Visual-Pigment Spectra: Implications of the Protonation of the Retinal Schiff Base[†]

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ABSTRACT: Various models of visual-pigment spectra are critically discussed in terms of the spectral properties of protonated Schiff bases and the common structural features of most proteins. The opsin apoprotein is capable of regulating visual pigment wavelengths in ways that are difficult or impossible to reproduce in model systems. Theories based on solvent effects of the spectra of protonated Schiff bases may be misleading. Careful parameterization using known polyene spectra allows accurate calculation of the spectral properties of protonated Schiff bases. It is shown that an isolated protonated Schiff base of retinal should absorb near 600 nm and that blue-shifted spectra seen in solution arise from associated counterions or solvent molecules. We conclude that the most plausible specific model of chromophore–protein interactions is one in which the protonated Schiff base is closely associated with its counterion and where additional negatively charged or polar groups are positioned by the protein in the vicinity of the ring half of the chromophore. Pigment absorption maxima, bandwidths, and the A_2–A_1 pigment absorption differences arise naturally from these simple models of pigment spectra.

The source of the color of visual pigments and the variability in their absorption maxima (λ_{max}) have been a subject of considerable interest. It is known that the 11-*cis*-retinal chromophore of rhodopsin is bound to the apoprotein opsin via a Schiff base linkage to an ϵ-amino group of a lysine (Bownds, 1967). However, since Schiff bases absorb in the ultraviolet, an explanation of pigment color requires additional assumptions. Because Schiff bases undergo a bathochromic shift upon protonation, Morton and co-workers (Morton and Pitt, 1955) proposed that the Schiff base in pigments is protonated. A variety of indirect experimental evidence has supported this hypothesis (reviewed in Honig and Ebrey, 1974) and, recently, resonance Raman studies (Lewis et al., 1973; Oseroff and Callender, 1974) have firmly established that the Schiff base is protonated. A persistent difficulty with using protonation to explain pigment spectra is that the bathochromic shift observed in retinal Schiff bases usually is to about 450 nm, while visual pigments absorb at wavelengths up to 580 nm (620 nm for vitamin A_2 based pigments).

One possible way to further shift the spectra might be through solvent effects. The absorption spectra of protonated Schiff bases has been shown to be sensitive both to the polarizability and polarity of the solvent (Irving et al., 1970). However, the sensitivity of the absorption maxima to these factors is limited and it has been impossible to produce an experimental model system which could shift absorption spectra to the λ_{max} observed for long-wavelength pigments. Moreover, theoretical calculations (Suzuki et al., 1974; Mantione and Pullman, 1971; Waleh and Ingraham, 1973) have demonstrated that a protein should be capable of regulating pigment spectra by the placement of polar or charged groups around the chromophore.

In this paper, we discuss a number of models for the chromophore–protein interactions and choose the one best able to explain the spectroscopic properties of visual pigments and which is compatible with the general structural properties of proteins. We provide both a qualitative and quantitative framework for a discussion of the sensitivity of pigment spectra to chromophore conformation and orientation in the binding site of the protein. Our results may then be used to consider both pigment color and variability, but also the spectroscopic changes that take place following light absorption. Our conclusion is that the principles which govern the absorption spectra of pigments are basically now understood.

Qualitative Aspects of Pigment Spectra

Spectroscopic Phenomena Related to Absorption Maxima. In a recent review (Honig and Ebrey, 1974), we have summarized a number of properties of pigment spectra besides the absorption maxima that an adequate theory must explain. In this paper, we consider the two observations that have received most attention: (1) the observed narrowing of absorption bands with increased λ_{max} of the pigment (Greenberg et al., 1975; Ebrey and Honig, in press); (2) the linear relationship between λ_{max} of a particular pigment and the magnitude of the red shift seen in replacing 3-dehydroretinal (A_2) for the original A_1 chromophore in the same protein (Blatz and Liebman, 1973). Both of these effects have been well documented; the former is usually described in terms of deviations from the Dartnall nomogram (Dartnall, 1953), which is based on the assumption that the bandwidth of visual pigment is independent of absorption maxima. The relevant experimental data is summarized in Table I and Figure 1.

Polyene Spectra. The factors that determine the spectroscopic properties of linear polyenes, such as retinal, are well understood (see, for example, Salem, 1966). It appears to be a general principle that long-wavelength absorption is correlated with increased electron delocalization and decreased single-double bond alternation. This behavior can be seen when comparing cyanine dyes, which have an odd number of atoms, to polyenes, which have an even number of atoms. Cyanines, whose π electrons are largely delocalized, have a long wavelength absorption band equal to about 1000 Å times the number of double bonds and have approximately equal bond lengths; that is, single and double bonds are equivalent.

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TABLE I: Absorption Maxima of A_1-Based Pigments and Their A_2 Analogues.

Species	λ_{A_1} (nm)	λ_{A_2} (nm)	Source[a] of A_1 to A_2 Change	Reference
Frog	432	438	Metamorphosis	Liebman and Entine, 1968
Turtle	442	450	Fresh vs. salt-water species	Liebman and Granda, 1971
Teleost	492	510	Environmental changes	See table in Lythgoe, 1972
Lamprey	497	518	Environmental changes	See table in Lythgoe, 1972
Teleost	499	522	Environmental changes	Many examples: see table in Lythgoe, 1972
Cattle	502	517	Regeneration	Wald, 1953
Frog	502	527	Metamorphosis	Liebman and Entine, 1968
Turtle	502	518	Fresh vs. salt-water species	Liebman and Granda, 1971
Teleost	503	527	Environmental changes	See table in Lythgoe, 1972
Teleost	507	535	Environmental changes	See table in Lythgoe, 1972
Teleost	509	536	Environmental changes	See table in Lythgoe, 1972
Goldfish	510	537	Regeneration	Liebman, 1973
Teleost	512	543	Environmental changes	See table in Lythgoe, 1972
Necturus	540	575	Regeneration	Liebman, 1972
Turtle	562	620	Species	Liebman and Granda, 1971
Chicken	562	620	Regeneration	Wald et al., 1955
Frog	575	620	Metamorphosis	Liebman and Entine, 1968
Goldfish	575	620	Regeneration	Liebman, 1973

[a] Only in the case of regeneration studies is it absolutely clear that the protein is identical for both chromophores.

Polyenes tend to absorb at much shorter wavelengths than cyanines of the same length and exhibit considerable bond alternation. The spectroscopic properties of conjugated hydrocarbon chains are related to the extent that their π electrons are delocalized (Labhart, 1957). *Any mechanism that increases delocalization and reduces bond alternation, even in a polyene with an even number of atoms, should induce bathochromic shifts in the absorption maxima.*

Spectra of Protonated Retinal Schiff Bases. When the Schiff base of retinal is protonated, a positive charge is partially delocalized throughout the π-electron system. Resonance structures, such as those of Figure 2a,b, will contribute (Kropf and Hubbard, 1958), and hence increase electron delocalization and decrease bond alternation (Honig and Ebrey, 1974). The latter effect can be seen from x-ray studies of unprotonated and protonated Schiff bases (Hamanaka and Mitsui, private communication), as well as from the frequency shift of the C=C stretching vibrations observed in resonance Raman studies (Heyde et al., 1971; Oseroff and Callender, 1974). The bathochromic shift that occurs upon protonation can be qualitatively understood in terms of increased electron delocalization. However, the magnitude of the shift is difficult to determine experimentally, since the protonated Schiff base cation does not exist in an isolated form and can only be measured in solution where significant environmental effects are to be expected.

Nevertheless, it is helpful to use the isolated cation as a starting point for a discussion of the spectra of solvated protonated Schiff bases. It can be treated theoretically using π-electron theorry, which has been successful in explaining the behavior of the main absorption band of polyenes. It is, thus, rather disconcerting that the calculated values for λ_{max} of the isolated protonated Schiff base of retinal reported in the literature range from about 440 to 620 nm. However, π electron calculations tend to be parameter dependent, and only Suzuki and his collaborators (Suzuki et al., 1974) have shown that their parameters are appropriate to a closely related series of molecules, the linear polyenes. They suggested that an isolated protonated Schiff base of retinal, in vacuo, would absorb at about 600 nm (see also Blatz et al., 1972). Our own results, reported below, are in close agreement with this prediction.

Solutions of protonated Schiff bases absorb much to the blue of this theoretical value. Irving et al. (1970) and Blatz and Mohler (1970) showed that this was at least partly due to the state of association of the counterion. In nonpolar solvents, where the salt is not dissociated, the effect of a counterion is to pull the positive charge to the nitrogen, increase the contribution of structure a in Figure 2, and thus induce a blue shift. Additional wavelength effects can be obtained by increasing solvent polarity, which induces blue shifts, or increasing solvent polarizability, which induces red shifts.

Models of Pigment Spectra. The discussion of the previous section suggests that it is possible to view pigment spectra as being blue shifted from the theoretical limit of the isolated retinal chromophore, or red shifted from the solution spectra of protonated Schiff base salts. Any combination of the solvent effects discussed above can be used to construct a model of pigment spectra. Models appearing in the literature have, however, emphasized one factor or another and it is useful to consider the extent to which a single environmental determinant is capable of accounting for the full range of pigment absorption spectra.

While solvent polarizability is known to induce spectral red shifts (Irving et al., 1970), we believe that related interactions in the protein are unlikely to play a major role in regulating wavelength. First, the magnitudes of the shifts observed in polarizable solvents are considerably smaller than the range of pigment absorption maxima; in fact, these shifts are difficult to correlate with index of refraction in polar and hydrogen-bonding solvents (Blatz and Mohler, 1970). Theoretical estimates also predict a relatively weak contribution from solvent polarizability (Mantione and Pullman, 1971). In addition, models based on polarizability are highly unsatisfactory from an intuitive standpoint. A highly polar molecule, such as a protonated Schiff base, can clearly have large electrostatic (e.g., dipolar) interactions with the many charged and polar amino acids present on the protein. For the pigment to regulate wavelengths with induced dipoles, when so many permanent dipoles are available, seems unreasonable. In fact, using analogies with bulk solvent properties to describe an essentially short range, highly specific protein-chromophore interaction seems highly questionable.

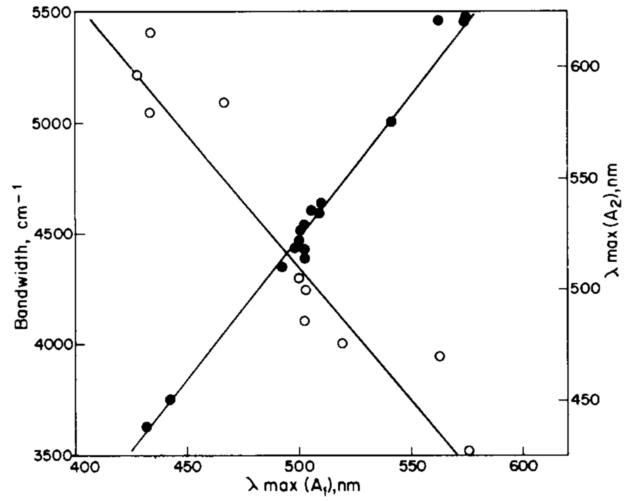

FIGURE 1: (○) Bandwidths of A_1-based visual pigments vs. absorption maximum of the pigment. Data summarized from Ebrey and Honig (in press). (●) Absorption maxima of A_2-based pigments vs. A_1-based pigments. Data taken from Table I.

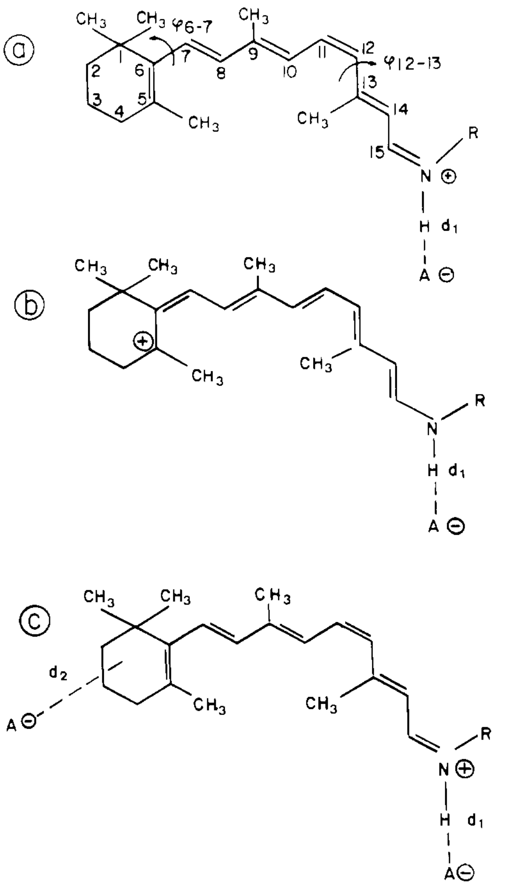

FIGURE 2: Resonance structures for the protonated Schiff base of 11-*cis*-retinal. External charges are included in the figure. (a,b) Counterion only, at distance d_1 from the nitrogen; (c) counterion plus an additional charge at distance d_2 from the ring.

Since twisting about C=C double bonds is known to induce spectral red shifts (400–600 nm in bianthrones, Kornstein et al., 1973), a structural distortion of this type must also be considered as a possible source of wavelength regulation. The range of transition energies spanned by visual pigments varies from 67 kcal/mol (430 nm) to 49 kcal/mol (580 nm). Taking the most favorable case that twisting stabilizes the excited state and destabilizes the ground state, then a minimum strain energy of 9 kcal is required to account for a pigment absorption of 580 nm (assuming a 440-nm pigment is planar about all double bonds). However, if torsional deformations of the chromophore are required for it to fit an opsin binding site, they are much easier to achieve by twisting about single bonds than double bonds. It is difficult to understand how the opsin could specifically introduce a deformation about the *least*-flexible segments of the polyene chain.

It appears that the most plausible model of wavelength regulation involves electrostatic interactions between the charged chromophore and charged or polar groups on the protein. There are obviously a large number of specific models that can be proposed in this context. One possibility that has received considerable attention is that wavelength is varied by regulation of the distance between the protonated nitrogen and its negatively charged counterion (Blatz et al., 1972). In this model, pigment spectra could, in principle, vary from a solution value of 440 nm, with a completely associated counterion, to 600 nm, with the counterion essentially removed to infinity. *However, the necessity of removing the ccounterion to large distances to account for red pigments makes this model extremely implausible.* Independent estimates (Blatz et al., 1972; Suzuki et al., 1974) require a counterion separation of about 10 Å to account for an absorption maxima of say, 580 nm (see also Table III). The Schiff base linkage is located in the interior of the opsin and is not accessible to solvent. But buried charges are not found in proteins; they must form a salt bridge or at least be solvated by surrounding polar groups. There can be no physical counterpart of a model of a protonated nitrogen removed much further than about 3.5 Å from a negative charge.

The most reasonable model for wavelength regulation is a slight modification of the one originally suggested by Kropf and Hubbard (1958). The positively charged nitrogen of the protonated lysine forms a salt bridge with its counterion (a charged amino acid Asp or Glu) producing a complex absorbing near 450 nm as is observed in solution. Wavelength regulation is then achieved by the positioning of *additional* negatively charged or polar groups in the vicinity of the chromophore which pull the positive charge into the chain thus increasing the contribution of resonance structures, such as structure b of Figure 2. We have chosen to represent this model (see Figure 2c) by assuming that a single charge, *in addition to the invariant counterion,* is responsible for wavelength regulation. This then is the simplest representation of the physical situation where the net environment in the immediate vicinity of the chromophore involves a predominance of negative groups.

In the following sections, we show, using π electron calculations, that important features of visual pigment spectra are easily understandable in terms of this model. First, we demonstrate that our calculations can accurately reproduce the absorption spectra of a number of conjugated systems, including a protonated Schiff base with an external charge. The calculations are then applied to the phenomena described above and for the case of the A_2–A_1 absorption difference, quantitative agreement with experiment is obtained. The implications of our results to the bleaching sequence of visual pigments are considered under Discussion.

Calculations

Calculations were performed on the π electron system of the protonated Schiff base using the Pariser-Parr-Pople (PPP) scheme. Additional charges were incorporated into the diagonal elements of the Fock matrix, $\mathbf{F}_{ii}$, so that

TABLE II: Theoretical Parameters.[a]

Atom	Bond	U_i	γ_{ii}	Z^{eff}	Z^{core}	α	g	β°
C	$C(C_3)$	11.16	11.13	3.250	1.0	1.0566	3.4246	2.003
C	$C(C_2H)$	10.62	10.86	3.170	1.0	1.0566	3.4246	2.003
C	$C(C_2Me)$	9.41	10.22	2.984	1.0	1.0566	3.4246	2.003
C	$C(CMeH)$	9.00	10.00	2.919	1.0	1.0566	3.4246	2.003
C	$C(CMe_2)$	8.11	9.49	2.770	1.0	1.0566	3.4246	2.003
C	$C(N^+CH)$	11.55	11.32	3.306	1.03	1.0566	3.4246	0.4980
C	$C(NCH)$	11.61	11.35	3.315	1.04	1.0566	3.4246	2.2700
N^+	$N^+{=}C$	27.42	16.28	4.228	1.93	1.53365	3.85052	0.4980
N	$N{=}C$	13.09	11.88	3.755	0.92	0.92834	3.16410	2.2700

[a] $\epsilon = 2.581$; $\delta = 0.122$.

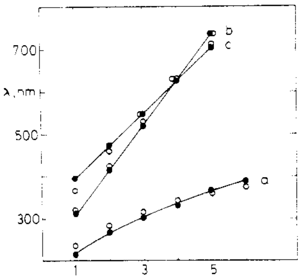

FIGURE 3: Plot of absorption maxima vs. number of double bonds for: (a) polyene hydrocarbons $H(CH{=}CH)_nCH{=}CH_2$ (Sondheimer et al., 1961); (b) cyanine dyes $(CH_3)_2N(CH{=}CH)_nCH{=}N^+(CH_3)_2$ (Malhotra and Whiting, 1960); (c) carbonium ions $(CH_3)_2C{=}CH(CH^+{=}CH)_nC(CH_3)_2$ (Sorenson, 1965). (●) Experimental; (○) calculated

$$F_{ii} = U_i + (\tfrac{1}{2})q_i\gamma_{ii} + \sum_{j\neq i}\{q_j - Z_i^{core}\}\gamma_{ij} + \sum_k Q_{ik} \qquad (1)$$

where U_i is the ionization potential of atom i, q_i is the π electron charge density on atom i, γ_{ii} and γ_{ij} are, respectively, the one and two center coulomb integrals; Z_i^{core} is the core charge, and Q_{ik} is the coulombic interaction between an external charge k separated by a distance R_{ik} from an electron in a p orbital (ϕ_i). Thus,

$$Q_{ik} = \left\langle \phi_i \left| \frac{1}{|r - R_{ik}|} \right| \phi_i \right\rangle \qquad (2)$$

Expressions to evaluate this integral are given by Roothaan (1951).

The effects of both methyl substitution and σ polarization due to heteroatoms were accounted for by adapting the work of Allinger and Tai (1965), and Dewar and Morita (1969). Thus, the ionization potential and one center coulomb integrals were written in the form

$$U_i = \alpha(Z_i^{eff})^2 \qquad (3)$$

and

$$\gamma_{ii} = g_i Z_i^{eff}$$

where Z_i^{eff} is the effective nuclear charge appearing in the orbital exponent and α_i and g_i are empirical parameters. These were determined from the standard values of ionization potentials, repulsion integrals, and orbital exponents (Dewar and Morita, 1969) listed in Table II. The method of Allinger and Tai (1965) was then used to calculate the corresponding values for the various methylated carbons. The core charges, ionization potentials, and repulsion integrals for heteroatoms and bonded carbons were taken from Dewar and Morita (1969). The effect of σ–π polarization was incorporated into the effective nuclear charge using the method of Brown and Heffernan (1958).

Two center repulsion integrals were obtained from the Mataga relationship. The resonance integral was written in the form

$$\beta_{ij} = \{\beta_{ij}^\circ + \epsilon(U_i + U_j - 21.24) - \delta(r_{ij} - 1.397)$$

The values of β_{ij}° for C—C and C—N were obtained by fitting the spectra of two of the cyanine dyes $(CH_3)_2N{-}(CH{=}CH)_3{=}N^+(CH_3)_2$ and $(CH_3)_2N(CH{=}CH)_{11}{=}N^+(CH_3)_2$ (Malhotra and Whiting, 1960). δ and ϵ were determined from the spectra of butadiene and dimethylbutadiene (a detailed study of methylation effects in retinals will appear in a future publication).

At each stage in the iteration cycle, bond lengths were adjusted by the bond order–bond length relationship, $r = r^\circ - ap$ using the parameters given by Dewar and Morita (1969). Excited state bond lengths were calculated from the same equation.

Unless otherwise specified, all torsional angles for the bond ij(ϕ_{ij}) were assumed to be planar trans ($\phi_{ij} = 180°$).

Results

Polyenes. Our results for a series of polyenes, cyanine dyes, and carbonium ions derived from polyenes are summarized in Figure 3. There is clearly an excellent fit for the three classes of molecules even though only two of the cyanines were used to obtain parameters. We were not able to obtain a fit of this accuracy by first determining parameters from aromatic molecules. For instance, parameters determined from benzene, pyrimidine, and pyridine led to a significant underestimation of the λ_{max} of cyanine dyes. The use of such parameters is probably the cause of the large discrepancies among previous calculations on protonated Schiff bases. Our results are very similar to those of Suzuki et al (1974), who used a series of polyenes to obtain parameters.

Protonated Schiff Bases. Effect of Counterion. Calculations were first carried out on the π-electron system of an all-trans protonated Schiff base with the inclusion of a counterion near the nitrogen, as indicated in Figure 2. Table II tabulates the absorption maxima, C=N bond distance, and bond order as a function of separation of the negative charge from the positive nitrogen. The accuracy of these calculations can be verified in a number of ways. First, when the counterion nitrogen separation is 3 Å, the calculated absorption maximum is 461 nm, in the range observed for the all-trans *N*-retinylidene-*n*-butylammonium hydrochloride in nonpolar solvents (Blatz et al., 1972). A recent crystallographic study of this molecule

TABLE III: Counterion Separation Distance, λ_{max}, $C{=}N^+$ Bond Length, and Bond Order for All-Trans Protonated Schiff Base, $\phi_{6-7} = 60°$.

d_1(A°)	λ_{max} (nm)	$r_{C=N^+}$ A°)	$p_{C=N^+}$
2.8	451	1.302	0.82
3.0	461	1.304	0.81
3.2	470	1.305	0.80
3.4	478	1.307	0.79
4.0	497	1.312	0.76
6.0	532	1.321	0.71
8.0	547	1.33	0.69
∞	595	1.35	0.63

(Hamanaka and Mitsui, private communication) determined an N^+-Cl^- distance of 3 Å and it is likely that this ionic bond length is close to the value in nonpolar solvents. Thus, our calculations are consistent with the absorption maximum of a known protonated Schiff base counterion complex. Moreover, the experimenaal red shift of λ_{max} of about 15 nm in going from a chloride to the larger iodide counterion (Blatz and Mohler, 1975) is close to the theoretical value obtained by increasing the counterion separation by 0.4 Å (approximately the difference between the ionic radius of I^- and Cl^-). Finally, the calculated unprotonated Schiff base λ_{max} of 347 nm (Greenberg et al. 1975) is almost identical to the experimental value.

Additional confidence in the calculations is provided by the theoretical values of the C=N bond distance of 1.27 Å for the Schiff base and 1.30 Å for the protonated Schiff base. Both of these values obtained from the bond order-bond length relationship are within the experimental error of the results available from x-ray crystallography (Hamanaka and Mitsui, private communication). The bond orders appearing in Table III may, in principle, be correlated with the observed $C{=}N^+$ stretching frequency. Unfortunately, this is extremely difficult, since the observed frequencies of different pure protonated $C{=}N^+$ double bonds ($p = 1$) vary by about 50 cm^{-1} (1650-1700 cm^{-1}) and are strongly dependent on substituent (Leonard and Paukstelis, 1963). Qualitatively, Table III would predict that small changes in the counterion separation (3-3.5 Å) would have little effect on the $C{=}N^+$ frequency, whereas the larger separations required to account for the spectra of red pigments should induce a significant reduction in stretching frequencies. That no such sensitivity is observed from resonance Raman studies may be taken as an additional argument against the counterion model (Oseroff and Callender, 1974) (see, however, Blatz and Mohler, 1975).

Effect of an Additional Charge. Figure 4 summarizes results for the model where two external negative charges are included in the calculation. One is kept fixed at 2.8 Å from the nitrogen (this reproduces the absorption maximum of the all-trans protonated Schiff base of the chloride salt in CCl_4 of 448 nm) and the second is placed along a line normal to the plane of the ring and extending out from its center (Figure 2c). The chromophore conformation is assumed to be twisted 12-s-trans based on studies of artificial pigments (Ebrey et al., 1975) and on a recent resonance Raman study of visual pigments (Callender et al., 1976). The general trend in Figure 4 is that absorption maximum is red shifted as the external charge approaches the ring. This results from its increased facility in pulling positive charge from the nitrogen as its distance from the π-electron system decreases.

Effects of Twisting. We have argued above that twisting about double bonds is unlikely to be responsible for the wavelength variation of visual pigments. However, since the isolated chromophore is known to exhibit a range of conformations with respect to twisting about single bonds (Honig et al., 1975), the spectroscopic consequences of this type of deformation need to be considered. Blatz and Liebman (1973) have, in fact, suggested that such twisting can regulate visual pigment wavelength. Figure 5 summarizes the dependence of λ_{max} on the torsional angle of the 6-7 single bond, which determines the ring-chain orientation; this bond is known to be extremely flexible in the isolated chromophore. Two curves are presented; in one (Figure 5a) a negative charge is kept 5 Å from the ring and in the other (Figure 5b) this additional charge is absent. (The counterion is present in both cases.) The lower curve illustrates that single bond twisting alone cannot account for the range of absorption maxima of visual pigments. However, the upper curve demonstrates that, once an additional charge has been placed near the ring, absorption maxima become quite sensitive to the ring-chain conformation. This is because the additional charge increases the contribution of structures, such as structure b of Figure 2, and thus increases the 6C-7C bond order. Thus, although it appears that single bond twisting cannot account for the entire range of pigment absorption maxima, it can be an important spectroscopic factor.

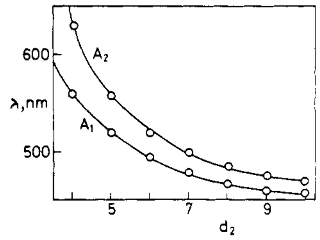

FIGURE 4: Calculated values of λ_{max} vs. distance of external charge from ring plane for A_1 and A_2 pigments. Counterion-nitrogen separation fixed at 2.8 Å, $\phi_{6-7} = 30°$, $\phi_{12-13} = 150°$.

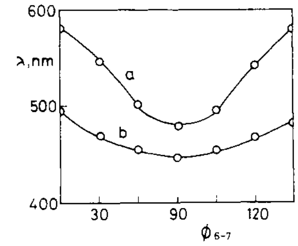

FIGURE 5: Dependence of absorption maximum on 6c-7c torsion angle ϕ_{6-7}; (a) Counterion plus external charge fixed 5 Å from the ring; (b) only counterion included in calculation.

A_2-A_1 Absorption Changes. We have argued previously (Honig and Ebrey, 1974) that the linear relationship between λ_{max} of a particular A_1 pigment and the red shift observed for the A_2 chromophore attached to the same opsin is consistent with the known behavior of conjugated systems. Delocalized systems, such as cyanines, exhibit an incremental shift of about 1000 Å/double bond (Salem, 1966), whereas polyenes display an asymptotic behavior with generally weaker dependence of λ_{max} on chain length. Thus, if we associate pigment red shifts with decreased bond alternation, red-absorbing pigments should behave more like cyanines and exhibit, as is observed, large wavelength shifts in going from A_1 to A_2 chromophores. On the other hand, blue pigments should behave like polyenes and show small wavelength shifts. In Figure 6, we plot λ_{A_1} vs. λ_{A_2} for a number of different chromophore conformations and charge separations. This was done to ensure that the calculated

TABLE IV: λ_{max} of A_0, A_1, and A_2 Pigments Calculated at Various Chromophore Conformations and Charge Separations.

ϕ_{6-7}[a]	ϕ_{12-13}[a]	d_2[b]	A_0	A_1	A_2
			465	498	517 (experimental)
45	150	6	468	494	516
45	60	5	464	497	524
45	30	6	462	493	518
30	150	7	465	501	529
60	150	5	469	497	514

[a] See Figure 2a. [b] See Figure 2c.

TABLE V: Charge Densities.[a]

Atom	Ground State	Excited State
5	0.11	0.13
6	−0.10	−0.09
7	0.03	0.10
8	−0.02	0.01
9	0.12	0.25
10	0.08	0.13
11	0.05	0.00
12	−0.04	0.14
13	0.22	−0.09
14	−0.09	−0.07
15	0.25	0.04
16	0.51	0.39

[a] d_1 = 2.8 Å, $d_2 = \infty$, λ = 463 nm.

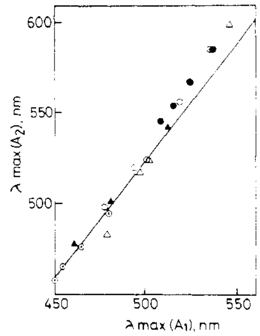

FIGURE 6: λ_{max} of A_2-protonated Schiff base plotted vs. λ_{max} of corresponding A_1-protonated Schiff base. Counterion–nitrogen separation d_1 fixed at 2.8 Å, distance d_2 of external charge from the ring varies. (○) $\phi_{6-7} = 45°$, $\phi_{12-13} = 150°$; (●) $\phi_{6-7} = 180°$, $\phi_{12-13} = 150°$; (⊙) $\phi_{6-7} = 120°$, $\phi_{12-13} = 150°$; (▲) $\phi_{6-7} = 45°$, $\phi_{12-13} = 120°$; (△) $\phi_{12-13} = 150°$, counterion as before, external charge fixed 5 Å from the ring, ϕ_{6-7} varies. Experimental line from Figure 1 included for comparison.

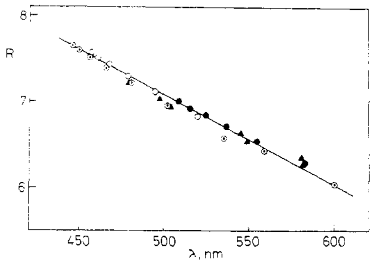

FIGURE 7: Bandwidth parameter R vs. maximum absorption wavelength for A_1 pigments. (○, ●, ⊙) as in Figure 6. (▲) $\phi_{12-13} = 150°$, other variables as in Figure 6.

trend was not the fortuitous result of a particular set of assumptions. The experimental line of Figure 1 is included for comparison. It is clear that the theoretical points and experimental line are in close agreement.

Experimental data are also available for the decrease in the absorption maxima observed for artificial pigments which have no double bonds in their rings (A_0 pigments). Since these studies have been done for only bovine opsin, a range of absorption maxima are not available. A value of 465 nm has been found for both 5,6-epoxyretinal and 5,6-dihydroretinal (see Kropf et al., 1973). In Table IV, we present the results for an A_0 pigment using conformations and charge separations that correspond to λ_{max} of about 500 nm for the A_1 pigment. Agreement with the experimental value is obtained, indicating, together with Figure 6, that our model provides a satisfactory explanation of the spectroscopic effect of adding or removing double bonds to the chromophores of visual pigments.

The 465-nm λ_{max} of A_0 pigments suggests that ring–cain twisting alone cannot explain the absorption maxima of blue pigments. Removing a double bond from the ring is spectroscopically equivalent to twisting 6C–7C by 90°, which would correspond to the shortest wavelength pigment in this model. The fact that there are natural pigments absorbing 35 nm to the blue of the artificial A_0 pigment based on bovine opsin proves that factors other than ring–chain orientation must be important.

Pigment Bandwidths. In a recent publication (Greenberg et al., 1975), we have shown that the parameter $R = (\Sigma_i \Delta r_i^2)^{1/2}$ (where Δr_i is the change in the ith bond length upon excitation) is closely related to the bandwidths of visual pigments. The correlation arises because the width of an electronic absorption band is due to symmetric vibrational progressions; these result from displacement of the equilibrium configuration in going from the ground to excited states. As the displacement R increases, more vibrations are observed and the absorption band becomes correspondingly broadened. Since in linear conjugated systems increased delocalization is associated with reduced geometry changes upon excitation, we showed that red-absorbing pigments should have narrower absorption bands than blue pigments. Theoretical calculations in which wavelength regulation was achieved by varying the charge on the nitrogen confirmed this result. In Figure 7, we plot the parameter R as a function of absorption maxima for the same chromophore conformations and charge separations used in Figure 6. It is clear that R decreases as λ_{max} increases, independent of the specific model we use to regulate wavelengths. Thus, we conclude that the deviations from the Dartnall nomogram, which have been described above, arise naturally from any model of pigment spectra based on associating spectral red shifts with increased π-electron delocalization.

Discussion

We have shown that the properties of the main absorption band of visual pigments may be understood in terms of the behavior of standard polyenes and cyanines. Our results have been discussed in terms of the resonance structures of Figure 2 but further insight is provided by considering the charge densities of Table V. These show that upon excitation a significant fraction of the positive charge of the retinylic cation is delocalized into the chain towards the β-ionone ring. In our calculations the buildup of positive charge begins at atom 10. A similar result has been recently obtained by Salem and Bruckman (1975) using ab initio calculations. Experimental verification of this charge delocalization is provided by the dipole moment studies of Mathies and Stryer (1976). It is thus clear that a negatively charged environment (such as the one represented in Figure 2c) near the half of the chromophore that contains the ring will stabilize the excited state and produce spectral red shifts.

As mentioned above, there are any number of ways to simulate a net negative environment around the chromophore. We have chosen the simplest representation, a single negative charge. We have also tested a model where the negative charge is part of a salt bridge with the positive charge placed 3 Å further than the negative charge from the ring. Our results were essentially unaffected by this additional factor, since the $1/R$ dependence of the Coulombic potential causes the proximal charge to dominate the interaction with the chromophore. In addition, an appropriately oriented group with a permanent dipole moment could clearly produce the desired effect.

The sensitivity of absorption maxima to charge position suggests that the spectral changes of bleaching intermediates may be due to a *reorientation* of the chromophore within the binding site that results from conformational changes in both the chromophore and protein. A cis-trans isomerization, for example, must lead to changes in the distance of external charges from the polyene chain and this effect, in addition to the conformational changes themselves, is likely to have significant spectroscopic consequences. It is interesting to speculate on the source of the red shift of bathorhodopsin in light of these considerations. One possibility is that the red shift results from a twisted conformation about double bonds (Yoshizawa and Wald, 1963), i.e., it is an intrinsic chromophore effect. A second type of model can be based entirely on electrostatic factors. For example, the red shift of bathorhodopsin could arise if the more extended conformation of its transoid chromophore brought the ring closer to an external negative charge than it is in the parent pigment. Alternatively, a small displacement of the protonated Schiff base away from the counterion would also produce red shifts.

Finally, it should be emphasized that the spectroscopic considerations presented here are equally relevant to the purple membrane protein of *Halobacterium halobium* (bacteriorhodopsin) whose all-trans chromophore is also connected to the protein via a protonated Schiff base (Lewis et al., 1974). It is of interest that the width of its main absorption band of about 3200 cm^{-1} is quite close to that of visual pigments absorbing at the same wavelength.

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