\xrightarrow{0.5\ \text{psec}}$ J → bR, in competition with I $\xrightarrow{0.5\ \text{psec}}$ K → L.

The occurrence of the very early intermediate, H, was suggested by the recent experiments of Mathies et al. (1988), and Dobler et al. (1988), in which bR was excited with ~60 fsec pulses. It was shown that the I intermediate is preceded by a state, denoted here as H, characterized by an absorption which is blue shifted in respect to bR but red shifted in respect to I. The H state is also characterized by an intense stimulated fluorescence around 690 nm. The H → I transition takes place over a ~200 fsec time scale and is associated with a blue shift in the (transient) increase in absorbance, and by a red shift

(coupled with a decay in intensity) of the stimulated emission. As to the decay of the K photoproduct, Shichida et al. (1983) and Milder and Kliger (1988) reported changes in the 1–10 nsec time range, associated with a 5–10 nm red shift of the transient absorption spectrum. Changes on a similar time scale are also observed in the resonance-Raman spectrum (Stern & Mathies, 1985). This process is much faster than the ~2 $\mu$sec growing-in of the L transient, suggesting the formation of a state, KL, which is intermediate between K and L.

It appears that the kinetic scheme associated with the primary photointermediates in bR may be more complicated than that monitored by time-resolved absorption spectroscopy. Recently, the early events in the bR photocycle were monitored with both (psec time-resolved) resonance-Raman spectroscopy as well as by fluorescence spectroscopy (Atkinson, 1987; Atkinson et al., 1988*a*,*b*). Both methods yielded distinct spectra for the J and K stages, but were also indicative of additional changes occurring over the 10–40 psec range. Namely, a change in the retinal "fingerprint" region of the Raman spectrum, and a substantial increase in the intensity of the (K) fluorescence, which are both faster than the K → KL transition as revealed by absorption spectroscopy. The results are interpreted in terms of an additional intermediate state (K′) between K and KL.

The basic analogy between K and BATHO calls for primary phototransients in the visual photocycle which are analogous to H, I and J in that of bacteriorhodopsin. An absorption decay in the red in the visual photocycle, analogous to the J state in bR was observed (at 4°K) by Peters et al. (1977) and assigned to a precursor of BATHO denoted as PBATHO (Dinur, Honig & Ottolenghi, 1980, 1981). Similar phenomena at room temperature were reported by Yoshizawa and coworkers (Shichida, Matuoka & Yoshizawa, 1984; Yoshizawa, Shichida & Matuoka, 1984) and assigned to a precursor (photorhodopsin, PHOTO, analogous to PBATHO) which is red-shifted by approximately 10 nm relative to bathorhodopsin. The room temperature lifetime for the PHOTO → BATHO process is 40 psec for bovine rhodopsin and approximately 200 psec in the cases of squid and octopus rhodopsin. The exact identity of PHOTO (room temperature) and PBATHO (4°K) still awaits experimental verification, but it appears that they both represent a precursor of BATHO. Steps preceding PHOTO or PBATHO, parallel to the I → J step in bR have not yet been observed in visual pigments. Finally, a complicating feature in the photocycles of several visual pigments has been associated with the observation, by continuous illumination at low temperatures, of an additional early intermediate absorbing at 430 nm (main band) and (weakly) at 540 nm (Yoshizawa & Horiuchi, 1973). The question arose as to the origin and nature of this species (termed as hypsorhodopsin-HYPSO) which thermally decays into BATHO, primarily as to whether HYPSO is also formed under low light-intensity and physiological conditions. It appears that at low temperatures HYPSO is generated from BATHO by the absorption of a second photon. Similarly, laser photolysis at room temperature shows that the main path leading to HYPSO involves a biphotonic mechanism (irrelevant under solar light intensities) in which a second photon is absorbed by PHOTO or by an earlier precursor (Shichida et al., 1984; Yoshizawa et al., 1984). However, some contribution from a direct dark (branching) reaction, from PHOTO to HYPSO, is suggested by the data of Kobayashi and coworkers for bovine rhodopsin (Kobayashi, 1980; Kobayashi, Ohtani & Tsuda, 1986). In any event, it appears that whenever observed, hypsorhorodpsin originates from PHOTO rather than being a primary photoproduct formed in a parallel early event (*see* review by Sharkov & Matveets, 1987).

## Identification of the Early Intermediates (H, I, J, K and PHOTO, BATHO)

### *K and BATHO*

The observations described in the previous section strongly suggest an essentially identical model for the primary photochemical events, i.e., for the structure of the red-shifted intermediates (BATHO and K) and their precursors, in both rhodopsin and bacteriorhodopsin. The currently accepted model for the structures of BATHO and K is based on primary polyene photoisomerization processes: 11-*cis* (Rh) → all-*trans* (BATHO) and all-*trans* (bR) → 13-*cis* (K), respectively. The model was proposed (Yoshizawa & Wald, 1963; Hurley et al., 1977; Rosenfeld et al., 1977) on the basis of indirect evidence. It was later confirmed by resonance Raman data, supporting basically isomerized, though distorted, chromophores: all-*trans* in the case of BATHO (Eyring et al., 1982) and 13-*cis* in the case of K (Braiman & Mathies, 1983). However, most convincing evidence for the isomerization model comes from work with artificial pigments. Thus, no BATHO or subsequent photoproducts, are observed upon blocking the $C_{11}$=$C_{12}$ isomerization in rhodopsin by preparing an artificial pigment with a $C_{10}$–$C_{13}$, seven-membered ring (chromophore **23**, Akita et al., 1980; Mao et al., 1981). Similarly, artificial bacteriorhodopsins in which the $C_{13}$=$C_{14}$ isom-

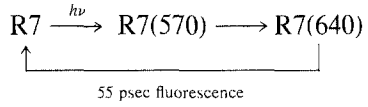
erization is blocked by five-membered (Fang et al., 1983; Chang et al., 1985*b*) or epoxy (Sheves et al., 1985) rings do not exhibit a photocycle.

While there appears to be no doubt concerning the requirement of a (specific) double bond isomerization in the generation of K and BATHO, the question arises as to the participation of other C=C bonds, and especially to the rotation about single C—C polyene bonds during the primary events. The involvement of any C—C rotation (up to $C_{13}$) or C=C isomerization (other than $C_{13}$=$C_{14}$) in the photocycle of bR, has been excluded on the basis of extensive studies with artificial pigments with blocked rotations or with short chained retinal analogues. (Sheves et al., 1985; Albeck et al., 1986). Of special interest is the observation that K is formed even with the synthetic chromophore derived from aldehyde **10**, which is constituted by a single C=C bond (analogous to $C_{13}$=$C_{14}$). [Evidence showing that neither $C_{14}$–$C_{15}$ rotation (Schulten & Tavan, 1978; Tavan & Schulten, 1986; Liu, Mead & Asato, 1985) nor $C_{15}$=N isomerization participate in the primary event in bR has been provided by resonance-Raman studies. (For a review, *see* Mathies et al., 1987.)] Unfortunately, due to substantial difficulties in preparing many artificial visual pigments, similar comprehensive information is still unavailable in the case of the visual photocycle. However, experimental evidence based on an artificial $C_9$–$C_{11}$ locked rhodopsin has been presented (Sheves et al., 1986*a*), indicating that rotational freedom about $C_{10}$–$C_{11}$ (Liu & Asato, 1985) is not required for the occurrence of the visual photoprocess. The same applies to the $C_{12}$–$C_{13}$ bond (our laboratory, *unpublished*). In conclusion, it appears that with all rhodopsins a *cis* → *trans* or *trans* → *cis* isomerization about one specific C=C bond is the only prerequisite for initiation of the photocycle. In the case of bR, the time resolved fluorescence and resonance Raman data (*see above*) indicate that the initially generated K (13-*cis*) photoisomer undergoes a subsequent, yet undefined, relaxation to the K′ state. The latter process is associated with changes in the fingerprint Raman spectrum and in the radiative decay of the excited state but not in the absorption spectrum. A similar ambiguity applies to the KL species which may differ from K′ (and K) in single bond conformations or in its interactions with the protein (Stern & Mathies, 1985; Rothschild, Roepe & Gillespe, 1985).

### *J and PHOTO*

Reaching the conclusion that at the stages of K and PHOTO the polyene has undergone *trans* → 13-*cis* and 11-*cis* → *trans* isomerizations, respectively, the question arises as to the nature of the preceding states. Namely, did the above isomerizations already occur in J and PHOTO? Are the latter ground states or excited states of the respective pigments? On the basis of: (i) the close resemblance of their absorption spectra with those of K and BATHO, respectively; (ii) the inconsistency of the rate of the (~4 psec) J decay with the much faster (<0.5 psec) fluorescence decay in bR, it was suggested (Dinur et al., 1980, 1981; Ottolenghi, 1982), that PHOTO and J are ground-state, rather than excited state, species, with polyene conformations essentially identical to the (isomerized) structures of BATHO and K, respectively. An undefined relaxation in the protein environment was suggested to account for both J → K and PHOTO → BATHO reactions.

The suggestion that isomerization has taken place at stages that precede J and PHOTO, has been confirmed by the failure to observe the two intermediates in artificial pigments in which isomerization (and thus the photocycles from the stages of K and BATHO) is blocked by rigid ring structures. Thus, Polland et al. (1984*a*,*b*) submitted a bR analog (9,12-Ph-bR), in which the $C_9$–$C_{12}$ region of the polyene is replaced by an aromatic ring, to ~3 psec laser excitation. An excited fluorescent state with a lifetime of ~10 psec is formed, relaxing to the ground state without generating the J (or any subsequent) intermediate. Buchert et al. (1983) carried out similar experiments with the analogous seven-membered, $C_9$–$C_{13}$ locked, analog of visual rhodopsin (R7, derived from **23**), suggesting the scheme:




in which R7(570) and R7(640) are excited states (nonfluorescent and fluorescent, respectively) of R7, basically retaining the 11-*cis* ground state configuration of R7. This model has been recently revised by Yoshizawa (1988) who identifies the R7(570) phototransient as a ground-state species, partially twisted around the $C_{11}$=$C_{12}$ bond. Accordingly, R7(570) is considered analogous to the PHOTO intermediate of the native pigment, while R7(640) is attributed to an excited state, generated from R7(570) by the absorption of a second photon. Analogously to 9,12-ph-bR, artificial rhodopsins, in which the seven-membered ring is replaced by a more rigid five or six-membered ring, exhibit only a ~100 psec fluorescence with no ground-state (R7(570)-like) photoproduct. The rigidity of the ring in this case is assumed to prevent the twist around

$C_{11}$=$C_{12}$ which leads to PHOTO in the native pigment, or to R7(570) in the more flexible ring system of R7. According to this interpretation PHOTO is assigned to a ground-state chromophore partially twisted along the $C_{11}$=$C_{12}$ coordinate leading from 11-*cis*(Rh) to all-*trans* (BATHO). This conclusion may be similar to that derived from a recent psec resonance-Raman investigation of the J intermediate in the bR photocycle (Atkinson et al., 1988*a*). It was shown that both J and K are characterized by a basically 13-*cis* structure, without providing clear identifications in terms of absolute configurations. However, the respectively "fingerprint" bands differ from one another, indicating that the retinyl moiety in J and K has different conformations. This may also be consistent with a substantial difference between the fluorescence properties of J and K (Atkinson et al., 1988*b*).

As mentioned above, a different approach, attributing the PHOTO → BATHO and J → K transitions to protein, rather than polyene, conformational changes, has been suggested. These may involve a variety of polyene-opsin intractions as discussed in the previous section. In view of the lack of an isotope effect in the J → K process (Polland et al., 1984*a*,*b*, 1986), it may be concluded that, at least in the case of bacteriorhodopsin, such changes probably cannot be associated with an intraprotein proton transfer. An alternative suggestion (Polland et al., 1986) interprets the J → K process in terms of dissipation of excess thermal energy generated in the retinal moiety following excitation, thus identifying J as a hot (13-*cis*) ground state species. Finally, on the basis of theoretical calculations, J has been recently assigned to a secondary excited state, formed from a primary excited state (identified as the I intermediates) in parallel to the I → K process (Birge, Findson & Pierce, 1987). This approach seems unlikely, mainly since there is no component in the bR fluorescence which matches the 4-psec decay of J. In conclusion, the exact changes occurring during the J → K process are still unknown. It is possible that the application of psec and fsec methods to artificial bR pigments of the kind discussed above may allow discrimination between the various approaches, primarily between those based on retinal conformational changes and those based on environmental protein relaxations. The same applies to PHOTO for which considerably less information is presently available.

### *The I Intermediate*

As a very early intermediate in the bR photocycle, I has been assigned to an excited state of bR. More specifically, to the relaxed (barrierless), "common excited state" (CES), along the reaction (isomerization) coordinate between the *trans* (bR) and 13-*cis* (J) configurations (Dinur et al., 1981; Ottolenghi, 1982; Sharkov et al., 1985; Polland et al., 1986; Mathies et al., 1988). The quantitative population of such a state, followed by partition to the two corresponding ground states, was suggested as the basis of the model shown in Fig. 4, accounting for the primary even in bacteriorhodopsin and, analogously, in visual rhodopsins (Hurley et al., 1977; Birge, 1981). According to this model the all-*trans* ground state of bR, depleted following light absorption, should be partially repopulated, at a rate which matches the 0.5-psec growing-in of the J absorption. The data of Mathies et al. (1988) are consistent with this requirement. The assignment of I as the relaxed excited state of bR calls for a comparison between the lifetime of the I → J process ($\tau_I$) and that of the spontaneous fluorescence of bR ($\tau_f$). Analysis of accumulated bR fluorescence data has indicated that $\tau_f$ is indeed in the same (subpicosecond) range as $\tau_I$, but no quantitative comparison with the $\tau_I = 0.5 \pm 0.2$ psec value (*see above*) could be carried out. (*See* discussions by Ottolenghi

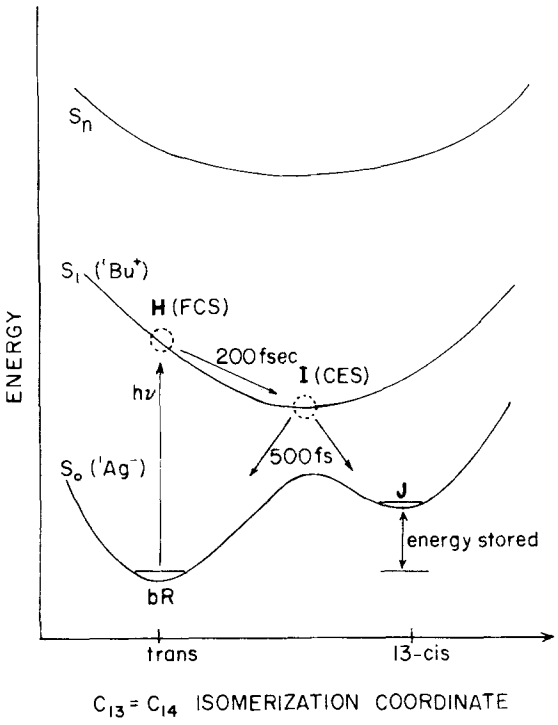



**Fig. 4.** Potential energy surfaces describing the primary events in bacteriorhodopsin. *FCS* and *CES* denote the Franck-Condon state and the "common excited state," respectively. (An analogous description appears to be applicable to visual pigments.) Note that the *K* intermediate is not included, since it involves an additional (protein or retinal) coordinate (*see* text)

(1982), Ottolenghi and Sheves (1987), Sharkov and Matveets, 1987.) However, new relevant information in this respect has been provided by the recent femtosecond experiments of Dobler et al. (1988). These show a decay of a (stimulated) fluorescence signal in bR at relatively long wavelengths ($\lambda > 850$ nm), which matches the rate of the I → J process, as monitored by absorption spectroscopy. These data constitute a direct experimental proof for the identification of I as the fluorescent CES in bR.

*The H Intermediate*

The H intermediate is characterized by an absorption spectrum which is red shifted in respect to that of I and by a stimulated fluorescence which is both blue shifted and more intense than that of I. On the basis of these properties, Mathies et al. (1988) and Dobler et al. (1988) identify H as the Franck-Condon excited state (FCS) of bR, retaining the original all-*trans* configuration of the ground state. Accordingly, the ~200 fsec H → I process is interpreted in terms of the tortional distortion of the molecule, from the all-*trans* to the intermediate 90°C configuration. In other words, one is directly observing, in real-time, the $C_{13}$=$C_{14}$ tortional isomerization of the retinal chromophore on the excited state potential surface. Motion on the $S_1$ surface is unidirectional (no oscillations in the stimulated fluorescence intensity are observed). This implies that the excess vibrational energy along the $C_{13}$=$C_{14}$ coordinate (between FCS and CES) is removed within ~200 fsec, most probably by redistribution with other polyene coordinates. We note that analogous phenomena, in respect to the $C_{11}$–$C_{12}$ coordinate, should also take place in the visual photocycle (Hurley et al., 1977; Birge, 1981). At present, precursors of PHOTO (or of PBATHO) representing the CES and the primary Franck-Condon excited state have not yet been directly detected. However, an analysis of the (very low) fluorescence quantum yields (~$10^{-5}$) observed for bovine and squid rhodopsins, suggested that motion along the $C_{11}$=$C_{12}$ tortional coordinate competes with fluorescence and takes place on the order of 100 fsec (Doukas et al., 1984). Accordingly, fluorescence is emitted from a configuration which is close to the Franck-Condon (0°, 11-*cis*) state (analogous to H) and is quenched by isomerization to the 90° CES (analogous to I in bR).

According to the model presented above it should be possible to reach the $S_1$ surface, and thus the CES, by exciting the J photoproduct. Experiments that will verify this hypothesis have not yet been carried out. However, the back photoreaction starting from the K intermediate, has been investigated with psec time resolution (*see* discussion by Sharkov and Matveets, 1987). The kinetics leading back to bR differ from that characterizing the forward H → I → J → K process. The results were interpreted (Ottolenghi, 1982; Iwasa et al., 1984; Ottolenghi & Sheves, 1987; Sharkov & Matveets, 1987) in terms of the sequence:

$$K \xrightarrow{h\nu} K^*(S_1\text{-FCS}) \rightarrow K^*(S_1\text{-CES}) \rightarrow J_K \rightarrow bR$$

in which $K^*(S_1$-FCS) and $K^*(S_1$-CES) are excited state species analogous to H and I, respectively, which have not yet been experimentally detected. $J_K$ (all-*trans*) is analogous to the J(13-*cis*) species of the forward photoreaction. It was proposed that both $K^*(S_1)$ and $J_K$ species retain the protein ("solvation") configuration characteristic of K, while H, I and J retain the protein configuration of bR. Accordingly (*see* discussion above), protein relaxations take place during the J → K and $J_K$ → bR steps.

## Changes in Chromophore-Protein Interactions in the Primary Event: The Spectral Red Shift and Energy Storage in BATHO and K

Apart from the high selectivity of the corresponding $C_{11}$=$C_{12}$ and $C_{13}$=$C_{14}$ isomerization processes, molecular models for the primary event in rhodopsin and bacteriorhodopsin must account for two other major observations: (i) The fact that, independently of the specific (either 11-*cis* → *trans* or *trans* → 11-*cis*) process, both photoreactions are associated with a bathochromic shift with respect to the parent pigment; (ii) The storage, in both intermediates, of a substantial fraction of the photon energy: 32–35 kcal/mol in BATHO (Cooper, 1979; Honig et al., 1979*b*; Boucher & LeBlanc, 1985; Schick et al., 1987) and ~14 kcal/mol in K (Birge & Cooper, 1983). Most of this energy is presumed to be subsequently used for the respective biological functions of the pigment. In all such respects the photocycles of the pigments basically differ from the photochemical behavior of simple protonated Schiff bases in solution (Becker, 1988). A model, qualitatively account for both observations was proposed (Honig et al., 1979*b*), based on the separation, induced by photoisomerization, between the protonated Schiff base moiety and its (protein) counterion. Accordingly, the photon energy is stored primarily by electrostatic interactions (Honig et al., 1979*b*; Birge & Hubbard, 1981; Rothschild et al., 1984) between the positively charged nitrogen and its protein counterion.

Evidence favoring a major change in the Schiff base environment at the stage of K has been derived from work with a large collection of artificial bR pigments. (Sheves et al., 1984*a*, 1985, 1987). Of primary importance is the parameter $\Delta\nu$(bR/K), which measures the value of the red shift associated with the formation of the primary photoproduct, K. Not only is a primary K species observed for all artificial pigments, but the value of $\Delta\nu$(bR/K) shows relatively little sensitivity to the nature of the specific pigment involved. Thus, the spectral shift associated with the bR → K transition (which is ~2500 $cm^{-1}$ in the native chromophore) does not show a trend parallel to that of $OS_{bR}$. For example, a substantial value of ~1500 $cm^{-1}$ is observed with the pigment derived from chromophore **10** in which only a single double bond is present. These observations lead to the unequivocal conclusion that the main contribution to the spectral shift in K cannot be due to changes in steric or electrostatic interactions of the opsin with the ring, or with other polyene sections up to $C_{13}$. It is thus evident that the primary event is directly associated with changes in the Schiff base environment of the chromophore. This may be due to charge separation between the positively charged nitrogen and its counterion (Honig et al., 1979*b*; Rothschild et al., 1984) and/or to changes in other kinds of interactions between the Schiff base and its opsin environment, such as hydrogen bonding (Warshel & Barboy, 1982; Baasov & Sheves, 1986).

The occurrence of major changes in the Schiff base environment at the stage of K is also supported by vibrational spectroscopy. Thus, as reviewed by Mathies et al. (1987) and by Ottolenghi and Sheves (1987), the C=N stretch frequency in bR as monitored by resonance-Raman or FITR methods undergoes a substantial drop (20–30 $cm^{-1}$) upon conversion to K. However, both resonance-Raman (*see also* Deng & Callender, 1987) and FTIR data indicate that the C=N stretch frequency in BATHO is identical to that of the mother rhodopsin pigment. Such observations imply (*see* discussion of the absorption spectra above) that the Schiff base environment in visual pigments does not change after photoisomerization. This conclusion questions the validity of any general model for the primary event which is common to visual rhodopsins and to bacteriorhodopsin. Specifically, it calls for a molecular model for the visual photocycle which will account for both spectral shift and energy storage in BATHO, without essentially invoking a single charge separation between the Schiff base and its counterion. Several approaches qualitatively accounting for the above observations have been proposed. As discussed by Ottolenghi and Sheves (1987), the primary event in the two systems may be interpreted as follows:

*Bacteriorhodopsin.* Isomerization induces charge separation and/or weakening of H-bonds, in keeping with the optical red shift, with the 20–30 $cm^{-1}$ drop in $\nu_{C=N}$, and with the (electrostatic) energy storage. Since charge separation is equivalent to neutralization of the counterion (due to an intraprotein proton transfer) it is possible that both red shift and energy storage are related to the protonation of a tyrosine group at the stage of K (Dollinger, et al., 1986; Rothschild et al., 1986).

*Visual Rhodopsin.* Since the Schiff base environment does not change during the primary event, two major approaches may be advanced: One possibility is that charge separation takes place (as in bR), accounting for energy storage and for the optical red shift. The fact that $\nu_{C=N}$ does not change in BATHO, may be attributed to the formation of strong H-bonds (with neutral protein moieties, or with $H_2O$), counterbalancing the effect caused by separation from the counterion (Ottolenghi & Sheves, 1987; Deng & Callender, 1987). Alternatively, assuming that there is no direct counterion to the Schiff base, electrostatic energy storage might be achieved if the Schiff base were displaced in an electric field produced by other structural elements of the protein, such as the dipole moment of an $\alpha$-helix (Rodman Gilson et al., 1988). Still another approach within this framework has been suggested by Birge et al. (1988). Here a specific negatively charged counterion is envisioned that lies below the positively charged chromophore, in such a way that its major electrostatic interactions are with the $C_{13}$–$C_{15}$ retinal carbons and not with the Schiff base moiety. While all these suggestions are still purely speculative, they do offer the possibility of retaining the attractive features of the charge-separation model while remaining consistent with the unchanged $\nu_{C=N}$ parameter. A different approach (Ottolenghi & Sheves, 1987) is to assume that charge dislocation does not take place during the Rh → BATHO transition and that nonelectrostatic effects, such as conformational distortion of the polyene chromophore, must substantially contribute to the energy storage mechanism (Birge & Hubbard, 1981; Warshel & Barboy, 1982; Palings et al., 1987). In fact, the increased $C_8$–$C_9$ and $C_{14}$–$C_{15}$ stretching frequencies and the anomalous properties of the hydrogen out-of-plane wagging vibrations in BATHO, are indicative of chromophore-protein intractions along the polyene chain (Eyring et al., 1982; Rothschild & DeGrip, 1986; DeGrip et al., 1987) that might be involved in the energy-storage mechanism (Palings et al., 1987). The disappearance of these anomalous vibrations at the LUMI stage many indi-

cate that during the BATHO → LUMI process energy stored in the perturbed polyene is transmitted to the protein. As to the red-shifted spectrum of BATHO, it must be interpreted in terms of changes in protein-polyene interactions which are not associated with the Schiff base region, e.g., with electrostatic or steric effects along the polyene chain.

Experiments that will measure energy storage both in K and in BATHO in a variety of artificial pigments carrying selective modifications along the polyene chain may prove valuable in establishing the molecular basis of the red shift and of the energy storage mechanism. Indirect evidence, in keeping with the conclusion that in the case of bR conformational changes in the polyene are not the main mechanism of energy storage, is provided by work with chromophores bearing a five or a six-membered ring along the polyene (Sheves et al., 1987): Normal photocycles and proton pumping activity are observed in spite of the major restrictions imposed on the polyene chain up to $C_{13}$. Unfortunately, analogous information is not yet available in the case of visual pigments. It is interesting to note, however, that at least part of the chromophore strain present in BATHO may be relieved, prior to the LUMI stage, by introducing relatively small modifications in the polyene. Thus, both 5,6 dihydro (derived from the 9-*cis* isomer of chromophore **22**) and 13-desmethyl artificial rhodopsins exhibit a new blue-shifted intermediate (BSI) between BATHO and LUMI which was not detected in the photocycle of native visual rhodopsin (Albeck et al., 1988). It was proposed that the retinal chromophore in the BATHO stage of these artificial pigments is relieved of the strain induced by the primary *cis* → *trans* isomerization, by undergoing a geometrical rearrangement. Such a rearrangement of the retinal moiety, which leads to BSI, may be less efficient in the native pigment due to a tight retinal-opsin (key-lock) configuration at the binding site. In light of these observations, early evidence indicating the occurrence of two BATHO species in bovine rhodopsin (Sasaki et al., 1980, at low temperatures, and Einterez et al., 1987, at room temperature) were recently re-examined using variable excitation power and wavelength (S. Hug et al., *submitted*), as well as a linear dichroism analysis (J. W. Lewis et al., *submitted*). The room temperature data were reinterpreted by assuming that even in the native pigment BATHO rapidly equilibrates with a BSI intermediate. Both species subsequently decay into LUMI over a ~100 nsec time scale.

## Later Stages in the Photocycles

Later stages in the photocycles of Rh and bR which follow the generation of BATHO and K, respectively, are shown in Fig. 3. (For a review, *see* Ottolenghi, 1982.) The scheme for bR includes the recently detected N intermediate (Kouyama et al., 1988), but omits several complicating features such as the occurrence of multiple forms of each of the K, L and M intermediates (Alshut & Stockburger, 1986). This leads to photocycle schemes which may originate in more than a single bR conformer (Ebrey, 1988; Stockburger, 1988). Similar arguments apply to the visual photocycle, for which two forms of the BATHO intermediate have been detected (Einterz, Lewis & Kliger, 1987). Coupling between the photocycles and the biological activity (transduction in Rh and proton translocation in bR) appear to be associated with the analogous blue shifted intermediates MII and M in which the Schiff base has undergone deprotonation. In the bR photocycle both M and N intermediates still maintain the isomerized 13-*cis*, retinal conformation. The back 13-*cis* → all-*trans* isomerization reaction occurs during the N → O transition (Fodor et al., 1988). It is beyond the scope of this review to discuss in detail the various stages of the photocycles and their coupling with the biological function (the reader is referred to the several review articles mentioned in the Introduction). We thus limit our discussion to several relevant conclusions derived from the application of synthetic retinals.

As discussed above, the freedom to rotate about $C_{13}$=$C_{14}$ appears to be a prerequisite for the formation of K in the bR photocycle. Work with a series of artificial pigments with a varying polyene chain lengths, has shown that parts other than the $C_{13}$ . . . N region must be associated with the formation of the L and M intermediates (Sheves et al., 1985). At least two C=C bonds (those corresponding to $C_{11}$=$C_{12}$ and $C_{13}$=$C_{14}$) are required for the formation of L. The M intermediate, which appears to be directly related to the initiation of proton translocation, is formed only in analogs retaining at least three C=C bonds ($C_{13}$=$C_{14}$, $C_{11}$=$C_{12}$ and $C_9$=$C_{10}$). However, specific isomerizations and rotations of bonds other than the $C_{13}$ . . . N region of the chromophore are not required for generation of these photocycle intermediates.

Of special relevance is the observation (Sheves et al., 1986*b*) that, while markedly affecting the Schiff base $pK_a$, $C_{13}$–$CF_3$ substitution has very little influence on the growing-in kinetics of the (deprotonated) M intermediate. This leads to the conclusion that proton transfer to a protein acceptor is not the rate-determining step in the Schiff base deprotonation process. Consequently, the formation of M must be controlled by a distinct process involving changes in the protein. Suggestions for such a rate-determining process (e.g., exposure to a protein positive charge, catalyzing deprotonation) have been discussed by Hanamoto et al. (1984), Sheves

et al. (1986*b*), and by Baasov and Sheves (1986)). We note in this respect that the suggestion that translocation of the Schiff base proton is directly involved in the proton pump (Honig et al., 1979*b*; Engelhard et al., 1985) is consistent with recent work involving methylated bR (Longstaff & Rando, 1987). Methylation of the Schiff base, which exchanges the $NH^+$ moiety with $NCH_3^+$, prevents the generation of the M intermediate, completely inhibiting the proton pump.

In the case of bovine rhodopsin it was shown that the complete integrity of the polyene is not a prerequisite for maintaining the secondary stages of the photocycle. This applies to the 5,6-dihydro pigment (9-*cis* isomer) of chromophore **22** (four C=C bonds), which exhibits a normal photocycle from the stage of LUMI (Albeck et al., 1988). Similarly, the 7,8-dihydro 11-*cis* retinal pigment (three C=C bonds) exhibits a normal light-induced G-protein activation as revealed by its GTPase activity (Calhoon & Rando, 1985).

An interesting observation in this respect involves studies with *Chlamydomonas reinhardii*, a unicellular alga whose phototaxis is governed by a retinal pigment similar to visual rhodopsins. Incorporation of over 80 synthetic retinal analogs led to the unexpected finding that the phototaxis of a blind mutant is always restored, even by analogs with blocked double bond isomerizations and by short acylic aldehydes lacking any C=C bond (Foster et al., 1988). It was concluded that activation of the photoreceptor does not require a photocycle based on C=C isomerization. Alternative mechanisms, based on excited-state charge redistribution along the $C{=}NH^+$ bond, or on a syn/anti isomerization of the same moiety, were proposed. It was also suggested that the same conclusion may be applicable to other, including visual, rhodopsins. This would not be in keeping with the observation (Longstaff, Calhoon & Rando, 1986) that methylation of the Schiff base lysine (296) of bovine rhodopsin not only prevents the formation of the (deprotonated) MII phototransient, but also the activation of the G protein. Further work, with a large variety of artificial visual pigments, should be carried out in order to establish the role of 11-*cis* → all-*trans* isomerization in inducing the conformational change in Rh which in turn activates the G protein.

## Summary and Concluding Remarks

The application of synthetic retinals to rhodopsins has provided unique tools for understanding the polyene-protein interactions in the binding site which determine the color or the pigments. Specific molecular models, based on protein-induced electrostatic and steric effects, are now available for bacteriorhodopsin and for several visual pigments.

Similar progress has been made in respect to the photocycles, which determine the biological function of the pigments. Due to the use of synthetic chromophores it is now evident that the primary photochemical events, occurring over a subpicosecond time scale, consist of a (~90°) rotation about a single specific C=C bond in the excited state, followed by an additional ~90° twist in the ground state. However, several questions are still open, especially those concerned with the exact structures of the early species PHOTO and *J*, as well with the mechanism of energy storage and of the spectral red-shift in the primary photoproducts. Work with artificial pigments suggests that in bR the latter are exclusively associated with polyene-protein interactions in the vicinity of the Schiff base. The picture in visual pigments in this respect is different and still obscure. It is plausible that future applications of artificial chromophores will help in constructing a complete molecular model for the primary photoprocesses in visual transduction.

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## References

Akhtar, M., Bloose, P.T., Dewhurst, P.B. 1968. *Biochemistry* **110**:693–702

Akhtar, M., Jallo, L., Johnson, A.H. 1982. *J. Chem. Soc. Chem. Commun.* pp. 44–46

Akita, H., Tanis, S.P., Adams, M., Balogh-Nair, V., Nakanishi, K. 1980. *J. Am. Chem. Soc.* **102**:6370–6372

Albeck, A., Friedman, N., Ottolenghi, M., Sheves, M., Einterez, C.M., Hug, S.J., Lewis, J.W., Kliger, D.S. 1988. *J. Am. Chem. Soc.* (*in press*)

Albeck, A., Friedman, N., Sheves, M., Ottolenghi, M. 1986. *J. Am. Chem. Soc.* **108**:4614

Alshut, T., Stockburger, M. 1986. *Photochem. Photobiol.* **53**:55–66

Applebury, M.L., Peters, K.S., Rentzepis, P.M. 1978. *Biophys. J.* **23**:375–382

Atkinson, G.H. 1987. *In:* Primary Processes in Photobiology. T. Kobayashi, editor. pp. 213–222. Springer-Verlag, Berlin

Atkinson, G.H., Brack, T.L., Blanchard, D., Rumbles, G. 1988*a*. *Chem. Phys.* (*in press*)

Atkinson, G.H., Blanchard, D., Lemaire, H., Brack, T.L., Hayashi, H. 1988*b*. *Biophys. J.* (*in press*)

Aton, B., Doukas, A.G., Narva, D., Callender, R., Dinur, U., Honig, B. 1980. *Biophys. J.* **29**:79–84

Baasov, T., Friedmann, N., Sheves, M. 1987. *Biochemistry* **26**:3210–3217

Baasov, T., Sheves, M. 1986. *Biochemistry* **25**:5249–5258

Bagley, K., Dollinger, G., Eisenstein, L., Singh, AK.K., Zimanyi, L. 1982. *Proc. Natl. Acad. Sci. USA* **79**:4972–4976

Balogh-Nair, V., Nakanishi, K. 1982. *In:* Comprehensive Biochemistry. Vol. 3. Stereochemistry, C. Tamm, editor. pp. 283–334. Elsevier Biomedical, Amsterdam

Becker, R.S. 1988. *Photochem. Photobiol.* **48:**369–399

Birge, R.R. 1981. *Annu. Rev. Biophys. Bioeng.* **31:**315–354

Birge, R.R., Cooper, T.H. 1983. *Biophys. J.* **42:**61–69

Birge, R.R., Einterz, C., Knapp, H., Murray, L. 1988. *Biophys. J.* **55,** 367–385

Birge, R.R., Findson, L.A., Pierce, B.M. 1987. *J. Am. Chem. Soc.* **109:**5041–5043

Birge, R.R., Hubbard, L.M. 1981. *Biophys. J.* **34:**517–534

Birge, R.R., Murray, L.P., Pierce, B.M., Akita, H., Balogh-Nair, V., Findson, L.A., Nakanishi, K. 1985. *Proc. Natl. Acad. Sci. USA* **82:**4117–4121

Blatz, P.E., Mohler, J.H. 1970. *J. Chem. Soc. Chem. Commun.* pp. 614–615.

Blatz, P.E., Mohler, J.H., Novargal, H.E. 1972. *Biochemistry* **11:**848–855

Boucher, F., LeBlanc, R.M. 1985. *Photochem. Photobiol.* **41:**459–465

Brainman, M., Mathies, R. 1983. *Proc. Natl. Acad. Sci. USA* **79:**403–407

Buchert, J., Stefancic, V., Doukas, A.G., Alfano, R.R., Callender, R.H., Pande, J., Akita, H., Balogh-Nair, V., Nakanishi, K. 1983. *Biophys. J.* **43:**279–283

Calhoon, R.D., Rando, R.R. 1985. *Biochemistry* **24:**6446–6452

Callender, R., Honig, B. 1977. *Annu. Rev. Biophys. Bioeng.* **6:**33–35

Chang, C.H., Chen, J.G., Govindjee, R., Ebrey, T. 1985*a*. *Proc. Natl. Acad. Sci. USA* **82:**396–400

Chang, C.H., Govindjee, R., Ebrey, T., Bagley, K.A., Dollinger, G., Eisenstein, L., Marque, J., Roder, H., Vittitow, J., Fang, J.O., Nakanishi, K. 1985*b*. *Biophys. J.* **47:**509–512

Chen, J.G., Nakamura, T., Ebrey, T.G., Ok, H., Konno, K., Derguini, F., Nakanishi, K., Honig, B. 1989. *Biophys. J.* **55:**725–729

Cooper, A. 1979. *Nature (London)* **282:**531–533

Crouch, R.K. 1986. *Photochem. Photobiol.* **44:**803–807

DeGrip, W.J., Gillespie, J., Bovee-Guerts, P.H.M., Rothschild, K.J. 1987. *In:* Retinal Proteins. pp. 133–143. VNU Science Press, Utrecht

De-Groot, H., Smith, S., Courtin, J., van der Berg, E., Winkel, C., Lugtenburg, J., Herzfeld, J. Griffin, R. 1988. *Biophys. J.* **53:**381*a*

Dencher, N.A. 1983. *Photochem. Photobiol.* **38:**753

Deng, H., Callender, R.H. 1987. *Biochemistry* **26:**7418–7426

Derguini, F., Nakanishi, K. 1986. *Photochem. Photobiophys.* **13:**259–283

Dinur, U., Honig, B., Ottolenghi, M. 1980. *In:* Developments in Biophysical Research. A. Borsellino, editor. pp. 209–221. Plenum, New York—London

Dinur, U., Honig, B., Ottolenghi, M. 1981. *Photochem. Photobiol.* **33:**523–527

Dobler, J., Zinth, W., Kaiser, K., Oesterhelt, D. 1988. *Chem. Phys. Lett.* **144:**215–220

Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K., Termini, J. 1986. *Biochemistry* **25:**6524–6533

Doukas, A.G., Junnarkar, M.R., Alfano, R.R., Callender, R.H., Kakitani, T., Honig, B. 1984. *Proc. Natl. Acad. Sci. USA* **81:**4790–4794

Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., Callender, R.H. 1982. *Biochemistry* **21:**4953–4959

Dupuis, P., Harosi, F., Sandorfy, C., Leclercq, J.M., Vocelle, D. 1980. *Rev. Can. Biol.* **39:**247–258

Ebrey, T. 1988. Abstract, 10th International Congress on Photobiology, Jerusalem, Israel

Ebrey, T.G., Honig, B. 1975. *Q. Rev. Biophys.* **8:**129–184

Einterz, C.M., Lewis, J.W., Kliger, D.S. 1987. *Proc. Natl. Acad. Sci. USA* **84:**3699–3703

Engelhard, M., Gerwert, K., Hess, B., Krentz, W., Siebert, F. 1985. *Biochemistry* **17:**2430–2435

Eyring, B., Curry, B., Broek, A., Lugtenburg, J., Mathies, R. 1982. *Biochemistry* **21:**384–393

Eyring, G., Mathies, R. 1979. *Proc. Natl. Acad. Sci. USA* **76:**33–37

Fang, J.O., Carriker, J., Balogh-Nair, V., Nakanishi, K. 1983. *J. Am. Chem. Soc.* **105:**5162–5164

Fischer, V., Oesterhelt, D. 1979. *Biophys. J.* **28:**211–230

Fodor, S.P.A., Ames, B.J., Gebhard, R., van der Berg, E.M.M., Stoeckenius, W., Lugtenburg, J., Mathies, R. 1988. *Biochemistry* **27:**7097–7101

Foster, K.W., Saranak, J., Derguini, F., Jayathirtha Rao, V., Zarrilli, G.R., Okabe, M., Fang, J., Shimizu, N., Nakanishi, K. 1988. *J. Am. Chem. Soc.* **110:**6588–6589.

Friedman, N., Albeck, A., Sheves, M., Ottolenghi, M. 1989. *Biophys. J. (in press)*

Fung, B.K.K., Hurley, J.B., Stryer, L. 1981. *Proc. Natl. Acad. Sci. USA* **78:**152–156

Fung, B.K.K., Stryer, L. 1980. *Proc. Natl. Acad. Sci. USA* **77:**2500–2504

Hanamoto, J.H., Dupuis, S., El-Sayed, M.A. 1984. *Proc. Natl. Acad. Sci. USA* **81:**7083–7087

Harbison, G., Herzfeld, J., Griffin, R.G. 1983. *Biochemistry* **22:**1–5

Harbison, G.S., Smith, S.O., Pardoen, J.A., Courtin, J.M.L., Lugtenburg, J., Herzfeld, J., Mathies, R.A., Griffin, R.G. 1985. *Biochemistry* **24:**6955–6962

Hildebrandt, P., Stockburger, M. 1984. *Biochemistry* **23:**5539–5548

Honig, B., Ebrey, T., Callender, R.H., Dinur, U., Ottolenghi, M. 1979*b*. *Proc. Natl. Acad. Sci. USA* **76:**2503–2507

Honig, B., Dinur, U., Nakanishi, K., Balog-Nair, V., Gawinowicz, M.A., Arnaboldi, M., Moto, M. 1979*a*. *J. Am. Chem. Soc.* **101:**7084–7086

Honig, B., Greenberg, A.D., Dinur, U., Ebrey, T.G. 1976. *Biochemistry* **15:**4593–4599

Hubbard, R., Kropf, A. 1958. *Proc. Natl. Acad. Sci. USA* **44:**130–139

Hurley, J., Ebrey, T.G., Honig, B., Ottolenghi, M. 1977. *Nature (London)* **270:**540–542

Ippen, E.P., Shank, A., Lewis, A., Marcus, M.A. 1978. *Science* **200:**1279–1281

Irving, C.S., Leermakers, P.A. 1970. *Biochemistry* **9:**858–864

Ito, M., Hiroshima, T., Tsukida, K., Schichida, Y., Yoshizawa, J. 1985. *J. Am. Chem. Soc. Chem. Commun.* pp. 1443–1445

Iwasa, T., Suzuki, Y., Nakayama, T., Tokunaga, F., Hirai, M. 1984. *J. Phys. Soc. Jpn* **53:**2851–2856

Kakitani, T., Kakitani, H., Rodman, H., Honig, B., Callender, R. 1983. *J. Phys. Chem.* **87:**3620–3628

Kimura, Y., Ikegami, A., Stoekenius, W. 1984. *Photochem. Photobiol.* **40:**641–646

Kobayashi, T. 1980. *Photochem. Photobiol.* **32:**207

Kobayashi, T. (editor) 1987. Primary Processes in Photobiology. Springer-Verlag, Berlin

Kobayashi, T., Ohtani, H., Tsuda, M. 1986. *In:* Ultrafast Phenomena. V. D.H. Auston and K.B. Eisenthal, editors, p. 416. Springer, Berlin

Komatsu, T., Suzuki, H. 1976. *J. Phys. Chem. Jpn.* **40:**1725–1732

Koutalos, Y., Ebrey, T.H., Tsuda, M., Okashima, K., Lien, T., Park, M.H., Shimizu, N., Derguini, F., Nakanishi, K., Gilson, H.R., Honig, B. 1989. *Biochemistry* **28:**2733–2739

Kouyama, T., Ikegami, A.N., Mathew, M., Stoeckenius, W. 1988. *Biochemistry* **27:**5855–5863

Lewis, A., Spoonhower, J., Bogomolni, R., Lozier, R., Stoeckenius, W. 1974. *Proc. Natl. Acad. Sci. USA* **71:**4462–4466

Liu, R.S.H., Asato, A.E. 1985. *Proc. Natl. Acad. Sci. USA* **82:**259–263

Liu, R.S.H., Mead, D., Asato, A.E. 1985. *J. Am. Chem. Soc.* **107:**6609–6614

Longstaff, C., Calhoon, R.D., Rando, R.R. 1986. *Proc. Natl. Acad. USA* **83:**4209–4213

Longstaff, C., Rando, R.R. 1987. *Biochemistry* **26:**6107–6113

Lopez-Garriga, J., Babcock, G.T., Harrison, J.F. 1986*a*. *J. Am. Chem. Soc.* **108:**7131–7133

Lopez-Garriga, J.J., Babcock, G.T., Harrison, J.F. 1986*b*. *J. Am. Chem. Soc.* **108:**7241–7251

Lopez-Garriga, J.J., Hanton, S., Babcock, G.T., Harrison, J.F. 1986*c*. *J. Am. Chem. Soc.* **108:**7251–7254

Lugtenburg, J., Muradin-Szweykowska, M., Heeremans, C., Pardoen, J.A. 1986. *J. Am. Chem. Soc.* **108:**3104–3106

Mantione, M.J., Pulman, B. 1971. *Int. J. Quant. Chem.* **5:**349–360

Mao, B., Tsuda, M., Ebrey, T.G., Akita, H., Balogh-Nair, V., Nakanishi, K. 1981. *Biophys. J.* **35:**543–546

Mathies, A. 1979. *In:* Chemical and Biological Applications of Lasers. C.B. Moore, editor. Vol. 4, pp. 55–99. Academic, New York

Mathies, R., Brito Cruz, C.H., Pollard, T.W., Shank, C.V. 1988. *Science* **240:**777–779

Mathies, R.A., Smith, S.O., Palings, I. 1987. *In:* Biological Applications of Raman Spectrometry. Vol. 2-Resonance Raman Spectra of Polymers and Aromatics. T.S. Spiro, editor. pp. 59–108. John Wiley & Sons, New York

Milder, S.J., Kliger, D.S. 1988. *Biophys. J.* **53:**465–468

Molevanger, L., Kentgens, A., Pardoen, J., Curtin, J., Vieeman, W., Lugtenburg, J., DeGrip, W. 1987. *Eur. J. Biochem.* **163:**9–14

Mowery, P., Lozier, R., Chae, Q., Tseng, Y., Taylor, M., Stoeckenius, W. 1979. *Biochemistry* **18:**4100–4107

Muradin-Szweykowska, M., Pardoen, J.A., Dobbelstein, D., Van Amsterdam, L.J.P., Lugtenburg, J. 1984. *Eur. J. Biochem.* **140:**173–176

Nakanishi, K. (editor) 1962. Infrared Absorption Spectroscopy. Holden-Day, San Francisco

Nakanishi, K., Balogh-Nair, V., Arnanoldi, M., Tsujimoto, K., Honig, B. 1980. **102:**7945–7947

Nuss, M.C., Zinth, W., Kaiser, W., Koling, E., Oesterhelt, D. 1986. *Chem. Phys. Lett.* **117:**1–7

Ottolenghi, M. 1980. The Photochemistry of Rhodopsins. *In:* Advances in Photochemistry **12:**97–200

Ottolenghi, M. 1982. Methods in Enzymology. Vol. 88. Biomembranes. Part I(II). L. Packer, editor. pp. 470–491. Academic, New York

Ottolenghi, M., Sheves, M. 1987. *In:* Primary Processes in Photobiology. T. Kobayahsi, editor. pp. 144–153. Springer-Verlag, Berlin

Oesterhelt, D., Stoeckenius, W. 1971. *Nature New Biol.* **233:**149–152

Packer, L. (editor). 1982. Methods in Enzymology, Vol. 88. Part I (I and II). Academic, New York

Palings, I., Pardoen, J.A., Van der Blig, E., Winkel, C., Lugtenburg, J., Mathies, R. 1987. *Biochemistry* **26:**2544–2556

Peters, K., Applebury, M.L., Rentzepis, P.M. 1977. *Proc. Natl. Acad. Sci. USA* **74:**3119–3124

Petrich, J.W., Breton, J., Martin, J.L. 1986. *In:* Primary Processes in Photobiology. T. Koyayashi, editor. pp. 52–60. Springer-Verlag, Berlin

Polland, H.J., Franz, M.A., Zinth, W., Kaiser, W. Kolling, E., Oesterhelt, D. 1984*b*. *Biochim. Biophys. Acta* **767:**635–639

Polland, H.J., Franz, M.A., Zinth, W., Kaiser, W., Koling, E., Oesterhelt, D. 1986. *Biophys. J.* **49:**651–662

Polland, H.J., Zinth, W., Kaiser, W. 1984*a*. *In:* Ultrafast Phenomena. D.H. Auston, K.B. Eisenthal, editors. p. 456. Springer, Berlin

Rao, V.J., Derguini, F., Nakanishi, K., Taguchi, D., Hosoda, A., Hanzawa, Y., Pande, C., Callender, R., 1986. *J. Am. Chem. Soc.* **108:**6077–6078

Rao, V.J., Zingoni, J.P., Crouch, R., Denny, M., Liu, R.S.H. 1985. *Photochem. Photobiol.* **41:**171

Rodman-Gilson, H.S., Honig, H.B., Croteau, A., Zarrilli, G., Nakanishi, K. 1988. *Biophys. J.* **53:**261–269

Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J., Ebrey, T. 1977. *Pure Appl. Chem.* **49:**341–351

Rothschild, K., Roepe, P., Ahl, P.L., Earnest, T.N., Bogomolni, R.A., Das Gupta, S.A., Mulliken, C.M., Herzfeld, J. 1986. *Proc. Natl. Acad. Sci. USA* **83:**347–351

Rothschild, K., Roepe, P., Gillespe, J. 1985. *Methods Enzymol.* **88:**470–491

Rothschild, K.J., Cantore, W.A., Marrero, H. 1983. *Science* **219:**1333–1336

Rothschild, K.J., DeGrip, W.J. 1986. *Photochem. Photobiophys.* **13:**245–258

Rothschild, K.J., Marrero, H. 1982. *Proc. Natl. Acad. Sci. USA* **79:**4045–4049

Rothschild, P., Roepe, P., Lugtenburg, J., Pardoen, J.A. 1984. *Biochemistry* **23:**6103–6109

Sasaki, N., Tokunaga, F., Yoshizawa, T. 1980. *Photochem. Photobiol.* **32:**433–441

Schick, G.A., Cooper, T.M., Holloway, R.A., Murray, L.P., Birge, R.R. 1987. *Biochemistry* **26:**2556–2562

Schreckenbach, T., Walckhopf, B., Oesterhelt, D. 1978. *Biochemistry* **17:**5353–5359

Schulten, K., Tavan, P. 1978. *Nature (London)* **272:**85–86

Sharkov, A.V., Matveets, Yu.A. 1987. *In:* Laser Picosecond Spectroscopy and Photochemistry of Biomolecules. V.S. Letochov, editor. pp. 56–136. Hilger-Bristol, Philadelphia

Sharkov, A.V., Pakulev, A.V., Chekalin, S.V., Matveets, Yu.A. 1985. *Biochim. Biophys. Acta* **808:**94–102

Sheves, M., Albeck, A., Baasov, T., Friedman, N., Ottolenghi, M. 1987. *In:* Retinal Proteins. N.Y. Abdulaev, editor. pp. 205–216. VNU Science, Utrecht

Sheves, M., Albeck, A., Ottolenghi, M., Bovee-Guerts, P.H.M., DeGrip, W.J., Einstein, C.M., Lewis, J.W., Schaechter, L.E., Kliger, D.S. 1986*a*. *J. Am. Chem. Soc.* **108:**6440–6441

Sheves, M., Baasov, T. 1984. *J. Am. Chem. Soc.* **106:**6840–6841

Sheves, M., Baasov, T., Friedman, N. 1983. *J. Chem. Soc. Chem. Commun.* pp. 77–78

Sheves, M., Baasov, T., Friedman, N., Ottolenghi, M., Feinmann-Weinberg, R., Rosenbach, V., Ehrenberg, B. 1984*a*. *J. Am. Chem. Soc.* **106:**2435–2437

Sheves, M., Friedman, N., Albeck, A. 1986*b*. *Proc. Natl. Acad. Sci. USA* **83:**3262–3266

Sheves, M., Friedman, N., Albeck, A., Ottolenghi, M. 1985. *Biochemistry* **24:**1260–1265

Sheves, M., Friedman, N., Rosenbach, V., Ottolenghi, M. 1984*b*. *FEBS Lett.* **166:**245–247

Sheves, M., Nakanishi, K. 1983. *J. Am. Chem. Soc.* **105:**4033–4039

Sheves, M., Ottolenghi, M. 1989. *In:* Chemistry and Biology of Synthetic Retinoids, CRC Press, SRI International. Menlo Park, CA (*in press*)

Shichida, Y., Matuoka, S., Hidaka, Y., Yoshizawa, T. 1983. *Biochim. Biophys. Acta* **723:**240–246

Shichida, S., Matuoka, J., Yoshizawa, T. 1984. *Photochem. Photobiophys.* **7:**221–228

Siebert, F., Maentele, W., Kreutz, W. 1982. *FEBS Lett.* **141:**82–87

Smith, S., Mathies, P. 1985. *Biophys. J.* **47:**251–254

Smith, S., Palings, I., Copil, V., Raleigh, D., Courtin, J., Pardoen, J., Lugtenberg, J., Mathies, R., Griffin, R. 1987*a*. *Biophys. J.* **51:**51a

Smith, S., Palings, I., Copil, V., Raleigh, D., Courtin, J., Pardoen, J., Lugtenberg, J., Mathies, R., Griffin, R., 1987*b*. *Biochemistry* **26:**1606–1611

Spudich, J.L., McCain, D.A., Nakanishi, K., Okabe, M., Shimizu, N., Rodman, H., Honig, B., Bogomolni, R.A. 1986. *Biophys. J.* **49:**479–483

Stern, D., Mathies, R. 1985. *In:* Time Resolved Vibrational Spectroscopy. A. Louberaeu and M. Stockburger, editors. pp. 250–254. Springer-Verlag, New York

Stockburger, M. 1988. Abstract, 10th International Congress on Photobiology, Jerusalem, Israel

Stoeckenius, W., Bogomolni, R. 1982. *Annu. Rev. Biochem.* **51:**587–616

Szundi, I., Stoeckenius, W. 1987. *Proc. Natl. Acad. Sci. USA* **84:**3681–3684

Szundi, I., Stoeckenius, W. 1988. *Biophys. J.* **54:**227–232

Tavan, P., Schulten, K. 1986. *Biophys. J.* **50:**81–88

Uhl, R., Abrahamson, E.W. 1981. *Chem. Rev.* **81:**291

Van der Steen, R., Biesheuvel, P., Mathies, R., Lugtenburg, J. 1986. *J. Am. Chem. Soc.* **108:**6410–6411

Walech, A., Ingraham, L.L. 1973. *Arch. Biochem. Biophys.* **156:**261–266

Warshel, A. 1978. *Proc. Natl. Acad. Sci. USA* **75:**2558–2562

Warshel, A., Barboy, N. 1982. *J. Am. Chem. Soc.* **104:**1469–1476

Warshel, A., Ottolenghi, M. 1979. *Photochem. Photobiol.* **30:**291–293

Warshel, A., Russell, S.T., Churg, A.K. 1984. *Proc. Natl. Acad. Sci. USA* **81:**4785–4789

Weisenfeld, J.R., Abrahamson, E.W. 1968. *Photochem. Photobiol.* **8:**487–493

Yoshizawa, T. 1988. Abstract, 10th International Congress on Photobiology. Jerusalem, Israel

Yoshizawa, T., Horiuchi, S. 1973. *In:* Biochemistry and Physiology of Visual Pigments. H. Langer, editor. p. 69. Springer-Verlag, Berlin

Yoshizawa, T., Shichida, Y., Matuoka, S. 1984. *Vision Res.* **24:**1455–1463

Yoshizawa, T., Wald, G. 1963. *Nature (London)* **197:**1279–1286

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