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# Primary Photochemical Event in Bacteriorhodopsin: Study with Artificial Pigments†

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ABSTRACT: Artificial bacteriorhodopsin (bR) pigments based on synthetic retinal analogues with selectively blocked single and double bonds are prepared and submitted to pulsed laser photolysis. Similar experiments are carried out with short-chain aromatic analogues. It is concluded that only the $C_{13}$=$C_{14}$ double bond can be isomerized in the primary photoprocess. It is shown also that this process is accompanied by separation of the Schiff base from its protein counterion. The effective dielectric constant at the binding site and the nature of the Schiff base counterion play an important role in determining the absorption maximum of bR.

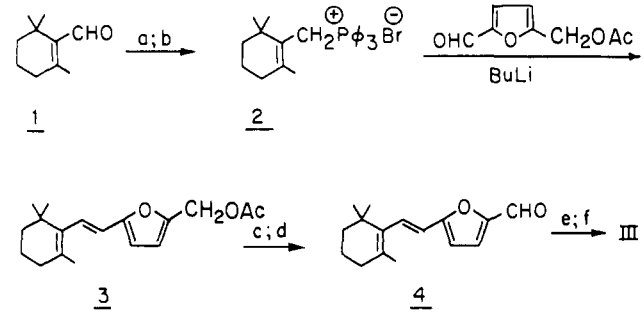
The biologically active, light-adapted, modification of bacteriorhodopsin (the protein pigment in the purple membrane of *Halobacterium halobium*) contains an *all-trans*-retinyl chromophore, bound to the bacteriorhodopsin protein via a protonated Schiff base linkage with a lysine residue. The light-driven proton pump in the pigment $bR_{570}$[1] is induced by a photoprocess centered in the polyene chromophore [see Stoeckenius et al. (1979) and Ottolenghi (1980) for reviews]. Light absorption is followed by a cascade of structural transformations involving both the polyene and the protein. Obviously, a detailed description of all these events is a prerequisite for formulating a molecular model for the function of bacteriorhodopsin.

Of primary importance is the primary event, associated with the red-shifted $K_{610}$ intermediate, which stores a substantial amount of the photon energy (Honig et al., 1979 Warshel et al., 1982). Accumulated, though indirect, evidence suggests that this process is based on isomerization about the $C_{13}$=$C_{14}$ double bond in the polyene and on the resulting charge separation between the protonated Schiff base and its counterion (Honig et al., 1979).

Looking for direct evidence supporting this model, we have previously carried out experiments with an artificial pigment made by recombining bacterioopsin with a synthetic retinal analogue in which the $C_7$=$C_8$ double bond rotation is blocked by a six-membered ring (Umadevi et al., 1983). In this work, this approach is extended to retinal derivatives in which all other C=C double bonds (i.e., $C_9$=$C_{10}$, $C_{11}$=$C_{12}$, and $C_{13}$=$C_{14}$) are alternatively blocked by appropriate five-membered epoxy rings. A basically similar work was first carried out by Akita et al. (1980), who tested the $C_{11}$=$C_{12}$ cis → trans photoisomerization model for visual pigments, using an artificial bovine rhodopsin based on a seven-membered ring retinal analogue.

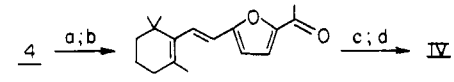
Parallel to the selective blocking of C=C bond rotations, we have carried out a complementary set of experiments with artificial pigments based on short-chain aromatic retinal

Scheme I[a]




[a] (a) $NaBH_4$/EtOH, 30 min, 0 °C. (b) $Ph_3P$·HBr/THF, 25 °C, 12 h. (c) KOH/EtOH, 0 °C, 2 h. (d) $MnO_2/CH_2Cl_2$, 25 °C, 12 h. (e) $(EtO)_2POCH_2CN$/NaH, THF, 25 °C, 1 h. (f) DIBAH/hexane, −78 °C, 1 h/silica, $H_2O$.

Scheme II[a]




[a] (a) MeLi/EtOH, 0 °C, 1 h. (b) $MnO_2/CH_2Cl_2$, 25 °C, 12 h. (c) $(EtO)_2POCH_2CN$/NaH, THF, 25 °C, 1 h. (d) DIBAH/hexane, −78 °C, 1 h/silica, $H_2O$.

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[1] Abbreviations: $bR_{570}$, bacteriorhodopsin, the subscript denoting the wavelength of maximum absorption; THF, tetrahydrofuran; DIBAH, diisobutylaluminum hydride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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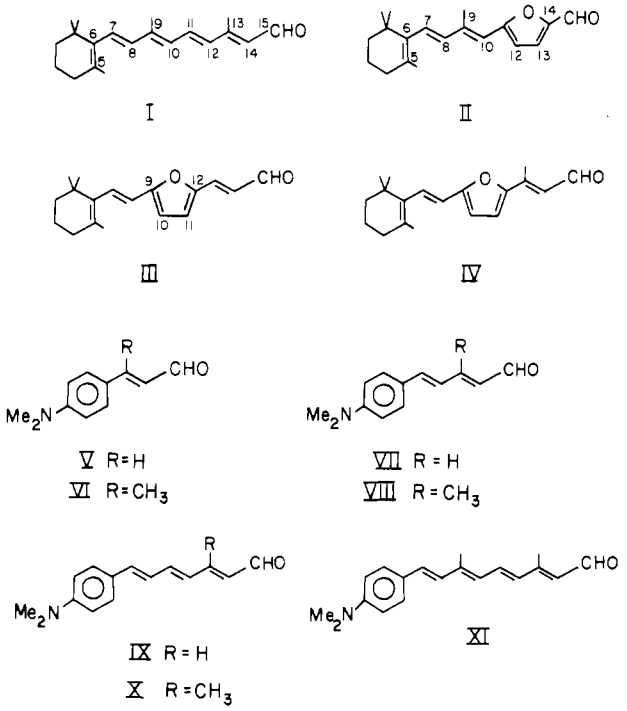
Chart I




analogues. These derivatives lack some of the basic C–C bonds of the original polyene chain, thus directly bearing on the role played by different parts of the molecule in the photocycle. The results are relevant to the nature of the primary event and on molecular aspects related to the biologically important intermediate $M_{412}$, characterized by an unprotonated Schiff base. Finally, spectroscopic information is also obtained, supporting the "point-charge" model for the retinal–opsin interactions in the binding site of bacteriorhodopsin (Nakanishi et al., 1980).

## Materials and Methods

*Synthesis of Compounds.* The structure of all compounds was confirmed by magnetic resonance spectroscopy and by absorption and mass spectrometry. Epoxyretinal II (Chart I) was prepared as previously described (Brock et al., 1983).

(*A*) *Epoxyretinals III and IV* (*Schemes I and II*). β-Cyclocitral (**1**) was reduced with $NaBH_4$ followed by overnight treatment at 25 °C with triphenylphosphine hydrobromide to afford the corresponding triphenylphosphonium salt, **2**. The latter was treated with BuLi and condensed with 5-(acetoxymethyl)-2-furaldehyde to give epoxy **3** in 70% yield. Hydrolysis under basic conditions followed by oxidation with $MnO_2$ afforded aldehyde **4**. Condensation with the sodium salt of $(EtO)_2POCH_2CN$ and reduction with DIBAH in hexane (−78 °C, 0.5 h), followed by a wet silica gel treatment, gave a mixture of two aldehyde isomers. The all-trans isomer III was separated by flash chromatography.

The epoxy derivative IV was prepared by treatment of aldehyde **4** with MeLi (Scheme II) in THF at 0 °C followed by oxidation with $MnO_2$ to give epoxy ketone 5. Wittig reaction with the sodium salt of $(EtO)_2POCH_2CN$, reduction with DIBAH, and separation of isomers gave *trans*-epoxy IV.

All the aromatic aldehydes (V–XI) were synthesized from *p*-(dimethylamino)benzaldehyde as a starting material. Aldehyde VI was prepared by treatment of the starting material with MeLi followed by oxidation with $MnO_2$ and condensation with $(EtO)_2POCH_2CN$. Reduction of the nitrile group with DIBAH afforded aldehyde VI. The other chromophores were prepared by standard procedures, i.e., by condensation with

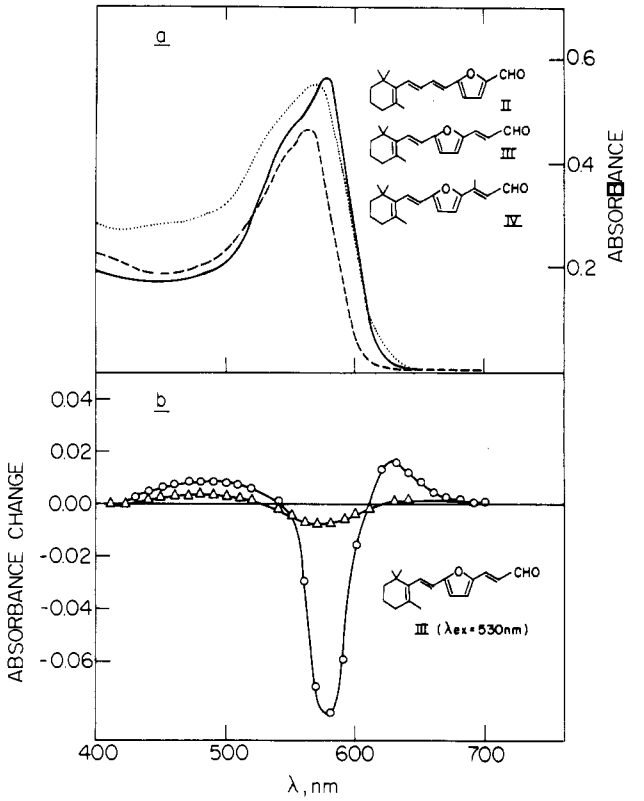



FIGURE 1: (a) Absorption spectra of bR pigments derived from epoxy analogues bR(II) (– –), bR(III) (···), and bR(IV) (—). (b) Laser-induced transient spectra of bR(III): (○) 1 μs; (△) 1 ms.

$(EtO)_2POCH_2CN$, $(EtO)_2POCH_2C(CH_3)=CHCN$, or $(EtO)_2POCH_2CH=CHCO_2Et$, followed by conversion of the ester or the nitrile into an aldehyde group.

(*B*) *Preparation of Artificial Pigments.* Artificial pigments were prepared by reconstituting the apomembrane with the synthetic retinals. The preparation of apomembrane and the reconstitution procedure were described previously (Tokunaga & Ebrey, 1978). The pigments were buffered with $2 \times 10^{-2}$ M HEPES at pH 7.0.

*Pulsed Laser Photolysis.* Pulsed laser photolysis studies of membrane suspensions were carried out on a UV 1000/DL-200 Molectron dye laser system (pulse energy, 0.5 mJ, pulse duration 8 ns) previously described by Kalisky et al. (1981). Data digitized with a Biomation 8100 transient recorder were averaged in a Nicolet 1170 computing system.

It is extremely difficult to obtain apomembrane preparations that are totally free of any $bR_{570}$ chromophore contamination. The contribution of such residual amounts of $bR_{570}$ to the observed phototransient patterns was taken into account by carrying out blank photochemical experiments with an apomembrane preparation under identical conditions.

## Results

*Five-Membered* (*Epoxy*) *Pigments.* The absorption spectra of the artificial bR pigments based on the synthetic analogues II–IV are shown in Figure 1a. All three molecules exhibit a principal absorption maximum close to the 570-nm maximum of $bR_{570}$ with a secondary shoulder around 540 nm. The exact nature of the secondary band may be associated with a low-lying upper excited state of the same pigment or with the contribution of a conformeric species [see, e.g., Sheves et al. (1984)]. Table I compares the spectra of the pigments with those of the free protonated Schiff bases of the related retinal analogues. The energy differences between the corresponding absorption maxima, defined by Nakanishi et al. (1980) as the

"opsin shift" [OS(bR)], are essentially identical with those of native bacteriorhodopsin.

The same pigments were also submitted to pulsed laser photolysis. No phototransients were observed (limited by the 50-ns time resolution of our apparatus) for pigments II and IV excited at 530 nm. In clear contrast to this behavior, excitation of III leads to transient phenomena shown in Figure 1b. The shape of the difference spectrum, observed 1 $\mu$s after the pulse, is due to depletion of the pigment's absorption at 570 nm, accompanied by the generation of two new bands. One is red shifted, while the second is blue shifted relative to the original absorption. In analogy with the intermediates $K_{610}$ and $L_{550}$, characteristic of the photocycle of $bR_{570}$ [see Ottolenghi (1982) for a comprehensive review], we attribute the two bands to two phototransients denoted as K(III) and L(III). Identification of K(III) as a species analogous to $K_{610}$ is based on its red shift relative to bR(III), quantitatively measured by the parameter $\Delta\nu(bR/K)$ (see Discussion). The notation of the 480-nm band as an L species is less meaningful since we have no information on the state of protonation of the Schiff base for this transient. Taking into account that the $L_{550}$ intermediate is characterized by a protonated Schiff base chromophore (Argade et al., 1983) and since it is unlikely for an unprotonated retinal Schiff base to absorb as high as 480 nm, we prefer the L notation over an M assignment. It should, however, be emphasized that our notations do not essentially imply a structural identity between L(III) and the $L_{550}$ intermediate of $bR_{570}$.

It is evident that, while $K_{610}$ is a precursor of $L_{550}$ in a simple consecutive scheme ($bR_{570} \rightarrow K_{610} \rightarrow L_{550} \rightarrow M_{410}$), the photocycle of bR(III) is more complex. Thus, up to 50 $\mu$s, the transient difference spectrum (Figure 1b) is time independent, indicating equilibration between the two intermediates, K(III) and L(III). Subsequently, the spectrum becomes time dependent, the decay of K(III) being faster than that of L(III) (see Figure 1b). It is thus indicated (possibly due to a structural transformation in the photocycling pigment) that the equilibration between L(III) and K(III) is lost on a longer time scale.

The following scheme appears to be in keeping with these experimental observations:

$$\text{bR(III)} \xrightarrow{h\nu} \text{K(III)} \rightleftarrows \text{L(III)}$$

followed by

$$\text{K(III)} \rightarrow \text{L(III)} \rightarrow \text{bR(III)}$$

or by the two independent processes

$$\text{K(III)} \rightarrow \text{bR(III)}$$

$$\text{L(III)} \rightarrow \text{bR(III)}$$

*Short-Chain Aromatic Pigments.* The role of specific regions of the polyene chain in the photocycle may also be studied by varying the chain length. There is, however, a basic difficulty with simple short-chain retinal analogues, which is due to their short wavelength absorption. Thus, in the case of only one or two C=C bonds, the main absorption band of the chromophore is expected to overlap the UV bands of the aromatic amino acids in the protein, leading to substantial experimental problems. We have circumvented this difficulty by working with the aromatic retinal analogues V–XI. In a previous work we have shown that the photocycle of an artificial pigment in which the ionone ring is replaced by an aromatic ring is essentially identical with that of $bR_{570}$ (Umadevi et al., 1983). The present series of molecules carries a dimethylamino ring substituent that substantially shifts the spectra of the pigments to the red. As shown in Figures 2–7 and Table I, this effect is especially pronounced in the case of the shorter chain derivatives, allowing spectroscopic and

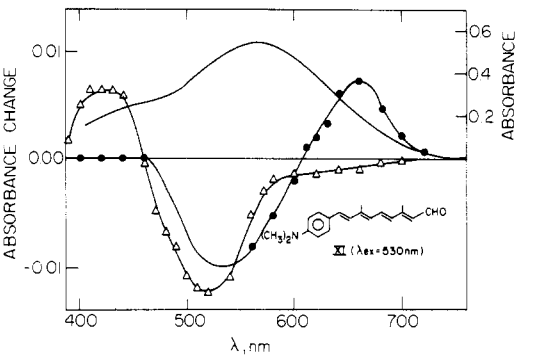



FIGURE 2: Absorption spectrum (—) and laser-induced transient spectra of bR(XI): (●) 1 $\mu$s; (△) 1 ms.

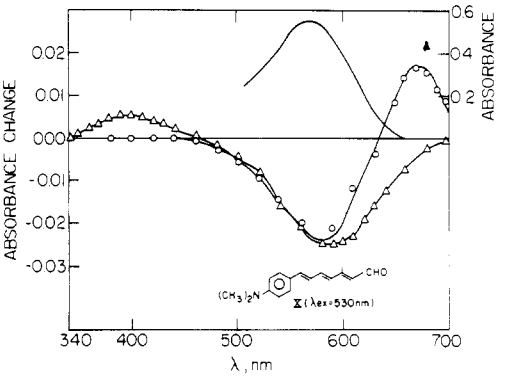



FIGURE 3: Absorption spectrum (—) and laser-induced transient spectra of bR(X): (○) 1 $\mu$s; (△) 1 ms.

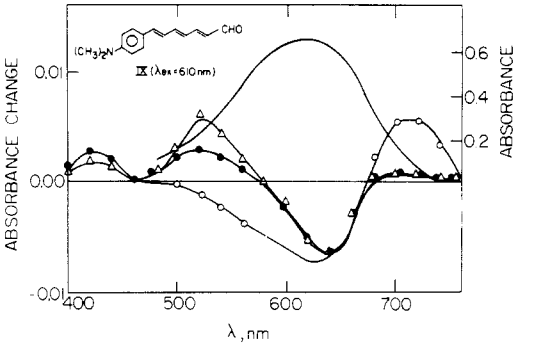



FIGURE 4: Absorption spectrum (—) and laser-induced transient spectra of bR(IX): (○) 1 $\mu$s; (●) 250 $\mu$s; (△) 1 ms.

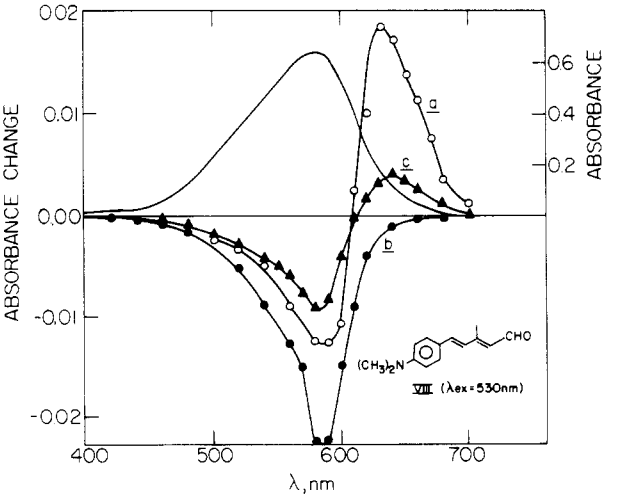



FIGURE 5: Absorption spectrum (—) and laser-induced transient spectra of bR(VIII): (a) 1 $\mu$s; (b) 250 $\mu$s; (c) 1 ms.

Table I: Spectral Data for Synthetic Retinals and Their Artificial Pigments

| chromophore | absorption maxima (nm) R | RSB | $RSBH^+(EtOH)^a$/ $RSBH^+(CH_2Cl_2)^b$ | pigment | photocycle | spectral shifts OS(bR)$^c$/ OS(bR)$^d$ | $\Delta\nu(bR/K)$ | OS(M) |
|---|---|---|---|---|---|---|---|---|
| I | 380 | 360 | 440/475 | 570 | + | 5180/3500 | 2440 | 3500 |
| II | 375 | 367 | 436/460 | 565 | - | 5240/4040 | | |
| III | 379 | 365 | 435/460 | 565 | + | 5600/4040 | 1520 | |
| IV | 386 | 364 | 442/460 | 576 | - | 5260/4380 | | |
| V | 390 | 352 | 460/480 | 510 | + | 2130/1225 | 1420 | |
| VI | 390 | 347 | 464/490 | 518 | - | 2245/1105 | | |
| VII | 418 | 384 | 509/565 | 582 | + | 2465/516 | | |
| VIII | 407 | 384 | 511/550 | 576 | + | 2210/820 | 1490 | |
| IX | 435 | 396 | 525/580 | 615 | + | 2787/980 | 2075 | 1440 |
| X | 414 | 398 | 524/580 | 590 | + | 2135/290 | 2240 | 0 |
| XI | 438 | 400 | 533/570 | 570 | + | 1217/0 | 2160 | 1745 |

[a] RSB (Schiff base) was protonated with HCl, and the spectrum was taken in EtOH. [b] RSB was protonated with periodic acid, and the spectrum was taken in methylene chloride. [c] Opsin shift relative to $RSBH^+$ chloride in EtOH. [d] Opsin shift relative to $RSBH^+$ periodate in methylene chloride.

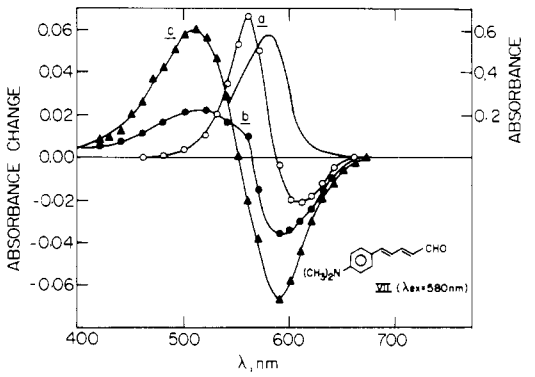



FIGURE 6: Absorption spectrum (—) and laser-induced transient spectra of bR(VII): (a) 1 μs; (b) 250 μs; (c) 1 ms.

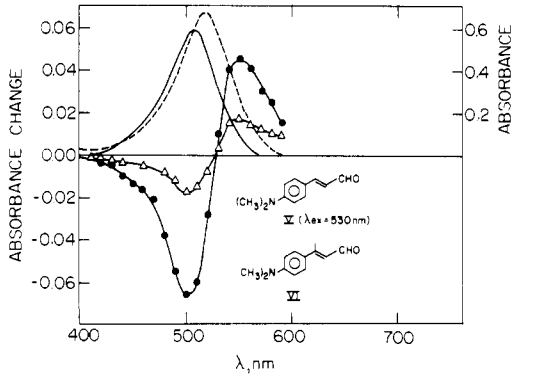



FIGURE 7: Absorption spectra of bR(V) (—) and bR(VI) (--) and laser-induced transient spectra of bR(V): (●) 1 μs; (Δ) 250 μs.

photochemical studies to be carried out in the visible range (Shkrob et al., 1981).

Figure 2 shows the transient phenomena observed following the 530-nm pulsed laser excitation of bR(XI). In complete analogy to the pigment derived from the aromatic analogue of retinal lacking the dimethylamino substituent (Umadevi et al., 1983), bR(XI) also exhibits the basic elements of the $bR_{570}$ photocycle. Thus, a primary red-shifted species identifiable as K is observed. It decays on a 10-μs time scale, yielding the characteristic blue absorption of M. Similar conditions apply to the pigments bR(X) and bR(IX) carrying only three C=C double bonds in their polyene chromophore. We note that no distinct L phase between K and M is observed in the case of the above-three pigments. This behavior, characteristic of several other artificial bR pigments, is encountered whenever the K decay and the M growing-in phases, in the scheme K → L → M, are not kinetically well separated (Umadevi et al., 1983).

While the photocycle of bR(X) (see Figure 3) is basically similar to that of $bR_{570}$, that of bR(IX) exhibits different patterns in the millisecond range. As shown in Figure 4, the formation of K(IX) ($\lambda_{max}$ 720 nm) is followed by generation of a long-lived transient absorbing around 520 nm. With respect to its generation time scale, this transient is reminescent of the long-lived species $O_{610}$, which follows $M_{410}$ in the photocycle of $bR_{570}$. However, spectroscopically, O(IX) is blue shifted (rather than red shifted) with respect to absorption of the parent pigment, bR(IX).

Photocycles are also observed in the case of derivatives bR(VII) and bR(VIII) characterized by two C=C bonds. In the case of bR(VIII), the formation of a red-shifted K species (Figure 5a) is followed by a transient spectrum of an intermediate [L(VIII)] similar to the $L_{550}$ stage of $bR_{570}$ (Figure 5b). No blue-shifted intermediate analogous to $M_{410}$ is observed. However, the L(VIII) stage is followed by the generation of a long-lived, red-shifted intermediate [O(VIII)], analogous to $O_{610}$ in the photocycle of $bR_{570}$ (Figure 5c). In contrast to this "$bR_{570}$-like" behavior, the pigment based on molecule VII, which lacks the methyl substituent, exhibits a photocycle basically different from that of the natural pigment. Thus, no red-shifted transient comparable to $K_{610}$ is observed within the 50-ns time resolution of our apparatus. Its existence would be established only by using equipment with better time-resolution power. As shown in Figure 6a, the first observable intermediate is a blue-shifted species, $L_1$(VII), which decays yielding a second species $L_2$(VII) whose absorption is further shifted to the blue (Figure 6c).

We finally consider the single C=C molecules bR(V) and bR(VI). bR(V) exhibits a single-step photocycle, characterized by a K-like intermediate (Figure 7). No L, M, or O species were observed. No photocycle was detected in the case of bR(VI), whose chromophore has an additional methyl substituent absent in the case of bR(V).

## Discussion

*Absorption Spectra.* All synthetic retinal analogues presented in Table I yield artificial pigments when combined with bacterioopsin. Of special importance is the formation of a pigment even in the case of the shortest chromophore maintaining only a single double bond [bR(V) and bR(VI)]. The data in Table I are best understood in terms of the opsin shift OS(bR), which measures the polyene–protein interactions in the binding site. The original definition of the opsin shift in visual pigments and in bacteriorhodopsin refers to the energy difference between the lowest energy absorption maximum of the pigment and that of the corresponding protonated Schiff base ($RSBH^+$) in ethanol (Nakanishi et al., 1980). The use

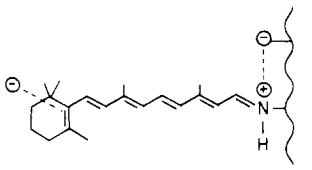


FIGURE 8: External point-charge model for bacteriorhodopsin.

of ethanol as the reference solvent for the spectrum of $RSBH^+$ has the advantage of yielding absorption maxima that are essentially independent of the specific acid used for protonating the Schiff base. In other words, the spectra are insensitive to the size of the counterion and thus to the effective separation between the negative charge and the protonated nitrogen (Blatz et al., 1972). It should be noted, however, that a polar solvent such as ethanol may not properly reflect the dielectric constant in the interior of proteins. Thus, in Table I we have presented spectral data in both ethanol and methylene chloride, the latter being characterized by a relatively low dieletric constant ($\epsilon = 9.08$).

In the case of pigments bR(II)–bR(IV), the values of OS(bR) are close to that of native bacteriorhodopsin with both reference solvents. This is in keeping with the "point-charge" model (Nakanishi et al., 1980) according to which the spectrum of bR is controlled by two charges, one acting as the counterion to the positively charged protonated Schiff base and the second being located in the vicinity of the ring (see Figure 8). Assuming that no major changes in the position of the polyene, relative to the neighboring protein charges, are induced by the addition of the epoxy rings, pigments bR(II)–bR(IV) are expected to behave similarly to $bR_{570}$. However, a complete elimination of the effects due to the ring charge (i.e., very small opsin shifts) is predicted in the cases of bR(V)–bR(X). This is to be expected due to the presence of the aromatic ring, which drastically decreases the effect of the ring charge (Sheves et al., 1984a), and, primarily, due to the increased separation between the polyene and the ring charge. As shown in Table I, only a negligible opsin shift is indeed observed for the short-chain moleculess bR(V)–bR(X) with methylene chloride as the reference solvent and periodic acid as the protonating agent. However, substantial opsin shifts are still observed when ethanol is used as a reference solvent. As previously postulated (Sheves et al., 1983), this indicates that, in addition to the presence of the two protein charges, the effective dielectric constant in the retinal binding site plays a role in determining the opsin shift. Thus, consistency with the point-charge model [i.e., small OS(bR) values for molecules bR(V)–bR(X)] is obtained with methylene chloride and periodate in the reference system. In contrast, substantial OS(bR) values are still observed with ethanol as a reference solvent.

We note that the above observations do not imply that the effective dielectric constant in the bR binding site is equal to that of methylene chloride nor that the counterion separation is as in the case of periodate. (Other nonpolar solvent–counterion combinations may yield $RSBH^+$ spectra similar to those observed in methylene chloride and periodate.) The present results do, however, support the point-charge model for $bR_{570}$ by providing direct evidence for the existence of a charge in the vicinity of the polyene ring.

*The Photocycles.* Some of the new artificial pigments presented in the present work exhibit photocycles that are very similar to those of $bR_{570}$ [e.g., bR(IX) and bR(XI)]. Others differ in details such as the occurrence of an M-like species, the formation of an O intermediate, etc. In the present discussion, we shall not attempt to rationalize in detail the reasons for such differences. We shall be mainly concerned with the basic question as to whether a photocycle, initiated by the formation of a red-shifted k species, is observed. The following discussion includes a first section dealing with the geometrical changes in the retinal skeleton required for photocycling. The second part deals with the electrostatic retinal–protein interactions associated with the primary event.

(*A*) *Retinal Isomerization.* Previous work with a synthetic analogue of retinal, in which all C–C and C=C bond rotations up to $C_9$ are blocked by six-membered rings, indicates that only the $C_9$–N part of the molecule may be directly involved in the primary photochemical event (Umadevi et al., 1983). We first point out that a photocycle associated with a primary red-shifted "K" intermediate is observed for the artificial pigment bR (V), characterized by only one C=C double bond (i.e., the bond analogous to $C_{13}$=$C_{14}$). Complementary to this observation is the occurrence of the same primary event in bR(III) in which $C_{13}$=$C_{14}$ is the only unblocked double bond in the $C_9$–N region of the molecule. Such observations are of primary importance since they obviously restrict the possible conformational changes associated with the primary photoprocess to the $C_{12}$–N region of the polyene molecule. In other words, photoisomerization may only involve the $C_{13}$=$C_{14}$ and $C_{15}$=N double bonds. Furthermore, failure to observe a photocycle in the case of bR(II) in which the $C_{13}$=$C_{14}$ bond is blocked by the epoxy ring directly supports the suggestion (Honig et al., 1979; Braiman et al., 1982) that the generation of the $K_{610}$ photoproduct of $bR_{570}$ is associated with a trans–cis isomerization involving the $C_{13}$=$C_{14}$ bond. This is also in keeping with the lack of any proton-pumping activity in a bR pigment based on a synthetic chromophore in which the $C_{13}$=$C_{14}$ rotation was blocked by a five-membered ring (Fang et al., 1983). Recent NMR (Harbison et al., 1984) and resonance Raman (Smith et al., 1984) experiments have indicated that the $C_{15}$=N conformation is "anti" in both $bR_{570}$ and $K_{610}$. This excludes isomerization about $C_{15}$=N in the primary event, providing further support to the unique role of the $C_{13}$=$C_{14}$ isomerization. Isomerization about the $C_{13}$=$C_{14}$ double bond does not exclude additional conformational changes involving the $C_{12}$–$C_{13}$ and $C_{14}$–$C_{15}$ single bonds. Additional work will be required to establish whether such bonds are involved in any of the steps of the photocycle. A similar approach, aiming at providing evidence for an 11-cis–all-trans isomerization in visual pigments, was first used by Akita et al. (1980), who made use of a derivative with a blocked $C_{11}$=$C_{12}$ bond in the case of bovine rhodopsin.

It is worthwhile noting the effects of $C_{13}$-methyl substitution on pigments bR (IV) and bR (VI), with respect to bR (III) and bR (V), correspondingly. In both cases, no photocycle is observed in the presence of the additional methyl group. This may be attributed to a steric restriction imposed on the $C_{13}$=$C_{14}$ isomerization whenever a $C_{13}$-methyl substituent is present along with a neighboring ring at $C_{12}$. A similar effect was recently observed by Kölling et al. (1984) with an analogue containing an aromatic ring at $C_{12}$.

While the freedom to rotate about $C_{13}$=$C_{14}$ appears to be a prerequisite for the formation of K, it seems that parts other than the $C_{12}$–N region must be associated with the formation of the $L_{550}$ and $M_{410}$ intermediates. Thus, the data of Table I suggest that at least two C=C bonds (those corresponding to $C_{11}$=$C_{12}$ and $C_{13}$=$C_{14}$) are required for the formation of $L_{550}$. The $M_{410}$ intermediate, which is directly related to the initiation of proton translocation [e.g., see Ottolenghi (1980)], appears to be formed only in analogues retaining at least three C=C bonds ($C_{13}$=$C_{14}$, $C_{11}$=$C_{12}$, and $C_9$=$C_{10}$). Finally, it

is important to note that although the charge in the vicinity of the ring has dramatic effects on the opsin shift [i.e., bR(X) and bR(XI)], it does not alter the basic features of the photocycle (Sheves et al., 1984b).

(*B*) *Charge Separation.* Of primary importance is the parameter $\Delta\nu(bR/K)$, measuring the value of the red shift associated with the formation of K. Thus, in addition to the observation of a primary K species in the cycling pigments [bR(III), bR(V), bR(VIII)–bR(XI)], it is evident that the value of $\Delta\nu(bR/K)$ shows little sensitivity to the nature of the specific pigment involved. In other words, the spectral shift associated with the bR → K transition does not show a trend parallel to that of the basic opsin shift OS(bR). The aromatic cycling pigments exhibit much smaller opsin shifts than natural bR due to lack of interaction between the polyene and the ring charge. However, their $\Delta\nu(bR/K)$ values are close to that of bR. This observation leads to the unequivocal conclusion that the spectral shift in K cannot be due to changes in the interaction between the polyene and the ring charge. The same applies to any other protein charge, up to the $C_{13}$ position, which may be possibly interacting with the polyene during the photocycle. These conclusions constitute direct experimental evidence for the model attributing the formation of K to separation of the Schiff base from its protein counterion (Honig et al., 1979; Rothschild & Marrero, 1982). It is this separation between charged moieties that accounts both for the red shift in K and for the storage of light energy in the primary event.

## Conclusions

Artificial bR pigments, based on new synthetic retinal analogues, provide important information relevant to the absorption spectrum of bacteriorhodopsin and to basic molecular aspects of the photocycle: (a) The spectral data support the point-charge model, involving a negative charge in the vicinity of the ring. Two additional factors, the effective dielectric constant at the binding site and the Schiff base–counteranion separation, are also important in determining the absorption maximum of the pigment. (b) Employing artificial pigments based on furanoid retinal analogues, which block rotation around single and double bonds, leads to the conclusion that only the terminal $C_{12}$–N part of the polyene is essential for initiating the bR photocycle. More specifically, the freedom to isomerize about the $C_{13}$=$C_{14}$ double bond is the major prerequisite for the generation of the primary K intermediate. (c) An analysis of the spectral shift associated with the bR → K transition provides direct evidence supporting the suggestion that the primary trans → cis photoisomerization is accompanied by separation of the protonated Schiff base from its protein counterion. (d) It appears that at least three polyene C=C bonds are necessary for the detection of the biologically important M intermediate.

**Registry No.** **1**, 432-25-7; **2**, 56013-01-5; **3**, 94348-01-3; **4**, 94348-02-4; **5**, 94348-03-5; I, 116-31-4; II, 84666-83-1; III, 94347-96-3; IV, 94347-97-4; V, 20432-35-3; VI, 94347-98-5; VII, 20432-36-4; VIII, 94347-99-6; IX, 55298-77-6; X, 94348-00-2; XI, 75859-94-8; $(EtO)_2POCH_2CN$, 2537-48-6; $(EtO)_2POCH_2C(CH_3)$=CHCN, 82648-70-2; $(EtO)_2POCH_2CH$=$CHCO_2Et$, 42516-28-9; $O_3P$·HBr, 6399-81-1; 5-(acetoxymethyl)-2-furaldehyde, 10551-58-3; *p*-(dimethylamino)benzaldehyde, 100-10-7.

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