Spectroscopic Model for the Visual Pigments. Influence of Microenvironmental Polarizability*

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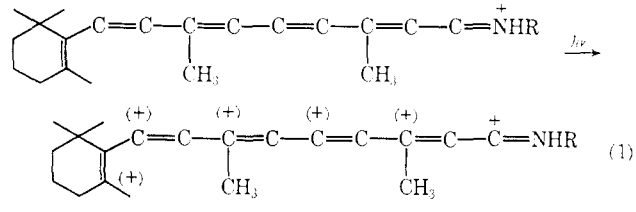
ABSTRACT: The spectroscopic properties of retinylidene iminium salts have been investigated in a variety of media. The results obtained by us and other workers on these "model" systems for the visual pigments are consistent with an hypothesis for the anomalous spectra of these compounds quite different from the point charge perturbation theories of Hubbard, Abrahamson, and others.

From solvent effects, counterion effects, concentration dependence of the spectra, and apparent molecular weight determinations, a physically rational spectroscopic model is proposed that invokes a significant role for induced dipole capacity (polarizability) within the microenvironment of the chromophore. An opsin matrix can provide such a local environment.

The origin of the colors of the visual pigments is a significant problem confronting research on the visual process. The phenomenon which to many physical scientists has been most perplexing in this regard is the spectral shift of the absorption maximum of retinal from 385 nm in aqueous solution to certain (species dependent) wavelengths between 443 and 562 nm, observed when the polyene aldehyde is bound to various opsins (reviewed by Bridges, 1967). Several theories have been proposed to explain these spectral shifts (*cf.* reviews by Abrahamson and Ostroy, 1967, Hubbard *et al.*, 1965, and Kropf and Hubbard, 1958); however none have gained general acceptance. In fact, Pitt (1964) has suggested that no simple theory exists which can explain these spectra. In the present paper we shall attempt to show how, in principle at least, the red-shifted absorption maxima of the visual pigments can be explained in the language of molecular orbital theory by simply considering various kinds of electrical (including dispersion or induced dipole) interactions between the opsin binding site and the ground and Franck–Condon excited states of protonated retinyliden-imine. The model that we shall propose is consistent with the experimentally observed spectroscopic behavior of protonated retinyliden imines in solution. Perhaps more importantly, it rationalizes the known spectroscopic properties of rhodopsin and its immediate photoproducts in the protein opsin as a function of conformational changes in opsin.

At present it is unclear whether in rhodopsin retinal is covalently bound (as the imine) to an ε-lysine group (Bownds and Wald, 1965) or to phosphatidylethanolamine (PE)[1] (Daemen and Bonting, 1969; Poincelot *et al.*, 1969). If the latter is the case, it is probable that the imine is protonated intramolecularly by the (monobasic) phosphate group of PE, with a phosphate diester as the appropriate anionic group. If instead it is bound to lysine, then the imine can be protonated by either a carboxylic acid group or by PE, resulting in carboxylate or phosphate anions, respectively. The nature of the anion is important since, as will be shown, it influences the extent of electrostatic interactions between the binding site and the ground state, and between the binding site and the Franck–Condon excited state of the chromophore.

The excitation of protonated retinylidenimine has been envisioned by some workers (Kropf and Hubbard, 1958; see also reviews by Bridges, 1967, and Abrahamson and Ostroy, 1967) in terms of valence bond or resonance theory which predicts that in the ground state the positive charge is located on nitrogen, whereas in low-lying excited states the charge is delocalized on alternate carbon atoms (eq 1). If



(1)

negatively charged groups, such as COO^-, O^-, or S^-, are distributed throughout the binding site, these electron-rich centers can lower the energy of the first excited state by stabilizing the delocalized positive charge of this state, and thus shift the absorption maximum toward the red.

This simple resonance model suffers several disadvantages, as does the more sophisticated model of Abrahamson (Wiesenfeld and Abrahamson, 1968) which also invokes perturbation by one or more point negative charges carefully positioned along the carbon chain. The highly hydrophobic character (Erickson and Blatz, 1968, and references therein) of opsins makes improbable the presence and involvement of abundant polar groups such as COOH, OH, and SH in the binding. Even if these are present near the binding site, they would most likely not be ionized although we realize that this sup-

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[1] Abbreviations used are: PE, phosphatidylethanolamine.

position cannot be easily tested. The model ignores the effect of the most important and probably only anionic group immediately in the binding site, the conjugate base of the acidic group which protonates the imine nitrogen in the ground state of the system. If protonation is to occur in a hydrophobic medium, the acidic group must be in close proximity to the imine nitrogen, so that the charges produced will not create a large, high-energy dipole. Thus, in our view, it is questionable whether an anionic group will be found in the immediate region of the binding site except adjacent to the imine linkage.

We thus propose an alternative spectroscopic model which attempts to predict the behavior of the chromophore in several specific environmental situations, after which experimental data of others and ourselves will be given and discussed in light of the model. Finally, results of solution studies will be extrapolated to real (protein) systems, *i.e.*, the visual pigments.

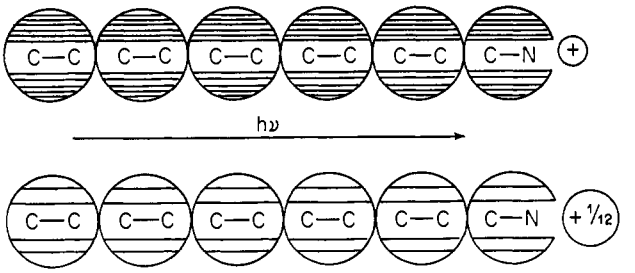


FIGURE 1: Schematic molecular orbital representations of the highest energy π orbital during excitation of retinylideniminium ion. Densely shaded areas above the arrow represent the orbital in the doubly (electron) occupied ground state. In the lower representation, the orbital is half-filled, nitrogen is nearly neutralized, leaving a residual charge of $+1/12$ on all atoms ideally. The arrow indicates the electric dipole transition moment accompanying photoexcitation of an electron to the (not shown) π^* orbital polarized primarily around nitrogen.

Proposed Model

In the discussion below, we shall be concerned with the behavior of electrons in the highest filled molecular orbital, a π-bonding orbital, and the lowest unfilled (in the ground state) orbital, a π^* antibonding molecular orbital. To simplify the arguments, let us assume that an electron in the π orbital is delocalized over all 12 atoms of the π system in the chromophore, and that in the π^* orbital the electron is essentially located around the iminium nitrogen, rendering it nearly neutral (in the Franck–Condon state, as a result of the electric dipole transition oriented in the long axis of the polyene; R. Levinson and H. B. Gray, private communication, 1969).[2]

In the ground state, two electrons occupy the highest π orbital and the iminium nitrogen carries a unit positive charge (we have previously confirmed this by experiment (Irving *et al.*, 1969)). In the excited π,π^* state, one electron occupies the π orbital while the other now occupies the π^* orbital with most of the charge density around nitrogen (*vide supra*). Thus, in the π,π^* state, each of the twelve atoms (including nitrogen) of the π system ideally carries a $1/12$ e positive charge (Figure 1).

It is then appropriate to consider what factors determine the energy needed to promote an electron in the π orbital to the π^* orbital, or in state terminology, the energy difference between the ground and π,π^* singlet excited state. The problem can be considered by one of two reasonable approaches. (1) The energies of the ground and excited π,π^* states are determined by the energy of the isolated chromophore plus the sum of electrostatic and induced dipole interactions between the state and its environment. (2) Alternatively, one can consider the energy of the system as a unit rather than the sum E(vacuum) and E(interactions). Thus the position and behavior of an electron are "localized" in such a way as to obtain the lowest possible potential energy with respect to electrical interactions. Both approaches yield the same qualitative results and predictions. For the purpose of this paper the first approach will be used.

Let us now determine in a qualitative way how the energy difference between the ground and π,π^* states changes as we successively place the cationic chromophore in (A) a nonpolar, nonpolarizable medium (isolated, as in a vacuum or vapor); (B) a polar, nonpolarizable medium; (C) a polarizable medium (one capable of significant induced dipole interactions); (D) a polar, polarizable medium; (E) a medium in which an anion is tightly bound to the iminium nitrogen; and (F) media where the anion is successively and gradually removed from the nitrogen atom.

A. Vacuum. Upon π–π^* excitation in a nonpolar, nonpolarizable medium, the electron undergoing promotion moves toward and resides on the iminium nitrogen within 10^{-15} sec. It is only this excited state, the Franck–Condon state, with a time scale much too short for vibrational relaxation (internuclear motion, solvent–solute reorganization), that plays the upper state role in determining the absorption spectrum. (Relaxed excited states, that have lived as long as 10^{-13} sec, are irrelevant to absorption spectroscopy.) The energy required for excitation can be crudely calculated by standard molecular orbital methods.

B. Polar Medium. When the chromophore is in an environment of polar molecules which possess permanent dipole moments, *but are not significantly polarizable*, the dipoles will in the ground state orient themselves around the positively charged nitrogen in a fashion in which maximum favorable electrostatic interactions can be achieved. Elsewhere along the chromophore the dipoles will be largely randomly oriented with little effect on the total energy. However, the net result will be a substantial *lowering* of the ground state energy due to favorable solvation around nitrogen. Excitation to the π,π^* Franck–Condon state results in a new charge distribution, as previously discussed, for the chromophore. Reorientation of the permanent dipoles cannot occur in 10^{-15} sec, thus the medium cannot accommodate the new charge distribution, and the excited state thus greets a hostile environment. With reduction of charge at the nitrogen atom, the electrostatic repulsion between the dipoles surrounding the nitrogen atom becomes considerably greater than their attraction to the smaller (ideally $+1/12$ e) charge. For this portion of the system the energy is clearly raised. The partial positive charges generated along the backbone of the polyene should meet a statistical, roughly equal distribution of positive and negative

[2] See Ceasar and Gray (1969).

ends of dipoles; the net change in potential energy of this portion of the system as influenced by the medium will thus be of small magnitude (because of cancellation of fortuitous favorable and unfavorable interactions). The result for the total system is an *increase* in energy of the excited state (relative to vacuum). Thus, in a polar, nonpolarizable medium the energy of the ground state is *lowered* and that of the excited state is *raised*, resulting in a significant blue shift of the absorption maximum relative to vacuum.

C. Polarizable Medium. When the chromophore is placed in a medium of polarizable molecules (or side-chain groups, if a protein), the positive charge on the iminium nitrogen in the ground state will generate induced dipoles in the medium. These will intereact favorably with the positively charged nitrogen and slightly lower the energy of the ground state relative to a vacuum. Upon excitation to the π,π^* state the positive charge on the nitrogen atom is greatly reduced, and the induced dipoles surrounding it will diminish in magnitude within the lifetime of the Franck–Condon state. Simultaneously, as partial positive charges develop along the polyene backbone, induced dipoles will be generated (this *can* be done in 10^{-15} sec since dipole induction requires no internuclear motion) which will interact favorably with the chromophore and lower the energy in this region. It is safe to assume that the energy of the excited state is in this way *lowered* about as much as the ground state; thus the energy difference will be approximately equivalent to that in a vacuum. Clearly, the energy difference will be *less*, with spectra to the red, than in the above situation with nonpolarizable media (Bayliss and McRae, 1954).

D. Polar, Polarizable Medium. Arguments in this section are basically a synthesis of parts B and C above. Since the net result of the two situations leads to opposite predictions with respect to spectral shifts in the two kinds of media, it is difficult to predict *a priori* which direction polar, polarizable media will cause absorption to shift, relative to vacuum. Importantly, however, it is very clear that spectra in polar, *polarizable* media will be to the *red* of spectra in polar, nonpolarizable media.

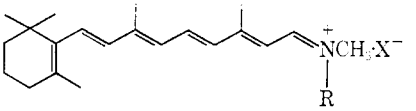
E and F. Position and Influence of Anion. Thus far, attention has been focused on the cationic chromophore, protonated retinylideniminium ion. The influence of the anion (the conjugate base of the protonating acid) obviously cannot be ignored since it is certainly a perturbing factor in the microenvironment. The presence of such a perturbing influence does not qualitatively alter the predicted shifting of the spectrum to the red in polarizable media, but theoretically and experimentally it does have a quantitative effect which may be of some importance in the biological systems. Consider the chromophore, under vacuum, with an anion bound electrostatically to the iminum nitrogen. The tighter the ion pair, the more stable the ground state due to coulombic attraction. The energy of the excited state should be lowered to a lesser degree, since the anion will be interacting ideally with only a $^1/_{12}$ positive charge. Thus the absorption spectrum will be blue shifted with respect to the dissociated ions. As the anion is systematically separated from the iminium nitrogen, the energy of the ground state should increase faster than that of the excited state, resulting in shifts to the red.

It can also be argued that as anion separates from cation, the nitrogen becomes increasingly electronegative. The electron, which is transmitted to nitrogen in the π–π^* transition, should become "easier" to excite, manifested in absorption at lower energies.

From the discussion in this general section of the paper, and based on the experimental data to be presented, our hypothesis considers polarizability (capacity for induced dipole interactions) to be the most important property of the microenvironment in a spectroscopic model that will satisfactorily account for the anomalous, low-energy spectra of the visual pigments. The highly individual and unusual structure of the chromophore has, *via* the fabric of the arguments presented, led us to this conclusion, although, as indicated above, the influence of the counterion is an important secondary consideration which must also be treated. In the next section is presented confirmatory experimental evidence or, in the case of the protein itself, strong circumstantial evidence for the involvement of polarizable amino acid residues in the region of the binding site. In a preliminary communication (Irving *et al.*, 1969), to which the reader is referred, is presented some of the experimental evidence along with a simple schematic picture invoking polarizability which requires little modification for the present purposes. Also, the occasionally proposed notion (*cf.* Akhtar *et al.*, 1968) that the anomalous spectra are due to charge-transfer transitions is definitively ruled out in our above cited communication.

Results and Discussion

Solvent Effects. In the first relevant work on solvent effects, Pitt *et al.* (1955) observed that the hydrochloride salt of *N*-methyl-*all-trans*-retinylidenimine (I) possesses an absorption maximum in ethanol at 440 nm and in chloroform at

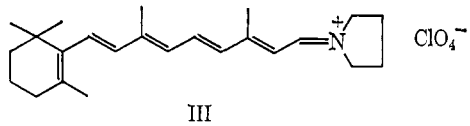


I, R = H; X = Cl
II, R = CH_3; X = I

466 nm; similarly the dimethyliminium iodide II has λ_{max} (ethanol) at 455 nm and λ_{max} (chloroform) at 485 nm. These workers did not elaborate on the ethanol → chloroform red shifts of 26 and 30 nm in I and II, respectively, but in fact chloroform is both more polarizable and less polar than ethanol. Thus our model predicts absorption maxima in chloroform to be to the red of absorption in ethanol.

Erickson and Blatz (1968) have observed that the perchloric acid salt of *N*-(2-hydroxypropyl)retinylidenimine possesses a λ_{max} in methanol at 443 nm and in 1,2-dichloroethane at 490 nm. When the above retinylideniminium ion was observed in mixtures of methanol and 1,2-dichloroethane, the authors report that the absorption maxima decreased in a nonlinear manner as the polarity and dielectric constant of the solvent pair increased. In view of these data, they concluded that the λ_{max} was a function of solvent polarity. In this specific experiment our approach would lead to the same conclusion, *but only because as the mole fraction of polar, nonpolarizable methanol increases, the mole fraction of the polarizable but much less polar dichloroethane decreases.* In this fortuitous case, the polarizability of the medium is obviously related to the reciprocal of the polarity or dielectric.

Recently Akhtar *et al.* (1968) and we (Irving *et al.*, 1969) have examined the absorption spectra of retinylideniminium salts in a variety of solvents (Table I) demonstrating without question that there is little or no correlation between λ_{max} and solvent polarity alone. Akhtar, whose data in Table I are for *N,N*-dimethylretinylideniminium ion, failed to note or mention the correlation with refractive index—a measure of the polarizability of the gross solvent, which admittedly cannot be equated to but certainly approximately reflects polarizability of the microenvironment in the solvent cage around the solute. (The table will again be referred to when the influence of the counterion is discussed.) To take one example from the table, it is seen that ether (D = 4.33) and chloroform (D = 4.80) have essentially the same polarity, but the absorption maxima in chloroform are shifted to the red, relative to ether, by 30–40 nm (depending on anion). This is consistent with the fact that chloroform (n = 1.444) is substantially more polarizable than ether (n = 1.353). Additionally, we have studied the absorption spectra of the perchlorate salt of III (10^{-5} M) in a somewhat wider variety of solvents (Irving *et al.*, 1969) which were grouped in two categories: nonpolarizable (alcohols, acetic acid, ethers, etc.), and polarizable (benzene, pyridine, thioanisole, alkyl, aryl, and vinyl halides). In all of the *nonpolarizable* solvents the absorption maxima fall within



III

a very narrow range, 454 ± 4 nm, and are not appreciably affected by either large changes in solvent polarity or the ability of the solvent to hydrogen bond. In the case of the polarizable solvents, spectra are anomalously and significantly red shifted, as demonstrated *experimentally* and predicted physically.

Inductive Effects. Rosenberg and Krigas (1967) and Erickson and Blatz (1968) have prepared *N*-retinylidenimines from amino acids which contain electron withdrawing groups. They observed that the absorption maxima of the protonated imines shifted to the red to an extent roughly proportional to the electron withdrawing power of the substituent (usually attached to the second carbon of the imino portion of the molecule). This effect may be explained in terms of our proposal. The electron withdrawing group increases the effective positive charge on nitrogen in the ground state, in the same way that is accomplished by partially removing the anion, described in part E,F of the previous (Proposed Model) major section of this report. The arguments presented earlier in part E,F also, therefore, predict red shifts in the present situation when nearby substituents create electron withdrawing inductive effects.

Effect of Size, Nature, and Proximity of Anion. As seen in Table I, for both nonpolarizable and polarizable solvents, a strong correlation exists between the size of the anion and the absorption maximum of the salt; spectra shift to the red with increasing size of the anion. This phenomenon is consistent with previous arguments, especially in low dielectric media in which the cation and anion can reasonably be expected to be intimate. A hypothetical point charge in a large anion (*e.g.*, perchlorate) will necessarily be more remote

TABLE I: Long-Wavelength Absorption Maxima of (*all-trans*-Retinyl)=NR_2X^- As a Function of Solvent Parameters and the Nature of the Anion X^-.[a]

Solvent	D	n	λ_{max} (mμ) X^- = ClO_4^-	X^- = I^-	X^- = Br^-
Ethyl ether	4.33	1.353	451	445	442
Methanol	32.36	1.326	453	448	444
Benzene	2.28	1.498	474	455	451
Chloroform	4.80	1.444	481	485	477
Dichloromethane	9.08	1.424	496	489	484

[a] R is methyl (identical with cation II), and X^- is halide, in Akhtar's data; and the R groups are part of a ring system, X^- is perchlorate, in our results (compound III, *vide infra*). For spectroscopic purposes, cations II and III are considered identical with no qualification necessary.

from the (ground state) positive nitrogen than in a smaller ion (*e.g.*, bromide). We cannot at this time explain the trend in methanol solvent in which the ions are apparently fully dissociated. Molecular weight determinations in ethanol indicate complete dissociation (*vide infra*); the same certainly should be true in the even more polar methanol. Yet the anion effect is present in methanol[3] as well as in nonpolar solvents where less dissociation occurs. The remarkable sensitivity of variations of protonating acid on the spectra of iminium ions (analogous to I, not the "quaternarized" systems II and III) is seen in Table II. The data on the butylimine IV are ours; those on the 2-hydroxypropylimine V are taken from Erickson and Blatz (1968). The data are easily rationalized on the basis of our overall proposal which, *for this specific experiment*, is partially equivalent to the discussion of Erickson and Blatz. In 1,2-dichloroethane, in which the protonated iminium cation is largely associated with the anion (as we have experimentally shown for the related system III in the same solvent, by molecular weight analyses, to be discussed below), the spectral maxima correlate excellently with acid strength. Strong acids (by definition) are easily dissociated, leading to stable anions. Accordingly, salts of strong acids (*e.g.*, iminium perchlorates) are more dissociated than those of weaker acids such as chlorides and acetates (or dichloroacetates). The chromophore cation, by arguments used earlier, will absorb at longer wavelengths as the degree of dissociation increases. Moving up the table to methanol solvent, there appears to be *no* effect on λ_{max} with variation of the nature of the acid. Since dissociation is virtually complete in this medium, an effect would not be expected since it is the cation that is the essential chromophore, and in this polar, nonpolarizable, blue-shifting solvent the resultant cation from all four acids should be identical.

In the case of the imine IV, in methylene chloride (dichloro-

[3] This particular and unexpected result is not consistent with data in methanol solvent observed in Table II. However, data on the iminium salts in Table I are subject to some uncertainty due to variations in sources, and thus to minor variations in experimental conditions.

TABLE II: Effect of Acid Strength on the Absorption Spectra of *N*-Butylretinylidenimine (IV) and *N*-(2-Hydroxypropyl)-retinylidenimine (V) in the Solvents Indicated.

Chromophore	Acid	Solvent	λ_{max} (nm)
IV[a]	H_2SO_4	CH_2Cl_2	488
	$HClO_4$		480
	HI		481
	HBr		465
	HCl		460
	CH_3COOH		451
V[b]	$HClO_4$	CH_3OH	445
	HCl		445
	$Cl_2CHCOOH$		445
	CH_3COOH		445
V[b]	$HClO_4$	$ClCH_2CH_2Cl$	490
	HCl		465
	$Cl_2CHCOOH$		452
	CH_3COOH		*c*

[a] Results from this laboratory. [b] Data of Erickson and Blatz (1968). [c] Spectrum apparently not determinable (the authors report no reaction).

methane), the spectra correlate reasonably well with acid strength. However, as an obvious exception, the spectral maximum with perchloric acid is 8 nm to the blue of that with sulfuric acid. Although this is not a large (perhaps not even significant) difference, it may reflect some other, minor perturbing effect (*e.g.*, the relative sizes and other properties of the HSO_4^- *vs.* the ClO_4^- ions in the relatively nonpolar methylene chloride solvent).

Dissociation of the Iminium Perchlorate III in Polar and Nonpolar Media. A semiquantitative determination of the extent of dissociation of the iminium salts is of obvious value with respect to the immediately preceding discussion of the role of the anion, and perhaps of even more value for the related subsequent section on concentration effects. Molecular weights of the iminium perchlorate III have been determined in ethanol, acetonitrile, and 1,2-dichloroethane by the lowering in vapor pressure utilizing a commercial vapor phase osmometer. Values in ethanol and acetonitrile were concentration independent and almost exactly one-half the molecular weight of the salt (438). Thus it can be safely assumed that in these and other related polar solvents (such as methanol, in which determinations were experimentally unfeasible) the ions are virtually completely dissociated. In relatively nonpolar but polarizable solvents this is *not* the case (Table III), powerfully reinforcing our basic argument as to the importance of induced dipole interactions. The data on anion and acid effects are internally consistent, but taken alone they would predict absorption of the iminium salts in dissociating media (*e.g.*, ethanol, methanol, etc.) to occur to the red of absorption in relatively nonpolar media in which the oppositely charged ions are much more closely bound. However, of course, the converse is true; absorption maxima of the salts in poorly ionizing, but polarizable solvents occur experimentally as much as *50 nm to the red of absorption in alcohols*. Yet the molecular weight data rule out aggregates and thus excitons; hence, to reiterate arguments in previous sections, the dominant influence reasonably appears to be polarizability (induced dipole interactions) of the microenvironment.

TABLE III: Molecular Weights of the Iminium Perchlorate III As a Function of Concentration in 1,2-Dichloroethane.

Concn (M)	App Mol Wt	Approx % Dissocn
3.4×10^{-2}	443	0
2.6×10^{-2}	402	9
5.3×10^{-3}	362	21
5.2×10^{-3}	366	20
1.3×10^{-3}	300	46
1.0×10^{-3}	307	43

TABLE IV: Concentration and Spectra of III in the Indicated Solvents.

Concn (M)	Solvent; λ_{max} (nm)				
	CH_3CN	CH_3OH	CH_2Cl_2	$C_2H_4Cl_2$	*o*-$C_6H_4Cl_2$
4×10^{-3}	454	457	480	484	485
2×10^{-3}	454	457	482	484	487
2×10^{-4}	451	452	482	488	490
2×10^{-5}	452	453	495	496	502
2×10^{-6}	453	453	506	498	520
2×10^{-7}			512	502	525

The equilibrium constant for dissociation of III in 1,2-dichloroethane, calculated from the data in Table III, has a value of approximately 2×10^{-4}. Thus, at a concentration of 10^{-5} M, the calculated extent of dissociation rises to approximately 95%.

Effect of Concentration of III in Polarizable and Polar, Nonpolarizable Solvents. Table IV illustrates the dependence of concentration of the iminium perchlorate III on its absorption maximum in several solvents: highly polar but relatively nonpolarizable acetonitrile and methanol, and relatively nonpolar but polarizable methylene chloride, 1,2-dichloroethane, and *o*-dichlorobenzene. It is obvious from the data that there is a profound difference in the spectroscopic behavior of III in the two "kinds" of solvents. Spectra in acetonitrile and methanol are, within experimental error, *concentration independent*, and occur at considerably shorter wavelengths than in methylene chloride, dichloroethane, and *o*-dichlorobenzene, in which *a significant concentration dependence is observed.*

Analysis of the results is relatively clear-cut within the framework developed in preceding sections. Even at the highest concentration, dissociation is virtually complete in acetonitrile and methanol, so that the insensitivity to concentration is entirely reasonable. In the red-shifting polar-

izable solvents, generally less polar than acetonitrile and methanol and specifically so in the present case, dissociation of the salt occurs to a substantially lesser degree and becomes important only at the lower concentrations. In the case of dichloroethane solvent the calculated percent dissociation increases from approximately 30% at 2×10^{-3} M to approximately 95% at 2×10^{-5} M. It is precisely in this concentration range that the absorption maximum of III shows its sharpest rise, as seen in Table IV, for dichloroethane. Thus the expected red shift, linked to the extent of dissociation as discussed earlier, increases with decreasing concentration of the chromophore III. In the aromatic solvent *o*-dichlorobenzene the absorption maximum moves as far as 525 nm at the lowest experimentally practical concentration, and there is no reason to preclude an additional shift to the red should experimental conditions permit essentially complete dissociation.[4] In summary, then, experiments strongly imply that the dominant consideration is polarizability of the medium. In such media counterion association is allowed to play a secondary, but not trivial, role.

Biological Applications and Hypothesis. The long-wavelength absorption spectra of the visual pigments depend intimately upon the conformation of opsin. Changes in conformation occur upon photobleaching, denaturation, and genetic substitution of amino acids (Matthews *et al.*, 1963). The polarizability, polarity, and related properties of the binding site of the protein are determined by the nature, proximity, and orientation of local side-chain groups. Analysis of amino acids in bovine opsin (Shields *et al.*, 1967; Heller, 1968) reveals an unusually high proportion of polarizable aromatic amino acids in this essentially hydrophobic protein.

We have experimentally demonstrated the sensitivity of the spectrum of the protonated iminium chromophore to gross polarizability of solvents and (as a secondary influence) to proximity of counterion. In bulk solvents the specific orientation of the solvent molecules around the chromophore, while not random, is nevertheless unlikely to be the *best* orientation for induced dipole interactions (polarizability). In aromatic solvents this is especially true since the magnitude of potential induced dipoles within an individual molecule varies greatly depending upon which of the three mutually perpindicular coordinate axes is chosen. Within the protein, however, the aromatic amino acids (phenylalanine, tyrosine, and tryptophan) can reasonably be expected to possess *very specific orientations with respect to the chromophore.* Our hypothesis infers that these orientations produce *large local polarizability* in the *longest wavelength* absorbing pigments, substantially better than obtained in bulk solvent. (This conformation may also be such as to favor moderate dissociation of the ion pair which, in a polarizable solvent, was seen to contribute to the spectral shift.) Systematic variations in opsin conformation produce orientations of the polarizable groups yielding different but still specific local polarizability. New, but specific and characteristic spectra are thus obtained. This hypothesis accounts for the variation in λ_{max} with species, and also for different, opsin-dependent, absorbing pigments within a given animal. It also accounts for the spectra of the chemical transients following photolysis of rhodopsin, where opsin undergoes several, distinct, conformational changes—each accompanied by a distinct, specific spectral shift. However, the alternative hypothesis of Hubbard, Abrahamson, and others, invoking point negative charge perturbations, also predicts qualitatively these genetically and photochemically generated specific spectral variations in the protein system.

At this time, neither hypothesis can be proved or disproved unambiguously. The structure of opsin is not known. Perhaps the answer will be found in a synthesis of both hypotheses which are not mutually exclusive. In any event, our arguments are based on experiment with reasonable model systems and developed as a consequence of experiment. The alternative hypothesis does not readily lend itself to experimental probe in model systems. The actual protein itself is a terrible system in which to test any spectroscopic hypothesis until its complete structure is known—an unlikely event in the near future due to its inability to crystallize.

[4] An agrument could be presented that there should be two absorption bands in dichloroethane, *o*-dichlorobenzene, and related solvents—one for the nondissociated and another for the dissociated ions. In fact, there is only a single long-wavelength band, though it is slightly broadened as it shifts to the red.

Experimental Section

Chemicals. Solvents were analytical reagent quality (Spectroquality when available) and were used without further purification with the exception of methanol and ethanol. *Methanol* (Spectroquality) and *ethanol* (analytical reagent) were each distilled from the corresponding lithium aluminum alkoxide. *all-trans-Retinal* (Eastman Kodak) was used as received. *N-Butyl-all-trans-retinylidenimine* (*IV*) was prepared as described previously (Irving and Leermakers, 1968).

all-trans-Retinylpyrrolidinium perchlorate (*III*) was prepared by a procedure analogous to that described by Leonard and Paukstelis (1963). Pyrrolidinium hydroperchlorate (170 mg, 1 mmole) and *all-trans*-retinal (283 mg, 1 mmole) were dissolved in 20 ml of absolute ethanol at room temperature. The solution was allowed to stand in the dark for 1 hr with the formation of a dark red precipitate. Recrystallization of the precipitate from ethanol with cooling to −56° yielded deep red crystals of the desired perchlorate, mp 194–1.95°. *Anal.* Calcd for $C_{24}H_{36}ClNO_4$: C, 65.8; H, 8.3; Cl, 8.1; N, 3.2. Found: C, 65.8; H, 8.2; Cl, 8.9; N, 3.2.

Absorption Spectra. All spectra were recorded on a Cary Model 14 spectrophotometer. Dilution studies were performed utilizing quartz cells with path lengths varying from 0.05 to 100 mm.

Colligative Vapor Pressure Measurements. Apparent molecular weights for *all-trans*-retinylpyrrolidinium perchlorate were determined in ethanol and 1,2-dichloroethane with a Hewlett-Packard Model 302B vapor-pressure osmometer at 39°.

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Structure of the Glycopeptide from Bovine Visual Pigment 500*

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ABSTRACT: A single glycopeptide containing nine amino acid residues was isolated from a peptic digest of the membrane protein bovine visual pigment$_{500}$ by chromatography on Dowex 50-X2. The carbohydrate composition of this peptide accounts for all the sugar present in visual pigment$_{500}$. A combination of chemical and enzymic methods established the sequence of the glycopeptide as Met-Asx(sugar)-Gly-Thr-Glu-Gly-Pro-Asn-Phe. All the carbohydrate was linked to Asp-2 through an alkali-stable bond, presumably an *N*-aspartylglycosylamine linkage. Digestion of the glycopeptide with β-acetylglucosaminase liberated two out of the three glucosamine residues as *N*-acetylglucosamine, leaving one glucosamine and all three mannose residues linked to the peptide. Digestion with α-mannosidase liberated 88% of the mannose residues present in the carbohydrate moiety as reducing sugar. It is concluded that bovine visual pigment$_{500}$ contains a single oligosaccharide moiety linked to the polypeptide chain through an *N*-aspartylglycosylamine linkage. Two of the *N*-acetylglucosamine residues and all the mannose residues are linked peripherally to the (*N*-acetyl)glucosamine that is linked to the asparagine residue. These experiments provide independent evidence that the molecular weight of visual pigment is approximately 28,000. It is suggested that the carbohydrate in visual pigment functions as a surface orientation marker assuring proper assembly of the molecule into the membrane structure. It is possible that the carbohydrate moieties which are often found in membrane proteins have a similar function.

Bovine visual pigment$_{500}$, a membrane protein which is a structural component of the rod outer segment disk system, was recently shown to be a glycoprotein containing three residues of glucosamine and three residues of neutral sugar per molecule (Heller, 1968). The present work attempts to define the type of linkage between the carbohydrate and the polypeptide chain and to determine the amino acid sequence around the linkage.

The experiments described in the present paper show that bovine visual pigment$_{500}$ contains a single oligosaccharide moiety linked to the polypeptide chain through an *N*-aspartylglycosylamine linkage.

Materials and Methods

Bovine visual pigment$_{500}$ was prepared and purified as previously described (Heller, 1968). Purified visual pigment, 5 μmoles, was dialyzed against deionized water to remove salts and was then denatured by adding four volumes of ethanol and incubating for 18 hr at 23°. The precipitated protein was collected by low-speed centrifugation and was then washed ten times with 80% aqueous ethanol at 23° over a period of 7

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