bubbling dry HCl gas into methanol solution of the Schiff base at −78 °C, under argon in the dark.

Each retinal analogue was incubated at room temperature with bovine opsin at pH 7.0, either in suspension or in 0.5% digitonin[18] for 18–20 h. In the case of suspensions, aliquots were taken at suitable intervals and centrifuged and the pellets were triturated with cold hexane to remove excess chromophore and dissolved in 0.5% digitonin, and the absorption maxima were measured. In the case of reconstitution in 0.5% digitonin, the maxima were simply measured at suitable time intervals. Appearance of a maximum red shifted from that of the corresponding SBH^+ was taken as indication of pigment formation. Support for the fact that the retinal analogues bind to the same site as do 11- and 9-*cis*-retinals was secured by the following experiments.

Rhodopsin analogues were formed in suspension, the hexane-washed pellets (see above) were resuspended in pH 7.0 buffer, and the suspension was incubated for 3 h either with 11- or 9-*cis*-retinal. In each case the amount of natural rhodopsin or isorhodopsin formed was only 2–3% as judged from the absorption maxima, i.e., 500 and 480 nm, respectively. Conversely, only a few percent of the rhodopsin analogues were formed upon reincubation of rhodopsin or isorhodopsin with the retinal analogues. In a competitive binding site study, the 7,8-dihydro-9-*cis*-retinal was coincubated with 9-*cis*-retinal in suspension. The hexane-washed pellet was analyzed for bound retinals by the CH_2Cl_2-denaturation extraction procedure[19] which showed that the pigment consited of a ~1:3 mixture of 7,8-dihydro-9-*cis*- and isorhodopsins.

The absorption data[20] of the chromophores and pigments thus prepared are summarized in Table I along with the data for 11-*cis*-retinal and bovine rhodopsin. The data in Table I shows several important tendencies: (a) since the λ_{max} of the pigment undergoes a progressive shift in going from **4** to **8** (and **9**), clearly it is the enal moiety and not the ene moiety which is responsible for the maxima; (b) since the maximum of the tetrahydroretinal (**9**) derived pigment is similar to that of dihydrorhodopsin, the through-space interaction **8a** can be disregarded; (c) in spite of the shorter chromophore of 11,12-dihydro- (and 9,10,11,12-tetrahydro-) rhodopsin, the difference between the maxima of SBH^+ and rhodopsin, in cm^{-1} (see Table I), is more than twice that observed for other cases, including natural rhodopsin.

Analysis of the Table I data has led to the external point-charge model, which in turn is supported by chemical models.[7,21]

Acknowledgments. We thank Mr. John D. Carriker for technical assistance.

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(3) In view of the recent preparations of various artificial pigments from synthetic model retinals and opsin, we propose the use of the generic name "rhodopsin" prefixed by the name of the specific chromophore, i.e., 5,6-dihydro-9-*cis*-rhodopsin.
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(8) In the case of 9,10- and 11,12-dihydroretinals the "all-trans" forms were capable of forming pigments by virtue of the conformational flexibility which enables them to adopt shapes mimicking 9-*cis*- and 11-*cis*-retinals, respectively. In the case of 7,8-dihydroretinal, however, it was necessary to prepare the 9-cis isomer **6**. Although DeGrip et al. have shown that 7-*cis*-retinal forms a pigment (W. J. DeGrip, R. S. H. Liu, V. Ramamurthy, and A. Asato, *Nature (London)*, **262**, 416 (1976), the rate of formation was only 1/150 that of rhodopsin derived from 11-*cis*-retinal; thus the "all-trans" form of 7,8-dihydroretinal, which already started to decompose after 3 h under the incubating conditions, was too unstable for the prolonged incubation.
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An External Point-Charge Model for Wavelength Regulation in Visual Pigments

Sir:

The chromophoric unit of visual pigments is known to consist of 11-*cis*-retinal covalently bound in the form of a protonated Schiff base to the ϵ amino group of a lysine in the apoprotein opsin.[1] Protonated Schiff bases of retinal absorb at ~440 nm in polar solvents while various salts formed in nonpolar solvents absorb at somewhat longer wavelength (~440–480 nm).[2] The visual pigment bovine rhodopsin has an absorption maximum of ~500 nm while other 11-*cis*-retinal-based visual pigments have maxima as far to the red as 580 nm. The mechanism through which the protein shifts the absorption maximum of the chromophore from its solution value to wavelengths ranging from 440 to 580 nm has been a question of major interest. In this communication we present the first experimentally based model which accounts for the absorption properties of a specific pigment, bovine rhodopsin.

There is considerable support for the suggestion[3] that electrostatic interactions between the chromophore and charged or dipolar groups on the opsin are responsible for wavelength regulation in visual pigments.[2-8] Although models

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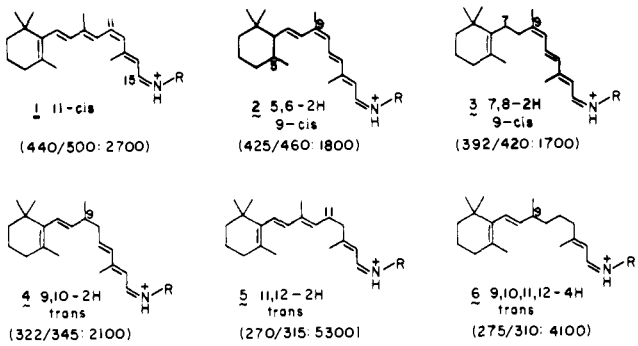


Figure 1. The structures of hydroretinals are represented in conformations resembling 9-*cis*- and 11-*cis*-retinals, i.e., conformations which they presumably adopt when binding to opsin. R stands for either *n*-Bu or opsin. The numerals in parentheses stand for λ_{max} of SBH^+/λ_{max} of pigment (in nm): shift between SBH^+ and pigment (in cm^{-1}).

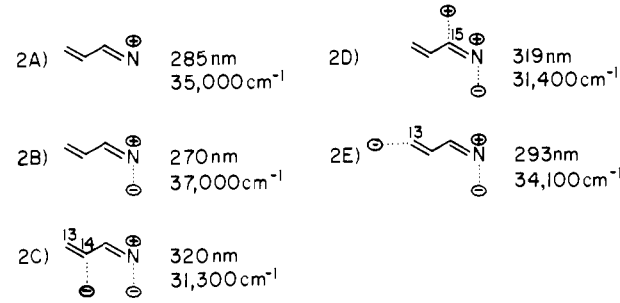


Figure 2. Calculated absorption maxima for model dienes. Dotted lines stand for a 3-Å distance.

of this type are capable of explaining many features of pigment spectra,[6,7] the data have not been available to allow the proposal of a specific model for the electrostatic interactions in the binding site of a particular pigment. However, the spectroscopic data of the dihydrorhodopsins[9] now make it possible for the first time to *locate* a group on the opsin which plays a major role in determining the absorption maximum of bovine rhodopsin.

Our model is derived primarily from the results summarized in Figure 1 which show that the largest shift of 5300 cm^{-1} between the λ_{max} in MeOH of the protonated *n*-butylamine Schiff bases and the λ_{max} (in 0.5% digitonin) of the pigments is encountered for the shortest chromophore, 11,12-dihydroretinal (**5**). The shift for the longer polyenal chromophores **2–4** is around the value of 2000 cm^{-1}, a value close to that observed for retinal **1** and rhodopsin itself. Since the magnitude of the shifts induced by external charges depends on their proximity to the π-electron system,[5,6] these data strongly imply the existence of significant electrostatic interactions in the vicinity of the chromophoric unit of 11,12-dihydroretinal, that is from C-13 to the nitrogen. Moreover, there are unlikely to be spectroscopically important electrostatic interactions near the β-ionone ring since the wavelength shift for rhodopsin **1** is no greater than that for the 7,8- and 9,10-dihydro pigments (**3** and **4**) whose π-electron systems are isolated from the ring.[10]

In order to further specify the position of the group (or groups) on the opsin that determines the λ_{max} of the pigment, we have attempted to account quantitatively for the large bathochromic shift observed for the 11,12-dihydro chromophore (270 to 315 nm, **5** in Figure 1). The magnitude of this shift and the small size of the chromophore provide the severest possible constraints on the construction of acceptable models. Semiempirical π-electron calculations[11] of absorption maxima were carried out on model diene protonated Schiff bases with external charges located as indicated in Figure 2. As is evident from 2B, the calculated absorption maximum (270 nm) of the diene with a single charge representing the counterion or solvent dipoles is identical with the experimental value of 11,12-dihydro protonated *n*-butylamine Schiff base (**5** in Figure 1). However, the most important observation evident from Figure 2 is that it was necessary to place, in addition to a counterion, a second negative charge close to C-14 (2C) or a positive charge close to C-15 (2D) in order to produce a shift as large as the experimental value of 5300 cm^{-1} (**5** in Figure 1). Calculations were carried out with external charges located in a variety of positions, but, with the exception of the general locations shown in 2C and 2D, the absorption maxima obtained were significantly blue shifted from the experimental value of ~315 nm (**5**). For example, a negative charge as located in 2E only shifted the maximum to 293 nm.

These calculations and the data summarized in Figure 1 have been used below to locate the position of a charge on the opsin that determines the chromophore absorption maximum. In doing so we have assumed that the orientation of the chromophore in the various dihydro pigments is similar to that of 11-*cis*-retinal (or when appropriate to 9-*cis*-retinal) in rhodopsin. A clear requirement of any model is that it explain the red shift not only of artificial pigments but of rhodopsin itself. This requirement is rather difficult to satisfy. For example, calculations[12] indicate that a positive charge near C-15 which could account for the λ_{max} of the 11,12-dihydro pigment (as in 2D) would on the other hand produce excessive red shifts for the other dihydro pigments and rhodopsin (λ_{max} of 560 nm). In contrast, a negative charge near C-14 (in the plane of C-13/C-15, as in 2C) yields a λ_{max} for rhodopsin somewhat blue shifted from its experimental value. However, larger red shifts could be obtained for rhodopsin by positioning this negative charge close not only to C-14 but to C-12 as well (since C-12 accumulates positive charge in the excited state[6]). This leads to the model for rhodopsin depicted in Figure 3 where a negative charge is located 3 Å above C-12 and ~3 Å from C-14. The model assumes a fixed counterion near nitrogen[6] and a twisted 12-*s*-transoid conformation for the chromophore as suggested by a number of experimental and theoretical results.[3-15]

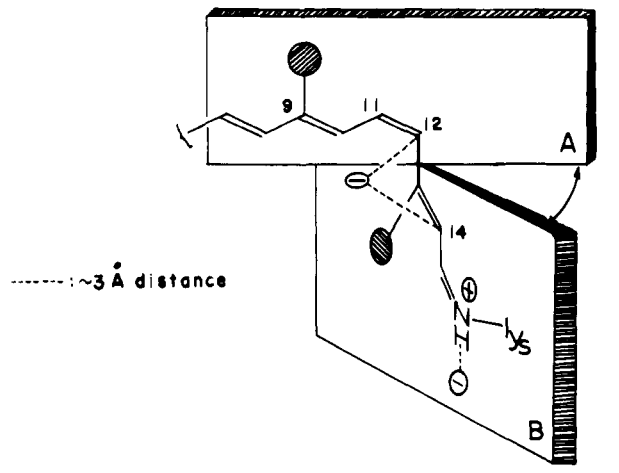
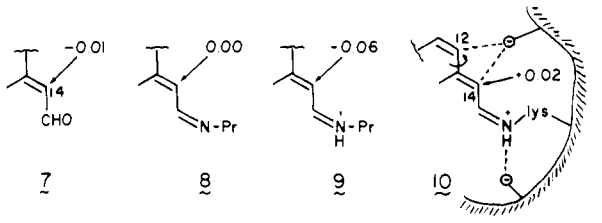


Figure 3. A model for electrostatic interactions in the binding site of bovine rhodopsin. The existence of a counterion near the protonated nitrogen is assumed. A second negative charge is located ~3 Å above carbon 12. This charge is presumably a member of a charge pair in a salt bridge or possibly the negative end of a neutral dipolar group.

We emphasize that there is fairly little flexibility in choosing the position of the external charge. The need to account both for the absorption maximum of the 11,12-dihydro pigment and the smaller but nearly constant wavelength shifts for the other



7 8 9 10

Figure 4. Calculated (see note 10) charges at C-14 for 11-*cis*-retinal (**7**), its Schiff base **8**, the protonated Schiff base **9**, and rhodopsin model **10**.

chromophores as well as rhodopsin requires a model such as the one that we have proposed above. The model as shown was developed in order to account for the wavelength shifts in bovine rhodopsin. Wavelength shifts in other rod and cone visual pigments could be produced from the positioning of one or more external charges in other orientations with respect to the chromophore (see also note 9).

Figure 4 provides an explanation of the C-14 ^{13}C NMR chemical shift reported by Shriver et al.[16] They found that the ^{13}C NMR peaks of C-14 in 11-*cis*-retinal and its propylamine Schiff base were around 130 ppm, whereas in the protonated Schiff base it is upfield shifted to 120.14 ppm. Since the ^{13}C NMR peak in rhodopsin generated from [14-^{13}C]retinal is at 130.8 ppm, it was concluded that the Schiff base in rhodopsin is unprotonated, a result which is in conflict with the resonance Raman data.[17] This apparent discrepancy can be resolved since a negative charge near C-14 would be expected to reduce the π-electron density at this position through coulombic repulsion and, as a result, cause a large deshielding effect. To estimate the magnitude of the effect we calculated π-electron charge densities at C-14 for 11-*cis*-retinal, its Schiff base, the protonated Schiff base, and the rhodopsin model shown in Figure 3. As is clear from Figure 4, the effect of an external charge near C-14 (as in **10**) is to reduce the charge density at this position from that of the protonated Schiff base **9**. Thus, taking into consideration the Raman evidence which favors protonation, the important NMR study of Shriver et al.[16] provides further support for positioning an external charge close to that shown in Figure 1.[18]

A further conclusion is suggested from our model. Rhodopsin, which has an 11-cis chromophore, is red shifted with respect to isorhodopsin (λ_{max} 485 nm), which has a 9-cis chromophore, and the artificial pigment (λ_{max} 450 nm) formed from 7-*cis*-retinal.[19] If we assume that the ring is approximately fixed in the same position in each pigment, then the presence of a cis linkage close to the ring will move the polyene chain from the immediate vicinity of the external charges. The effect will be particularly strong for the 7-cis isomer and weaker in isorhodopsin, thus accounting for the respective shifts to shorter wavelengths.

We have assumed through this paper that external charges positioned around the chromophore are responsible for wavelength regulation in visual pigments. That such effects are possible and of the proper magnitude is supported by absorption data of simple synthetic models.[20] We note that an alternative model[21] for the wavelength shift in rhodopsin involving twisting about the 11-ene is not supported by the 11,12-dihydrorhodopsin data since large red shifts are encountered in the absence of this double bond.

Finally, we emphasize that the presence of a charged group so close to the 11,12 double bond is likely to have a strong effect on the torsional potential for cis-trans isomerization at this position. The high quantum yield (0.67) for photoisomerization of rhodopsin relative to protonated Schiff bases in solution[22] (0.05–0.2) may be due in part to the same electrostatic interactions that we have shown here to account for the absorption maximum of this pigment.[23]

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(11) See ref 6 for a detailed description of the calculations: $\beta_{ij} = \{\beta_{ij}^0 + \delta(U_i + U_j - 21.24)\exp[-\epsilon(r_{ij} - 1.397)]\}$. A distance dependent dielectric constant given by $r - 2$ was used in the calculation of the coulombic interaction with the external charge Q_{ik}. This function is similar to that normally used in calculations on proteins: cf. A. Hopfinger, "Conformational Properties of Macromolecules", Academic Press, New York, 1973, pp 59–63. The magnitude of the shifts induced by the external charges in various positions was found to be independent of the parameterization scheme employed. CNDO/S calculations involving extensive configuration interaction were also carried out (unpublished results) but had no significant effect on the results given in Figure 2.
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Through-Space Electrostatic Effects in Electronic Spectra. Experimental Evidence for the External Point-Charge Model of Visual Pigments

Sir:

The protonated Schiff base (SBH^+) formed between *n*-butylamine and 11-*cis*-retinal (**1**) absorbs at 440 nm in methanol;[1] however, in cattle rhodopsin, in which the retinal moiety is linked to the terminal amino function of a lysine residue **2**, the λ_{max} is shifted to 500 nm.[2] This red shift upon

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