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Chemistry

Retinal has a highly dipolar vertically excited singlet state: Implications for vision*

(electric field effects/dipole moments/rhodopsin/visual excitation)

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ABSTRACT **We have measured the effect of an intense electric field on the absorption spectrum of solutions of all-*trans* retinal, its unprotonated Schiff base with *n*-butylamine, and the Cl^- salt of this protonated Schiff base. The field-induced change in extinction coefficient as a function of wavelength was analyzed to determine the ground-state dipole moment (μ_g), the change in dipole moment on excitation ($\Delta\mu$), and the direction of μ_g and $\Delta\mu$. These experiments have shown that all three species become highly dipolar upon excitation to the first allowed excited singlet state ($|\Delta\mu| = 15.6, 9.9, 12$ D, respectively). The ground-state and excited-state dipole moments are nearly parallel to the long axis of these molecules. Excitation is accompanied by a shift of negative charge toward the carbonyl or Schiff base terminus, making the ionone end of these molecules positively charged. The large excited state dipole moment of all-*trans* retinal indicates that the vertically excited state, which is of ^1B_u parentage (C_{2h}), has become significantly mixed with even-parity states. On the basis of previous theoretical calculations, this mixing is expected to facilitate isomerization in the singlet manifold. We have also found that 11-*cis* retinal has a large $\Delta\mu$ (12.7 ± 1.4 D) on excitation. In the visual pigments, the interaction of the excited-state dipole moment of retinal with a suitably located charged group could control the position of the absorption maximum. Also, the large shift in charge density upon excitation of retinal may lead to new electrostatic interactions between the chromophore and the protein that would act as a driving force for the initial conformational changes in visual excitation.**

The chromophore in all known vertebrate visual pigments is 11-*cis* retinal (or dehydroretinal) which is isomerized by light to all-*trans* retinal (1). In rhodopsin, 11-*cis* retinal is attached to a specific lysine residue of opsin by a protonated Schiff base linkage (2–6). The known properties of this chromophore have recently been reviewed (7, 8). A more detailed knowledge of the excited state properties of retinal should facilitate the elucidation of its function since its absorption spectrum and mechanism of isomerization depend on the character of its excited electronic states. However, relatively few direct measurements have been made on the excited states of retinal because of the difficulty of such experiments.

We report here the first determination of the ground and excited state dipole moments of all-*trans* retinal, its Schiff bases, and 11-*cis* retinal. This was accomplished by measuring the effect of an intense electric field on the absorption spectrum of solutions of these molecules. The Schiff bases of the all-*trans* isomer were chosen for this initial study because the interpretation of their electric field spectra is more direct than for those of the 11-*cis* isomer. The striking finding is that excitation of all-*trans* retinal and its Schiff bases is accompanied by a large shift in negative charge away from the ionone ring end of the molecule. These observations help to characterize the excited states and isomerization mechanism of retinal. Furthermore, these measurements give insight into the interactions between retinal and opsin during the initial stages of visual excitation.

Abbreviation: D, debye (10^{-18} esu cm).

* A preliminary account of this work was presented at the 20th Annual Meeting of the Biophysical Society, February 1976.

ANALYSIS OF ELECTRIC-FIELD SPECTRA

Quantitative electric-field perturbation studies were performed independently by Labhart (9) and Czekalla (10) in 1961. Since then, several laboratories have determined both ground and excited-state dipole moments (11, 12) and polarizabilities (13–15) by studying the effects of intense electric fields on absorption and fluorescence (16, 17). For a comprehensive review, see Liptay (18).

An external electric field has two effects on the absorption spectrum of a solution of dipolar molecules. First, the energy of the electronic transition changes if the ground-state dipole moment μ_g is different from the excited-state dipole moment μ_e. The shift in the energy of the transition ΔE is given by

$$\Delta E = -(\mu_e - \mu_g)\cdot F = -\Delta\mu\cdot F \qquad [1]$$

where $\Delta\mu$ is the change in dipole moment and F is the applied electric field. Second, the molecules tend to be oriented by the electric field if they possess a ground-state dipole moment. This generates an anisotropic molecular distribution, and consequently the sample exhibits linear dichroism. Liptay (19) has derived an expression for the field-induced change in extinction coefficient $\Delta\epsilon$ as a function of the frequency ν of the incident light, the internal field strength F_{int}, and the angle χ between the direction of the applied electric field and the polarization vector of the incident light.

$$\Delta\epsilon(\nu, F, \chi) = {F_{\text{int}}}^2 \times \epsilon\left[A_\chi + \frac{B_\chi}{15h}\frac{\frac{\partial}{\partial\nu}\left(\frac{\epsilon}{\nu}\right)}{\frac{\epsilon}{\nu}} + \frac{C_\chi}{30h^2}\frac{\frac{\partial^2}{\partial\nu^2}\left(\frac{\epsilon}{\nu}\right)}{\frac{\epsilon}{\nu}}\right] \qquad [2]$$

where

$$A_\chi = \frac{\beta^2}{30}[3(\hat{p}\cdot\mu_g)^2 - \mu_g^2](3\cos^2\chi - 1) \qquad [3]$$

$$B_\chi = 5\beta(\Delta\mu\cdot\mu_g) + \beta[3(\hat{p}\cdot\Delta\mu)(\hat{p}\cdot\mu_g) - \Delta\mu\cdot\mu_g](3\cos^2\chi - 1) \qquad [4]$$

$$C_\chi = 5\Delta\mu^2 + [3(\hat{p}\cdot\Delta\mu)^2 - \Delta\mu^2](3\cos^2\chi - 1) \qquad [5]$$

In these equations, $\hat{p}$ is a unit vector in the direction of the molecular transition moment, h is Planck's constant, and $\beta = (kT)^{-1}$, where k is Boltzmann's constant and T is the temperature.

The physical basis of the three terms in Eq. 2 can be described qualitatively. The A_χ term results from the field-induced orientation of the ground-state molecules, giving it the shape of the unperturbed absorption spectrum. The C_χ term

arises from the band shift effect. For each molecule oriented so that the field lowers its transition energy by a certain amount there is another molecule oriented so that the field raises its transition energy by an equal amount. Consequently, the electric field induces a symmetric broadening of the absorption spectrum, giving the C_χ term, which is a second-derivative signal. The orientation effect and the band shift effect interact to give a first-derivative term. This B_χ cross-term arises because the orientation effect unbalances the number of molecules having their transition energies raised and lowered by the band shift effect. All three contributions depend on the square of the effective field.

In using these equations, it is necessary to convert the externally applied field F_{ext} to an internal field F_{int}. We have used the spherical cavity approximation, which gives

$$F_{int} = \frac{3\xi}{2\xi + 1} \cdot F_{ext} \qquad [6]$$

where ξ is the dielectric constant of the solvent. No corrections have been made for the effect of dipole reaction fields or for the contribution of molecular polarizability to the electric field signals. Electric field experiments on diphenyldecapentaene, which should have nearly the same polarizability as retinal, suggest that the polarizability contribution to the field-induced $\Delta\epsilon$ for retinal alters the derived dipolar molecular parameters by less than 5%. Recent electric-field measurements (20) comparing solution and gas phase results show that the *combined* error due to the spherical cavity approximation and the lack of a reaction field correction is less than 10% for molecules like *p*-nitroanisole. The calculated internal field for an ellipsoidal cavity containing the more elongated retinal is nearly the same as that given by Eq. 6. Our experiments measure the condensed phase dipole moment, which is the sum of the gas-phase dipole moment and that induced by the reaction field. The reaction field corrections needed to derive gas-phase dipole moments from the observed ones may be appreciable because of the large polarizability of retinals. However, since there is likely to be a comparable reaction field for retinals in solution and in visual pigments, our observed dipole moments in the condensed phase are in fact the pertinent ones for understanding the photochemistry of these systems.

The dipole moments of the ground and excited states and their relationship to the transition moment direction were determined by measuring the field-induced change in extinction coefficient $\Delta\epsilon$ as a function of wavelength at three values of χ. The absorption spectrum in the absence of an electric field was also measured and its first and second derivatives with respect to frequency were computed numerically (21). The values of A_χ, B_χ, and C_χ were then obtained by making a least-squares fit of the observed $\Delta\epsilon$ spectrum to a sum of the absorption spectrum and its first and second derivatives. Finally, the molecular parameters were calculated from the slopes and intercepts of least-squares plots of A_χ, B_χ, and C_χ versus $(3\cos^2\chi - 1)$. The statistical errors were computed using a standard error matrix method for a linear least-squares fit (22).

MATERIALS AND METHODS

Electric Field Spectrometer. The electric field apparatus must be capable of detecting changes of about 1 part in 10^6 in the intensity of the transmitted light because the field-induced changes in the extinction coefficient are very small. This sensitivity was achieved by simultaneously applying a 20 kV dc potential and a 10 kV (root mean square) 200 Hz ac potential and detecting $\Delta\epsilon$ with a phase and frequency-sensitive amplifier (PAR HR-8). The value of the effective external field F_{ext} is given by $F^2_{ext} = 2 \cdot F_{ac} \cdot F_{dc}$, where F_{ac} and F_{dc} are the values of the applied ac and dc fields.

The essential electronic and optical features of this spectrometer have been described previously (23, 21). Our electric field cell is similar to the one designed by Czekalla (16), except that in our cell quartz spacers defined the path length and eliminated fringing fields from the electrodes. These spacers were pressed into the Teflon body, giving an optical path of 1.85 cm. The gap between the stainless steel electrodes was 0.45 cm. The hollow electrodes were maintained at 22 ± 1 °C.

Materials. All-*trans* retinal (Eastman) was used without further purification. Thin-layer chromatography (24) showed that the amount of other isomers was about 1%. 11-*cis* Retinal was a gift of Hoffmann-LaRoche and Co. All samples were handled under dim red light and stored under N_2. 1,4-Dioxane (MCB Spectroquality) was stored over 4-A Molecular Sieves (MCB). The *n*-butylamine Schiff base of all-*trans* retinal was prepared by the method of Irving and Leermakers (25). After the reaction was completed, the mixture was evaporated and the residue was thoroughly dried in a vacuum dessicator. The Schiff base was then dissolved in dioxane for immediate use or stored in hexane under N_2 at −20°C. The protonated *n*-butylamine Schiff base of all-*trans* retinal was prepared by adding a solution of HCl in dioxane to the Schiff base in dioxane.

Absorption spectra were taken before and after each electric field experiment to check for isomerization and degradation. The concentration of retinal and its *n*-butylamine Schiff base decreased slightly (<5%) during the electric field run, presumably because of adsorption to the cell. The absorption spectrum was virtually unaltered; in particular, there was no evidence of *cis* peaks. In addition, experiments were performed comparing the electric-field results of all-*trans* retinal with and without a recirculating sample system (6). The results were nearly identical, indicating that the extent of isomerization in the light beam was very small. The electric-field spectra of 11-*cis* retinal were also obtained using this recirculating sample system. The protonated Schiff base samples were run within 1 hr of formation because of their instability. The concentration of the protonated Schiff base dropped by as much as 6% during an electric-field experiment because of adsorption to the cell and deprotonation. This loss was corrected by monitoring the peak value of $\Delta\epsilon$ (470 nm and $\chi = 0°$) at several points during the electric-field run and scaling the data accordingly.

RESULTS

The electric field spectra of all-*trans* retinal, its unprotonated Schiff base with *n*-butylamine, and its protonated Schiff base are shown in Figs. 1, 2, and 3, respectively. The data points in these figures are the observed values of $\Delta\epsilon$ at three settings of the polarization angle χ. The continuous lines in these figures were computed from the least-squares values of A_χ, B_χ, and C_χ using Eq. 2. The good agreement between the observed and calculated electric field spectrum shows that Eq. 2 adequately accounts for the field-induced spectral changes. The molecular parameters are summarized in Table 1.

Let us first consider the results for all-*trans* retinal. Its ground state dipole moment parameters are small ($|\mu_g| = 3.5 \pm 3.3$, $|\hat{p}\cdot\mu_g| = 4.3 \pm 0.8$) indicating that it is a relatively non-polar molecule. This value is similar to the μ_g of 1.99 and 2.33 D found by Chen and LeFèvre (26) for all-*trans* retinol and all-*trans* retinyl acetate, respectively. Our electric-field results for these molecules agree within experimental error with these values. In contrast to these small ground state parameters, there is a very large change in dipole moment upon excitation to the first allowed singlet state of retinal ($|\Delta\mu| = 15.6 \pm 1$ D). Thus,

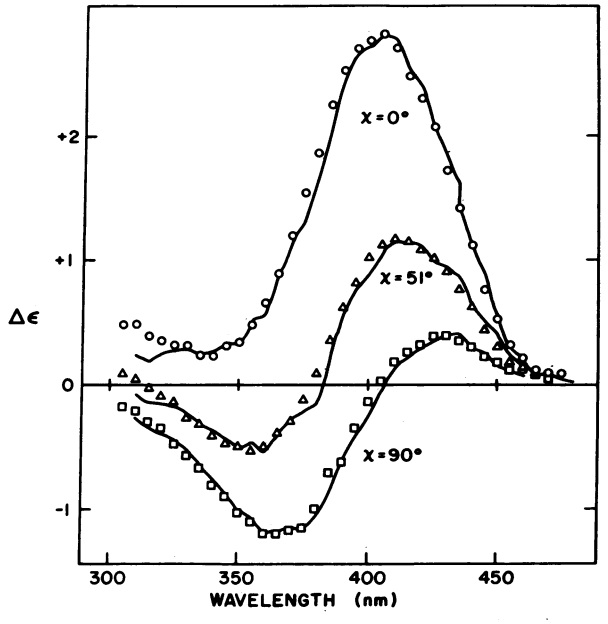

FIG. 1. Electric-field spectrum of all-*trans* retinal in *p*-dioxane. $F_{ext} = 4.48 \times 10^4$ V/cm.

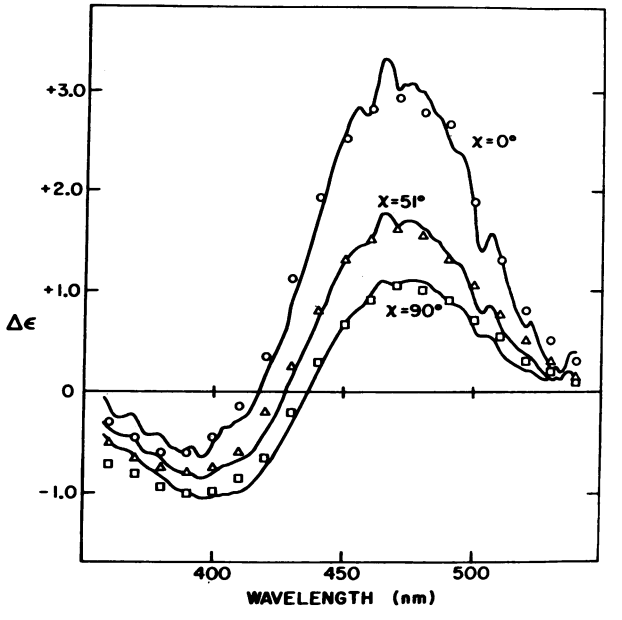

FIG. 3. Electric-field spectrum of the Cl^- salt of the protonated *n*-butylamine Schiff base of all-*trans* retinal. $F_{ext} = 4.12 \times 10^4$ V/cm.

all-trans retinal becomes highly dipolar on excitation. The orientation of the shift in charge on excitation can be deduced in the following way. (*1*) The value of $|\Delta\mu|$ (15.6 ± 1 D) is nearly the same as that of $|\hat{p}\cdot\Delta\mu|$ (14.7 ± 2.1), revealing that $|\Delta\mu|$ is nearly parallel to the transition moment direction, which is known to be along the long axis of the molecule (27). This is consistent with the observation that the product of $|\hat{p}\cdot\mu_g|$ with $|\Delta\mu|$ is nearly equal to $\Delta\mu\cdot\mu_g$. (*2*) The positive sign of $\Delta\mu\cdot\mu_g$ shows that $\Delta\mu$ points in the same direction as the projection of μ_g on the long axis of the molecule. Do $\Delta\mu$ and μ_g point toward the ionone ring or the carbonyl end of the molecule? It seems likely *a priori* that the oxygen atom of the carbonyl group bears a partial negative charge, which would mean that μ_g and hence $\Delta\mu$ point toward the ionone ring. This expectation is supported by theoretical calculations of ground and excited states of retinals (H. Suzuki; D. S. Kliger; personal communications). We conclude that ***negative charge moves away from the ionone ring when all-trans retinal is excited.***

The molecular parameters for the unprotonated and protonated Schiff bases of retinal and *n*-butylamine are qualitatively similar to those of all-*trans* retinal (Table 1). The protonated Schiff base has a larger ground-state dipole moment ($|\mu_g| = 6.2 \pm 2.4$) than does retinal, whereas the unprotonated Schiff base has a smaller one ($|\mu_g| = 1.3 \pm 0.7$). The association of a counter ion with the protonated Schiff base introduces an uncertainty in interpreting the value of μ_g derived from its electric field spectrum. A charge contributes to the orientation effect only if it is fixed to the framework containing the transition moment. Part of the contribution of the Cl^- counter ion would be lost if it changes its position relative to the transition moment in responding to the electric field. This uncertainty is not encountered in interpreting $\Delta\mu$, which arises only from the shift in electrons caused by π-electron excitation. In fact, ***the protonated and unprotonated Schiff bases, like retinal, exhibit a large $\Delta\mu$ on excitation*** ($|\Delta\mu| = 12.0 \pm 2.0$ and 9.9 ± 0.5 D, respectively), which lies parallel to the long axis of the molecule. The assignment of the direction of $\Delta\mu$ for the unprotonated and protonated Schiff bases is based on a comparison with theoretical calculations on their electronic states (H. Suzuki; D. S. Kliger; private communications) which predict that $\Delta\mu$ points toward the ionone ring in correspondence with all-*trans* retinal.

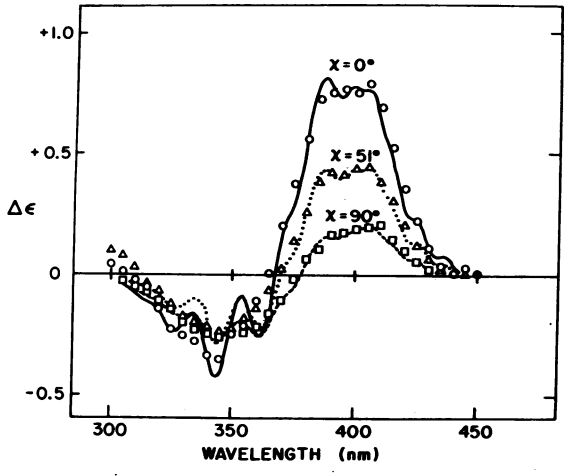

FIG. 2. Electric-field spectrum of the *n*-butylamine Schiff base of all-*trans* retinal in *p*-dioxane. $F_{ext} = 4.80 \times 10^4$ V/cm.

The electric-field spectra of 11-*cis* retinal are given in Fig. 4, and the results are summarized in Table 1. ***11-cis Retinal, like all-trans retinal exhibits a large change in dipole moment upon excitation*** ($|\Delta\mu| = 12.7 \pm 1.4$ D). As expected for a less symmetric molecule, the analysis of the vector projections indicates that $\Delta\mu$, $\hat{p}$, and μ_g are not colinear. Also, the ground state values for 11-*cis* retinal ($|\hat{p}\cdot\mu_g| = 6.9 \pm 2.8$, $|\mu_g| = 10.3 \pm 5.6$) indicate that it is probably more polar than the all-*trans* isomer.

DISCUSSION

These electric field experiments reveal that all-*trans* retinal, its unprotonated Schiff base with *n*-butylamine, and its protonated Schiff base become highly dipolar upon excitation to the first allowed singlet state. The ground state and the excited state dipole moments are nearly parallel to the long axis of each of these molecules, which is also the direction of the transition

Table 1. Ground and excited state dipole moments (debyes) of all-*trans* retinal, its Schiff bases, and 11-*cis* retinal

	all-*trans*	Unprotonated[a] Schiff base	Protonated[b] Schiff base	11-*cis*
$\lvert\Delta\mu\rvert$	15.6 ± 1.0	9.9 ± 0.5	12.0 ± 2.0	12.7 ± 1.4
$\Delta\mu\cdot\mu_g$	62.0 ± 1.3	12.1 ± 0.5	96.2 ± 2.0	58.3 ± 1.7
$\lvert\mu_g\rvert$	3.5 ± 3.3	1.3 ± 0.7	6.2 ± 2.4	10.3 ± 5.6
$\lvert\hat{p}\cdot\Delta\mu\rvert$	14.7 ± 2.1	10.2 ± 0.9	15.2 ± 2.9	8.9 ± 3.2
$\lvert\hat{p}\cdot\mu_g\rvert$	4.3 ± 0.8	1.3 ± 0.2	4.5 ± 1.1	6.9 ± 2.8

[a] Schiff base of all-*trans* retinal and *n*-butylamine.

[b] Cl^- salt of the protonated Schiff base of all-*trans* retinal and *n*-butylamine. All samples were in dioxane at 22°C ($\beta = 2.455 \times 10^{13}$ erg^{-1} or 2.455×10^{6} joule^{-1}). $F_{int} = 1.224\ F_{ext}$. The error limits correspond to ± 1 standard deviation. Uncorrectable systematic errors of ± 8% in $\Delta\epsilon$, which arise during the determination of the derivatives and other numerical factors in Eq. 2, have not been propagated.

moment. Excitation of these molecules is accompanied by a shift of negative charge toward the carbonyl or Schiff base terminus making the ionone end of the molecule positively charged. The observed $|\Delta\mu|$ of 12.0 D for the protonated Schiff base corresponds to a shift of 0.21 e^- over the length of the molecule (about 12 Å). 11-*cis* Retinal also exhibits a large change in dipole moment on excitation. These results have implications for (*1*) the mechanism of photoisomerization of retinals, (*2*) the position of the absorption maximum of visual pigments, and (*3*) the interactions between the chromophore and opsin that occur on excitation.

Excited state dipole moment measurements on retinals provide information about the electron distribution and hence bond order in the excited state. In symmetric polyenes, the first allowed transition is to the ^1B_u state; a forbidden state (^1A_g) probably lies near or below this allowed state (28). Although this ^1A_g state cannot be readily populated by direct absorption, it could be important in determining the fluorescence and photochemical properties of these molecules (28, 29). The assignment of *g* and *u* parity for these states requires that the chromophore have a center (or pseudocenter) of electronic symmetry. A consequence of parity is that the ground and excited states cannot have a dipole moment. The small value of μ_g for all-*trans* retinal and its Schiff bases is consistent with this picture. In contrast, the large value of $\Delta\mu$ demonstrates that centrosymmetry is broken and that significant mixing of the *polyene* ^1B_u and ^1A_g states has occurred in the vertically excited retinals. These even-parity ^1A_g states are predicted to have

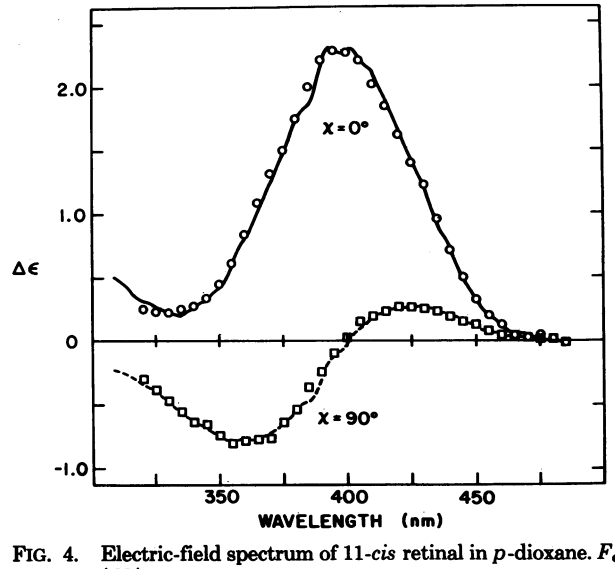

FIG. 4. Electric-field spectrum of 11-*cis* retinal in *p*-dioxane. $F_{ext} = 4.38 \times 10^4$ V/cm.

a lower degree of bond alternation than the ^1B_u state because of a large contribution from doubly excited configurations (29). Therefore, the observation of a large excited-state dipole moment tells us that the vertically excited state has a shifted electron distribution in which the bond order of the double bonds is reduced. This reduced double bond strength of the vertically excited state will be important if isomerization occurs from a partially or completely thermally relaxed form of this state. This inference is supported by the calculations of Salem and Bruckmann (30, 31) showing that *polar* excited singlet states of retinals are associated with a twisted 11–12 double bond which has a lower bond order.

The absorption maximum of 11-*cis* retinal is near 380 nm and that of its protonated Schiff base is near 440 nm. In rhodopsin, λ_{max} is 500 nm, and in the visual pigments of vertebrate cones, λ_{max} ranges from 432 to 562 nm (32). Our finding that the excited states of 11-*cis* retinal and the protonated Schiff base of all-*trans* retinal are much more dipolar than their ground states supports an electrostatic mechanism for generating these large spectral shifts. The ionone ring of the excited state of the protonated Schiff base of all-*trans* retinal has become positively charged and can be qualitatively described by resonance structures such as form II in Fig. 5. The ground state lacks such a large charge displacement and is best described by form I with the positive charge on the nitrogen. This difference in charge density suggests that the protein could shift λ_{max} to the red by stabilizing forms of the protonated Schiff base in which the positive charge is located near the ionone ring. Kropf and Hubbard in 1958 (33) proposed this mechanism on the basis of a postulated charge shift, which we have now experimentally verified. Also, theoretical calculations by Waleh and Ingraham (34) predict that this electrostatic interaction should be capable of regulating λ_{max}. The excited-state dipole could interact with fixed charges, oriented dipoles, or polarizable groups (35) on the protein. For example, let us place a negative charge 7 Å from the center of the chromophore positioned on the dipole axis near the ionone ring. The shift in the energy of the vertical transition is given by $\Delta E = Q\,|\Delta\mu|/(\xi r^2)$, where ξ, the effective dielectric constant at optical frequencies, is taken to be 2. For $|\Delta\mu| = 12$ D, we calculate that $\Delta E = 8.4$ kcal (35.1 kJ) or 2939 cm^{-1}. This would correspond to a shift in λ_{max} from 440 to 505 nm. This means of controlling λ_{max} does not preclude the action of other factors such as the location of the Schiff base counter ion (36) and the twisting of retinal (37). However, a mechanism based on an electrostatic interaction between a highly dipolar excited retinal and a charged, polar, or polarizable group on the protein is strongly suggested by our measurements. In addition to generating a red shift, these postulated electrostatic interactions might catalyze photoisomerization by stabilizing a polar transition state.

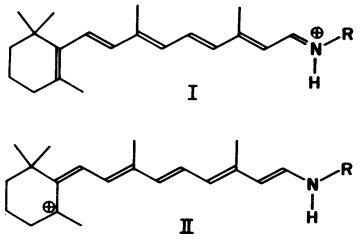

FIG. 5. Two possible resonance structures for the protonated Schiff base of all-*trans* retinal.

The creation of a highly dipolar excited state of retinal could provide the driving force for a conformational change in retinal and its immediate protein environment. A chromophore with a large shift in charge density upon excitation should have an equilibrium excited-state configuration that is significantly altered from that of its vertically-excited state. Similarly, some nearby amino acid residues would also be far from their equilibrium configuration in the vertically excited visual pigment. This electrostatic induction of conformational changes in the protein has also been proposed by Salem and Bruckmann on the basis of their molecular orbital calculations (31). A highly dipolar form of retinal would probably have to last for nanoseconds or longer to elicit appreciable conformational changes in the protein. It is significant in this regard that prelumirhodopsin, the first photolytic intermediate, also appears to contain a dipolar form of retinal. The 1539 cm^{-1} ethylenic stretching vibration in the resonance Raman spectrum of prelumirhodopsin (5) shows that there is a reduction of C=C bond order relative to the ground state of rhodopsin. As illustrated in Fig. 5, this reduction in bond order is consistent with an increased contribution from resonance structures like form II that have positive charge shifted to the ionone region. Our prediction of electrostatic interactions between the excited or dipolar state of retinal and its immediate protein environment in visual pigments may be testable with newly developed resonance Raman techniques (4–6). It will be interesting to see whether dipolar excited states are generally important in energy transduction processes (38, 39).

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