Effect of Selected Hydrogen-Bonding Solvents on the Absorption Maxima of *N*-Retinylidene-*n*-butylammonium Salts†

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ABSTRACT: *N*-Retinylidene-*n*-butylammonium chloride, bromide, and iodide were prepared, and their spectra were determined in $CHBr_3$, $CHCl_3$, CH_2Cl_2, CH_3CHCl_2, CH_3CH_2Br, CCl_4, and CH_3CCl_3. The first five solvents all have a hydrogen on the halogen-bearing carbon. For these solvents the λ_{max} increases linearly as the dipole moment of the solvent decreases. CCl_4 and CH_3CCl_3 have no hydrogen on the halogen-bearing carbon and do not induce a dipole moment dependence on the salts. The effect is interpreted as a binding of the anion by the partially charged proton, and this leads to greater dissociation of the ion pair. In addition to the hydrogen-bonding effect, the solvents exert a bulk dipole effect.

A general introduction to the problems concerning the bathochromic shift is given in Blatz *et al.* (1972). In the same paper it was also concluded that the separation between the centers of charge in the cation and anion controls the fractional charge on the nitrogen of the *N*-retinylidene-*n*-butylammonium cation. The remaining fractional charge on nitrogen not engaged in ionic bond formation polarizes the π system which gives a resulting excitation energy lower than the unprotonated system. This is called the anion effect since the anionic distance controls the excitation energy of the cation. It was also noted in the same article that solvent plays a major role. It was shown that in certain solvents such as methanol and ethanol there is no anion effect; however, in most other solvents the anion plays a major role in determining the λ_{max} of the cation.

A solvent like methanol was said to be a leveling solvent since it leveled the action of the anion. It was proposed that in methanol, *N*-retinylidene-*n*-butylammonium salts are fully dissociated and the cation might be expected to absorb at a long wavelength. However, because of the strong dipole–ion interaction between the electron-rich oxygen and the polarizable nitrogen, a significant charge is located on the nitrogen available for electrostatic interaction. Consequently, with respect to the retinylic cation the excitation energy is quite large.

In the same article it was noted that the λ_{max} of *N*-retinylidene-*n*-butylammonium chloride in CCl_4 is 442 nm, whereas it is 460 nm in $CHCl_3$. Thus, the λ_{max} values in three solvents CCl_4, $CHCl_3$, and CH_3OH are 442, 460, and 442 nm, respectively, whereas the dipole moments are 0, 1.54, and 1.70 μ and the dielectric constants are 2.238, 4.806, and 32.6. This investigation was undertaken to elucidate the role of certain solvents in the bathochromic shift.

Experimental Section

all-trans-N-Retinylidene-n-butylamine's preparation and the preparation of pure salts have been reported in the preceding article.

Solvents. Purification of $CHCl_3$ and CCl_4 was reported previously. $CHBr_3$ was purified by washing with sulfuric acid, then with water, next with dilute NaOH and finally three times with water. The washed solvent was dried with K_2CO_3, then P_2O_5 and distilled at reduced pressure. CH_2Cl_2 was purified similarly to $CHBr_3$. CH_3CCl_3 was washed with concentrated HCl, then with 10% K_2CO_3 and then with 10% NaCl solution. It was dried with K_2CO_3 and distilled over P_2O_5. CH_3CHCl_2 was washed twice with saturated $NaHCO_3$ and then with water. It was dried over K_2CO_3 and distilled over P_2O_5. CH_3CH_2Br was distilled from P_2O_5.

Absorption spectra were recorded in a Beckman DK-2A ratio recording spectrophotometer at room temperature.

Results

By determining the effect of solvent parameters on the spectra of visual chromophore models, we should be able to learn by analogy about the chromophore environment in the visual pigment. The solvent classifications (leveling and nonleveling) of Erickson and Blatz (1968) were confirmed by this investigation, and a third group of solvents not fitting either of their categories was discovered. From analysis of data reported here, it was found that halogenated hydrocarbon solvents must be subdivided into three groups: (1) those with no hydrogen bound to a halogen-bearing carbon, (2) those with hydrogen bound to a single halogen-bearing carbon, and (3) those with hydrogen bound to two adjacent halogen-bearing carbons. The third class is being investigated presently and will be reported on later. These subclasses are thought to be made necessary by *specific solvent–solute association* rather than by bulk solvent effects.

Samples of pure monoprotonated salts were dissolved in purified solvents and spectra were recorded in a Beckman DK-2A spectrophotometer. The spectra of the chloride, bromide, and iodide salts were determined in CCl_4, $CHBr_3$, $CHCl_3$, CH_2Cl_2, CH_3CCl_3, CH_3CHCl_2, and CH_3CH_2Br. The λ_{max} of the salts and the literature values of the dipole moments and the refractive indexes of these solvents are reported in Table I. The data from this table were plotted in Figures 1–3.

The change in excitation energy of a solute has been related to the solvent polarizability, and the solute polarizability in turn is proportional to the square of the refractive index, n^2 (Longuet-Higgins and Pople, 1957). Inspection of Table I indicates there is no relationship between the λ_{max} of the salt

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TABLE I: The Absorption Maximum of *all-trans-N*-Retinylidene-*n*-butylammonium (NRBA) Chloride, Bromide, and Iodide in Halohydrocarbon Solvents of Groups 1 and 2.

Solvent	n_D[b]	Dipole (D)	Ref[a]	NRBA-HCl λ_{max} (nm)	NRBA-HBr λ_{max} (nm)	NRBA-HI λ_{max} (nm)
$CHBr_3$	1.5976	0.90	1	469	475	480
$CHCl_3$	1.4433	1.15	1	460	468	478
CH_2Cl_2	1.3348	1.57	2	453	463	471
CH_3CHCl_2	1.4160	2.07	3	443	451	463
CH_3CH_2Br	1.4239	2.02	4	443	452	461
CCl_4	1.4607	0.00	5	442	452	460
CH_3CCl_3	1.4199	1.66	6	439	450	462

[a] References for dipole moments: (1) Timm and Mecke (1936), (2) Barclay and LeFevre (1950), (3) Ghosh *et al.* (1929), (4) Smyth and McAlpine (1934), (5) Watson and Ramaswamy (1936), and (6) Klanges and Langpape (1960). [b] Weast (1966).

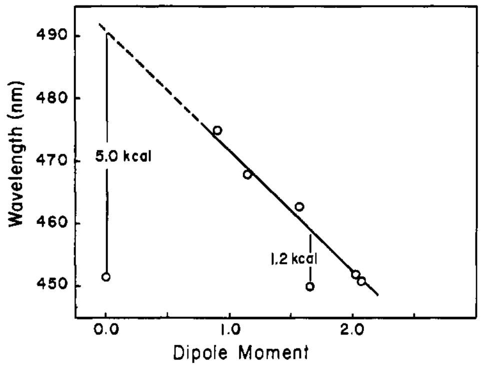

FIGURE 2: The effect of solvent dipole moment and hydrogen bonding ability on the λ_{max} of *all-trans-N*-retinylidene-*n*-butylammonium bromide.

and the refractive index of the solvent. Consequently, in spite of recent speculation by Irving *et al.* (1970), solvent polarizability does not play a major role in the wavelength shifts of *N*-retinylidene-*n*-butylammonium salts. Closer examination of the data in Table I brings out the fact that the wavelength of the salt is longest when there is a hydrogen on the carbon containing a halogen, a group 2 solvent.

Separate plots were made for the chloride, bromide, and iodide in which the absorption maxima of the salts were plotted *vs.* the dipole moment of the pure solvents. It is clear that there is a linear correspondence between these two parameters over the range of the dipoles examined for solvents of a single structural type fitting into group 2. CCl_4 and CH_3CCl_3 which belong to a different solvent type (group 1) deviate unmistakably from this linear relationship. It has been shown that those solvents which are classed in group 2 and obey the linear relationship between dipole moment and wavelength can participate as hydrogen donors in hydrogen bonding (Pimentel and McClellan, 1960; Glasstone, 1937). Klemperer *et al.* (1954) found that the carbonyl stretching frequency of *N*-ethylacetamide was shifted approximately 20 cm^{-1} by $CHBr_3$, $CHCl_3$, and CH_2Cl_2 compared to CCl_4 and only 2 cm^{-1} by CH_3CCl_3. By comparison to this study and simply from consideration of the solvent structures involved it seems safe to postulate that CCl_4 and CH_3CCl_3 deviate from the linear dipole moment–wavelength relationship due to their inability to hydrogen bond.

Data presented in the preceding article indicate that anion withdrawal causes a bathochromic shift in the spectra of *N*-retinylidene-*n*-butylammonium salts. It is not unreasonable, therefore, to postulate that weakening of the anion–cation interaction by solvent hydrogen bonding to the anion should likewise produce a bathochromic shift. Hydrogen bonding between halide anions and solvents such as $CHCl_3$ or $CHBr_3$ (group 2 solvents) has been demonstrated by infrared studies (Allerhand and Schleyer, 1963). The C–D stretching frequency of X_3C–D is lowered in the presence of either quaternary ammonium halides or metal halides in CCl_4 or acetone. The change in C–D stretching frequency is sensitive to anion identity; the order of apparent hydrogen bonding strength is $Cl^- > F^- > Br^- > I^-$. The direct relationship that was shown previously between ion interaction and energy of absorbed light should likewise permit a direct correlation between solvent–anion hydrogen-bonding energy and light energy. The energies indicated in Figures 1–3 represent the change in light absorption energy due to hydrogen bonding. These energy quantities fit nicely into the energy range expected for hydrogen bonding, and the order of hydrogen-bonding energies observed, $F^- > Cl^- > Br^- > I^-$, corresponds to the order observed in the above infrared study.

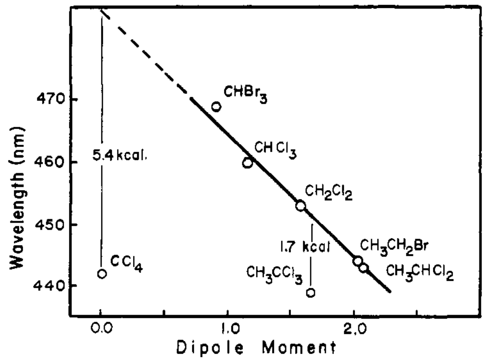

FIGURE 1: The effect of solvent dipole moment and hydrogen bonding ability on the λ_{max} of *all-trans-N*-retinylidene-*n*-butylammonium chloride.

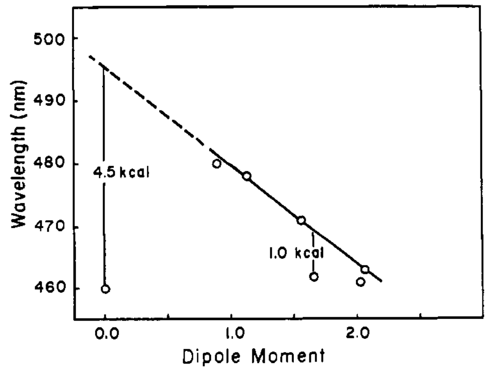

FIGURE 3: The effect of solvent dipole moment and hydrogen bonding ability on the λ_{max} of *all-trans-N*-retinylidene-*n*-butylammonium iodide.

TABLE II: Charge in esu × 10^{10} on the Solvent Atoms[a] (per Atom).

Solvent	E_{c_1}	E_{c_2}	E_{H_1}	E_{H_2}	E_x
$CHBr_3$	1.120		0.145		−0.421
$CHCl_3$	1.370		0.178		−0.615
CH_2Cl_2	1.110		0.145		−0.700
CH_3CCl_3	1.250	0.428		0.056	−0.616
CH_3CHCl_2	0.985	0.336	0.128	0.044	−0.790
CH_3CH_2Br	0.544	0.186	0.071	0.024	−0.945

[a] Charge distributions were calculated by the method of Eyring *et al.* (1951).

It may be well to summarize the interpretation of the relationship illustrated in Figures 1–3. In the solution spectra of *N*-retinylidene-*n*-butylammonium salts in nonleveling solvents, there are two main, simultaneously operative solvent effects—a dipole moment effect and a solvent–anion hydrogen-bonding effect. The solvents obeying the linear relationship between the dipole moment and wavelength are approximately equal in hydrogen-bonding ability while those which deviate significantly from the linear relationship do so because of their inability to hydrogen bond with the anion. The quantitative deviations from the line of CCl_4 and CH_3CCl_3 fit nicely in the range of expected hydrogen-bonding energies; furthermore, the order of hydrogen-bonding energies for the salts is in agreement with the order of hydrogen-bonding ability revealed by infrared analysis.

The assumption that all of the group 2 solvents are approximately equal in their ability to hydrogen bond with a given anion can be examined on the basis of the electrostatic model of hydrogen bonding. From this model, one can estimate the relative hydrogen-bonding strength between solvent and anion by examining the charge distribution of the solvent (Eyring *et al.*, 1951). The amount of positive charge on the bonding hydrogen is of primary importance. The charge distributions of all of the solvents except CCl_4, shown in Figures 1–3, are given in Table II. With the exception of bromoethane, the charge distributions in all of the solvents obeying the linear dipole moment–wavelength relationship appear similar enough to assume approximately equal hydrogen-bonding ability. 1,1,1-Trichloroethane, on the other hand, would not be expected to be a good hydrogen-bonding solvent.

Discussion

The results obtained with nonleveling solvents which are either hydrogen bonding or nonhydrogen bonding appear to be quite straightforward. The maximum absorption wavelength exhibited by *N*-retinylidene-*n*-butylammonium salts in these type solvents was found to be controlled by solvent dipole moment and hydrogen-bonding ability.

Hydrogen-bonding solvation of the anion of *N*-retinylidene-*n*-butylammonium salts would be expected to reduce its electrostatic interaction energy [$E_i = Z_C Z_A l^2/(r_c + r_a)$, where l = electron charge and r_c and r_a are the radii of cation and anion, respectively] with the cation. A reduction in E_i should cause a proportionate decrease in ΔE. Thus, the λ_{max} of an *N*-retinylidene-*n*-butylammonium salt should be longer in a hydrogen-bonding solvent than in a nonhydrogen-bonding solvent having the same dipole moment. As already mentioned in the results, hydrogen-bonding solvents shift the λ_{max} of *N*-retinylidene-*n*-butylammonium salts to longer wavelengths and in proportion to the expected relative hydrogen-bonding energy of the chloride, bromide, and iodide anion. Bufalini and Stern (1961) found that solvent–anion hydrogen bonding is proportional to anion charge density, in agreement with the preceding statement, but also that solvent–anion hydrogen bonding is absent with the picrate anion. From the data presented in the preceding article, it is seen that the λ_{max} of the halogen salts shifted about 18 nm, or 2.6 kcal, going from CCl_4 to $CHCl_3$, whereas the picrate salt shifted only 4 nm, or 0.5 kcal. This provides an example of a nonhydrogen-bonding anion, so to speak, the *N*-retinylidene-*n*-butylammonium salt of which exhibits a very small bathochromic shift on going from a solvent classed as nonhydrogen bonding to one classed as hydrogen bonding. The size of the spectral shifts attributed to hydrogen bonding (1.0–5.4 kcal) appears to be in good agreement with expected energies of hydrogen bonding.

We previously showed that the distance between nitrogen and the anion controls the λ_{max} of the cation in an *N*-retinylidene-*n*-butylammonium salt in a nonleveling solvent. In this study we showed that certain nonleveling hydrogen-bonding solvents induce a shift in the λ_{max} by binding the anion and causing the dissociation of the anion pair. In addition we found a bulk dipole effect in these solvents. The question naturally arises as to which effects are found in visual pigments. A detailed discussion of the anion effect has already been given. Since probably the anion in visual pigments is covalently bound, a random, pure solvent effect such as hydrogen bonding would not be operative. However, a bulk effect such as dipole moment would be expected to influence modestly the λ_{max} of the cation. Thus, in a matrix with a low dipole moment the wavelength would be longer than it would in one of high dipole moment. This is consistent with the notion that the chromophore is in a lipid environment of low dipole.

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Effect of Light Scattering on the Circular Dichroism of Biological Membranes†

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ABSTRACT: In order to establish the origin of the anomalies observed in the circular dichroism spectrum of biological membranes, we studied this property in *Streptococcus faecalis* plasma membranes, under conditions of varying particle size. This membrane exhibits the anomalies generally associated with membrane circular dichroism spectra. Reduction in particle size, effected by sonication for varying lengths of time, led to reduction in turbidity and an elimination of the anomalies, whereas Ca^{2+} induced aggregation of sonicated particles led to increased turbidity and a reappearance of the membrane associated anomalies. $[\theta]_{222}$ appears to be less sensitive to these optical artifacts than shorter wavelength ellipticities and reliable estimates of helicity in unsonicated preparations can be obtained using this value. $[\theta]_{208}$ is found to be very dependent on the turbidity of the membrane suspension and only yields reliable estimates of α-helical content in sonicated preparations under which conditions both $[\theta]_{222}$ and $[\theta]_{208}$ yield values of 27% helicity. In addition, infrared measurements performed on dried membrane films show a shoulder at 1630 cm^{-1}, suggesting the presence of β structure in the protein of this membrane. ATPase activity measurements and density gradient experiments indicate that neither extensive denaturation nor liberation of protein from the membrane surface has occurred during the sonication procedure.

The application of physical techniques to the problem of structural analysis in biological membranes has brought varied success. Circular dichroism has been used successfully to determine structural parameters in globular proteins (Timasheff *et al.*, 1967); obtaining similar data for membrane associated proteins would provide valuable information for formulating and confirming various models of membrane structure. The application of this technique to membrane systems has produced ambiguous results due to certain anomalies which are observed in the circular dichroism spectra; these anomalies are characterized by a red shift in the position of Cotton effects associated with the $n-\pi$ and $\pi-\pi^*$ transitions of the amide group and a loss of intensity of the $\pi-\pi^*$ transitions at 208 and 190 nm (Lenard and Singer, 1966; Wallach and Zahler, 1966; Urry *et al.*, 1967). A variety of explanations have been put forward to explain these observations; they have been attributed by various investigators to be the result of hydrophobic protein–lipid interactions (Wallach and Zahler, 1966), protein–protein interactions (Lenard and Singer, 1966), and optical artifacts caused by the particulate nature of the membrane system (Urry and Ji, 1968; Urry and Krivacic, 1970).

In order to make use of the information available from circular dichroism measurements, the origin of these anomalies must be clarified. Several attempts have been made to treat scattering corrections for membrane systems quantitatively (Gordon and Holzwarth, 1971; Schneider, 1971; Glaser and Singer, 1971; Gordon, 1972; Urry, 1972); in this paper we assess the effect of the particulate nature of membrane systems on the circular dichroism spectrum of the plasma membrane of *Streptococcus faecalis* bacterium under conditions where the membrane particle size was varied. Our results lead us to conclude that light scattering, which results from the particulate nature of membrane suspensions, is largely responsible for the anomalies observed in the membrane circular dichroism spectra. In addition, it appears as though reasonable estimates of helicity can be obtained using $[\theta]_{222}$, since this region of the spectrum shows very little dependence on particle size.

Experimental Section

Membrane Preparation. ATPase-depleted membranes of *S. faecalis* (ATCC No. 9790) were prepared by the procedure of Schnebli and Abrams (1970). After release of ATPase the membranes were further treated with 0.01 mg/ml of RNase and 8 mM EDTA to assure removal of rRNA, then washed. Samples were spun down at 100,000*g* for 30 min, then resuspended by homogenization in fresh Tris buffer for the next wash. The initial wash showed a burst of 260-nm absorbing material. Subsequent washing was continued until no discernible 260-nm peak was observed in the wash supernatant and the absorbance in the 280-nm region remained constant in two successive washes, generally at about 5% of the original absorbance in the first wash solution. Membrane samples in 0.02 M Tris buffer (pH 7.5) were sonicated for varying lengths of time in a jacketed, glass sonication vessel thermostated at 2°, in a nitrogen atmosphere. Sonication was accomplished with a Branson S-12 automatic tuning sonifier set for a power output of 90–100 W. Turbidity readings at 325 nm were employed to establish the length of time required to reach minimum particle size. All samples were stored under nitro-

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