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Alteration of pK_a of the Bacteriorhodopsin Protonated Schiff Base. A Study with Model Compounds†

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ABSTRACT: Factors influencing the pK_a value of retinal protonated Schiff base ($RSBH^+$) are examined by using fluorinated alcohols and series of retinals bearing nonconjugated positive charges along the polyene. It is shown that the effective pK_a of $RSBH^+$ is increased by a solvent, forming a strong hydrogen bond, that stabilizes the anion but weakly interacts with the Schiff-base proton. A positive charge in the vicinity of the Schiff-base linkage markedly reduces the effective pK_a. The effect is significantly enhanced in fluorinated alcohols in which positive charges are weakly solvated. It is suggested that drastic pK_a reduction might take place during bacteriorhodopsin (bR) photocycle either by elimination of hydrogen-bonding stabilization or by a positive charge approaching the Schiff-base linkage. Weak solvation of the positively charged Schiff-base nitrogen (relative to ethanol solution) and strong solvation with its counterion lead to a red shift in the absorption maximum of retinal protonated Schiff base up to ca. 2400 cm^{-1} in hexafluoro-2-propanol relative to ethanol. This mechanism of introducing red shift in the absorption maximum of $RSBH^+$ might play a role in determining part of the opsin shift found in bR and the red shift observed in the transformation from the bR_{570} to K_{610} intermediate following light absorption. Nonconjugated positive charges shift the absorption maximum of $RSBH^+$. Their influence is further enhanced with fluorinated alcohols as solvents.

The purple membrane of *Halobacterium halobium* functions as a light-driven proton pump due to its pigment bacteriorhodopsin, a substance comprised of a retinal chromophore bound covalently at an ϵ-aminolysine residue of a protein via a protonated Schiff base [see Stoeckenius et al. (1979), Ottolenghi (1980), and Birge (1981) for reviews]. The pigment was found to exist in two forms: a light-adapted form absorbing at 570 nm (bR_{570})[1] with an *all-trans*-retinal chromophore and a dark-adapted modification absorbing at 560

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[1] Abbreviations: bR_{570}, bacteriorhodopsin, the subscript denoting the wavelength of maximum absorption; HFIP, hexafluoro-2-propanol; RSB, retinal Schiff base; $RSBH^+$, retinal protonated Schiff base; TFE, trifluoroethanol; FT-IR, Fourier transform infrared spectroscopy.

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nm (bR_{560}) which contains a mixture of the *all-trans*- and 13-*cis*-retinal. Following light absorption bR_{570} undergoes a photochemical cycle involving several intermediates: K_{610}, L_{550}, M_{412}, and O_{640} characterized by absorption maxima at 610, 550, 412, and 640 nm, respectively, including a nonprotonated intermediate (M_{412}). The deprotonation of the Schiff base is probably directly associated with operation of the proton pump. Stoeckenius et al. (1979) and Kalisky et al. (1981) suggested that this proton loss resulted from a marked reduction of the protonated Schiff-base pK_a as a consequence of light absorption. The pK_a of bR_{560} (dark adapted) was measured by Druckmann et al. (1982), who reported a value of 13.3 ± 0.3 and showed the formation of a nonprotonated Schiff base species upon titration. We recently provided proof that the pK_a they measured reflects a direct titration of the Schiff base rather than the deprotonation of a protein residue that induces protein conformational changes exposing the Schiff base (Sheves et al., 1986). The apparent pK_a value of retinal protonated Schiff base ($RSBH^+$) in MeOH–H_2O solution is considerably lower [~7.4 (Schaffer et al., 1975)] than that of bR_{560}. By constructing a bacteriorhodopsin analogue from a retinal derivative with an intrinsic pK_a value even lower than 7.4, we were able to show a similar reduction in the pK_a of the bR analogue an effect not expected if the measured pK_a involved any other group than the Schiff base.

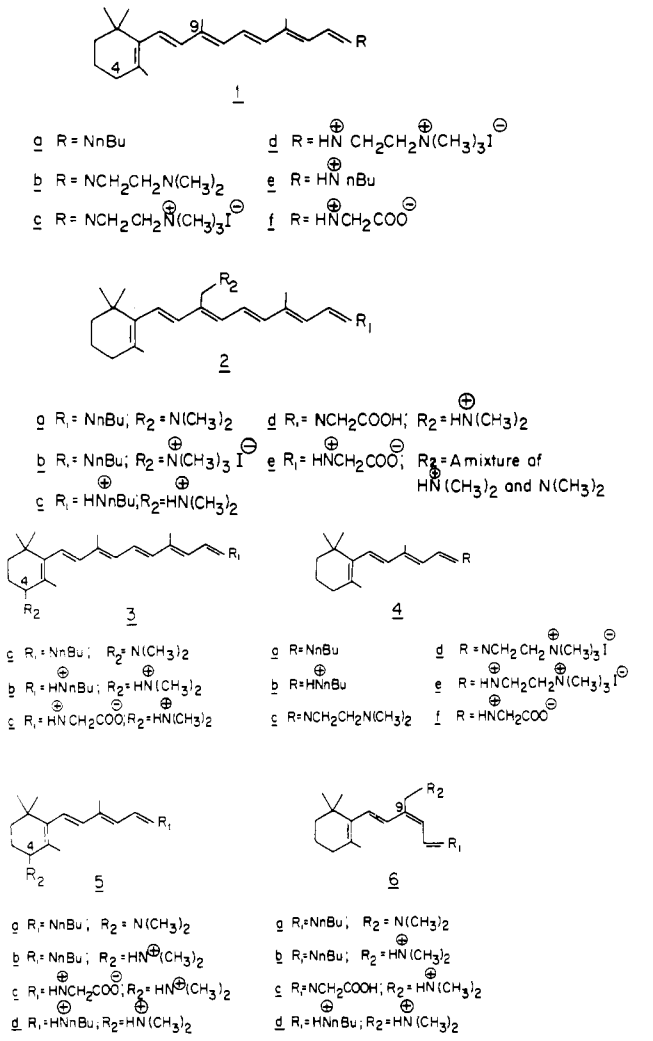
The significant difference between solution and bR pK_a values, as well as the change in pK_a taking place during the photocycle, focuses our present studies on the factors influencing the pK_a value of retinal protonated Schiff base itself. Understanding of these factors is prequisite for clarifying the proton pump mechanism in bR.

Several suggestions have been made to account for the high apparent pK_a of bR relative to that of $RSBH^+$ in solution and the reduction in pK_a due to light absorption, which assumedly alters the configuration of the retinal. Warshel et al. (1984) proposed a relatively polar protein environment surrounding the protonated Schiff base that stabilizes the protonated Schiff base–counterion pair. Hildebrandt and Stockburger (1984) attributed the stabilization to hydrogen bonding due to water. A different approach was taken by Schulten and Tavan (1978), who suggested a change in the pK_a during the photocycle due to twisting around the C_{14}–C_{15} single bond of the retinal chromophore. Destabilization of the ion pair due to charge separation was proposed by Honig et al. (1979), whereas Scheiner and Hillenbrand (1985) proposed a change in the relative orientation of the protonated Schiff base and its counterion.

In the present study we examine factors influencing the pK_a of $RSBH^+$ in a solvent (e.g., fluorinated alcohols) that strongly hydrogen bonds and stabilizes the dissociated anion but only weakly interacts with the Schiff-base proton. We show that the pK_a is markedly reduced by the presence of a nonconjugated positive charge on the retinal in the vicinity of the Schiff-base linkage. In addition to studying the pK_a, we also demonstrate that the absorption maximum of protonated retinal Schiff base is red-shifted in this family of solvents. The position of the absorption maximum is also found to be shifted by electrostatic interaction with the nonconjugated positive charges. This shift is further enhanced when measured in fluorinated alcohols.

Materials and Methods

Preparation and Titration of Schiff Bases. The required aldehydes were obtained as previously described (Baasov & Sheves, 1985). The Schiff bases (except **1b** and **4c**) were prepared by dissolving the aldehyde in dry ethanol and mixing



it with 1.5 equiv of *n*-$BuNH_2$ at 25 °C for 30 min. The solvent and excess *n*-$BuNH_2$ were evaporated under high vacuum to yield the target Schiff base. Compounds **1b** and **4c** were prepared similarly by condensing the aldehyde with 1 equiv of *uns*-dimethylethylenediamine for 30 min at 25 °C, followed by evaporation of the solvent under high vacuum.

Titration of Schiff bases was carried out in 50% MeOH–H_2O solutions at 0 °C with using appropriate buffers. The apparent pK_a values were determined by recording quantitative changes in the visual absorptions of the protonated Schiff base band and that of the corresponding deprotonated form. The chromophores are very sensitive to water, requiring rapid work. Schiff base **1b** was too unstable even at 0 °C; thus the measurement was carried out at −20 °C. Similar measurement of the protonated Schiff base derived from **1a** at −20 °C revealed an increase of 0.3 unit (7.9) in the pK_a value relative at 0 °C (7.6). The spectra were recorded on a Kontron 810 spectrophotometer.

Schiff bases **1b**, **2a**, and **4c** were mixed with 3 equiv of methyl iodide in MeOH at 25 °C for 12 h in the presence of Na_2CO_3. Filtration and solvent evaporation afforded **1c**, **2b**, and **4d**, respectively.

Condensation with Glycine. The required aldehyde was mixed with 1.2 equiv of glycine in purified trifluoroethanol (TFE) or hexafluoro-2-propanol (HFIP) at 25 °C for 12 h. The reaction was followed by monitoring changes in the absorption maxima of the mixture.

Spectra of the condensation product in ethanol or chloroform were measured by evaporation of the fluorinated alcohol and

resolution of the residue in the desired solvent.

The fluorinated alcohols (spectroscopic grade) were purified by elution through a basic alumina column followed by distillation. Protonated Schiff bases were obtained by dissolving the Schiff bases in EtOH, treating with HCl, evaporating the EtOH, and dissolving in the required solvent.

Results

In our search for factors influencing the pK_a of retinal protonated Schiff base in bR, we initially examined whether mechanisms previously suggested to explain the shift in absorption maximum of bR relative to retinal protonated Schiff base ($RSBH^+$) in ethanol might also account for the pK_a data. The red shift was recently attributed to a combination of factors: (1) weak interaction between the Schiff base nitrogen and its counterion in the protein, due to the relatively large distance between the two [first suggested by Blatz et al. (1975), and more recently by Harbison et al. (1983), Muradin-Szweykowska (1984), Sheves et al. (1985), and Spudich et al. (1986)]; (2) the planar s-trans ring-chain conformation that is present in bR (Harbison et al., 1985; Spudich et al., 1986; Akhtar et al. 1982; Schreckenbach et al., 1978); (3) interaction of the polyene skeleton with an ion pair on the protein in the vicinity of the β-ionone ring (Harbison et al., 1985; Spudich et al., 1986; Lugtenburg et al., 1986).

To clarify whether the latter two factors, operating in the vicinity of the ring moiety, also affect the pK_a value, we looked for a system in which these effects are significantly reduced. Recently, it was found that artificial bR pigments based on synthetic retinals bearing a substituent at the C_4 position exhibit a main band with only a small red shift (~460 nm) (Sheves et al., 1984). This weak red shift might result from an unnatural nonplanar ring-chain conformation adopted by the chromophore ring as a result of C_6–C_7 single bond rotation or from changes in charge distribution around the chromophore. Despite the unusually small red shift, the artificial pigment derived from 4-methylretinal has an apparent pK_a of 12.5 ± 0.2 not very different from that of natural bR. This leads to the conclusion that the factors determining the high pK_a value of bR do not include those involved with the ring moiety of retinal which red shifts the absorption maximum.

We next considered the possibility that ion–counterion interactions might account for the raised pK_a in bR and sought model systems in solution capable of stabilizing the pair of ions via a hydrogen bonding. Fluorinated alcohols were chosen as an ideal solvents for this purpose as they efficiently stabilize ion pairs due to strong hydrogen bonding (Evans et al., 1971). In view of their pK_a's [12.5 for TFE (Takahashi et al., 1972 and 7.4 for *n*-butylamine $RSBH^+$ in MeOH–H_2O solution (Schaffer et al., 1975)], a direct protonation of RSB by TFE is not expected. However, RSB was protonated in TFE, presumably due to an ionic stabilization resulting from hydrogen bonding with TFE, a process effectively raising the pK_a of $RSBH^+$. Moreover, this protonation effect was concentration dependent, indicative of solute–solvent interaction. A pK_a alteration in TFE solution was also observed by Carre and Devynck (1981) for various amines. Retinal Schiff base **1a** underwent almost complete protonation at a concentration of 1×10^{-3} M (>95%), whereas in 1×10^{-2} M a 1:1 mixture of RSB and its protonated form ($RSBH^+$) was observed (Figure 1). Protonation in TFE was followed by the characteristic red-shifted absorption maximum of the protonated species (467 nm) and by FT-IR spectra of $RSBH^+$ in CF_3CH_2OH and CF_3CD_2OD. The C=N^+ stretching shifted from 1650 cm^{-1} in CF_3CH_2OH to 1632 cm^{-1} in CF_3CD_2OD, indicating a protonated Schiff base linkage (Lewis, 1982). Gradual addition of ethanol to a solution of $RSBH^+$ in TFE led to deprotonation, as monitored by the Schiff-base band at 360 nm (Figure 2). Almost complete deprotonation, probably caused by weakening of hydrogen bonding (leading to a lower pK_a value of $RSBH^+$), was observed at 40% EtOH (in 1×10^{-5} M $RSBH^+$). Intense ionic stabilization was achieved with hexafluoro-2-propanol (HFIP) as a solvent, due to its stronger hydrogen-bonding capacity than TFE. Its pK_a is lower as well [9.5 (Takahashi et al., 1971)], and thus, complete protonation of RSB is observed even at a concentration of 10^{-1} M, with the species exhibiting absorption maximum of 492 nm.

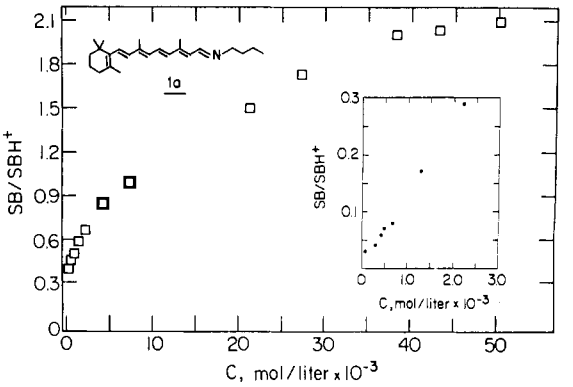


FIGURE 1: Absorbance ratio of Schiff base and its protonated species bands in different concentrations of retinal Schiff base **1a** in trifluoroethanol.

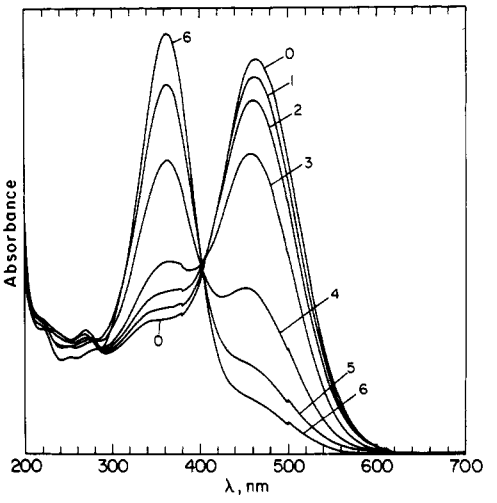


FIGURE 2: Addition of ethanol to a solution of 0.5×10^{-5} M retinal protonated Schiff base **1e** in trifluoroethanol. 0: the protonated species (**1e**) band. 1–6: addition of 5%, 10%, 20%, 30%, 40% and 50% ethanol correspondingly.

A positive charge in the vicinity of the retinal chromophore is yet another possible cause for lowering the pK_a value of $RSBH^+$ (Hanamoto et al., 1984), a mechanism parallel to that suggested to explain the reduction of tyrosine pK_a during bR photocycle by Kalisky et al. (1981) and Hanamoto et al. (1984). We have investigated the influence of a positive charge on the pK_a of $RSBH^+$ in 50% MeOH–H_2O using chromophores **1–6**, bearing dimethylamino groups at various locations along the polyene. The higher basicity of the dimethylamino group than that of the Schiff base function ensures the presence of a nonconjugated positive charge located at the quaternary amine while titrating the Schiff-base nitrogen. The existence of this ammonium salt is supported by the shifts in the absorption maxima [red when the charged species are located in the vicinity of the Schiff-base bridge and blue when they are in the vicinity of the ring moiety (Baasov & Sheves, 1985)] observed during the titration.

Table I: Apparent pK_a Values of Protonated Schiff Bases in 50% MeOH–H_2O

chromophore	1e	2c	3b	1d	4b	5d	6d	4e
pK_a	7.6 ± 0.1	6.5 ± 0.1	7.4 ± 0.1	5.5 ± 0.1[a]	7.5 ± 0.1	6.9 ± 0.1	4.7 ± 0.1	5.2 ± 0.3

[a] The measurement was carried out at −20 °C.

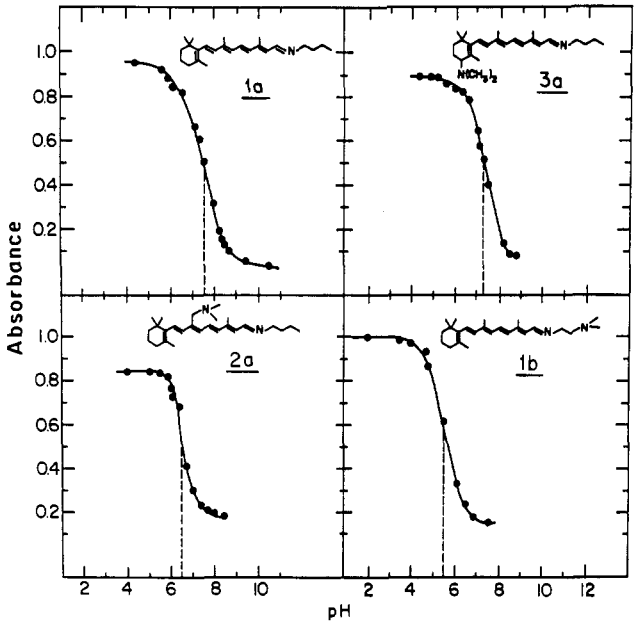


FIGURE 3: Titration curves of retinal Schiff base in 50% MeOH–H_2O at 0 °C (−20 °C in the case of **1b**). Absorbance of protonated species.

Table I and Figures 3–5 summarize the apparent pK_a values for the various retinal chromophores, revealing a reduction of the protonated Schiff base pK_a values due to the presence of a positive charge in the vicinity of the retinal chromophore. The influence is increased as the distance between the nonconjugated positive charge and the positively charged Schiff-base nitrogen decreases. In **1d** we observed ca. 2 units in the reduction of the pK_a value relative to that for **1e**, whereas for **3b** a reduction of only 0.2 unit (see Table I).

To get further insight into this influence of nonconjugated positive charge, we studied the protonation of charged chromophores in fluorinated alcohols. In contrast to **1a** (RSB), the Schiff base of chromophores **1c** and **2b** did not undergo effective protonation in TFE, presumably due to the nonconjugated positive charge. Protonation of **1c** and **2b**, however, was achieved in HFIP in which, as mentioned earlier, hydrogen bonding is stronger than in TFE. The positive charge of **1d** reduces the pK_a of the protonated Schiff base more than that of **2c**, where it is located near carbon 9. Only a small fraction of **1c** (ca. 10%) underwent protonation in TFE to give **1d** at 3×10^{-5} M. However, at a similar concentration, **2b** was almost 50% protonated (Figure 6). We note that the protonated species derived from **1b** and **2a** (in contrast to **1c** and **2b**, which carry quaternary salts) exhibited shifted absorption maximum (relative to protonation with HCl in TFE) due to partial protonation of the nonconjugated dimethylamino group. In chromophore **3a** the nonconjugated dimethylamino group is located relatively far from the Schiff-base bridge (ca. 12 Å). Thus, both the Schiff base and the dimethylamino group were protonated by both TFE and HFIP. However, with respect to the effect of concentration on protonation, there is a difference between **3a** and **1a**, the latter being protonated *almost completely in* TFE at a concentration of 0.5×10^{-3} M, whereas only a fraction of the Schiff-base function in **3a** (ca. 30%) took up a proton at this concentration (Figures 1 and 7). We note that, in the protonated fraction, the nonconjugated dimethylamino group is completely protonated in TFE. This is evident from the blue-shifted absorption maximum, as would be expected for $RSBH^+$ bearing a nonconjugated positive charge (Baasov & Sheves, 1985) as well as from the lack of change in absorption upon addition of HCl.

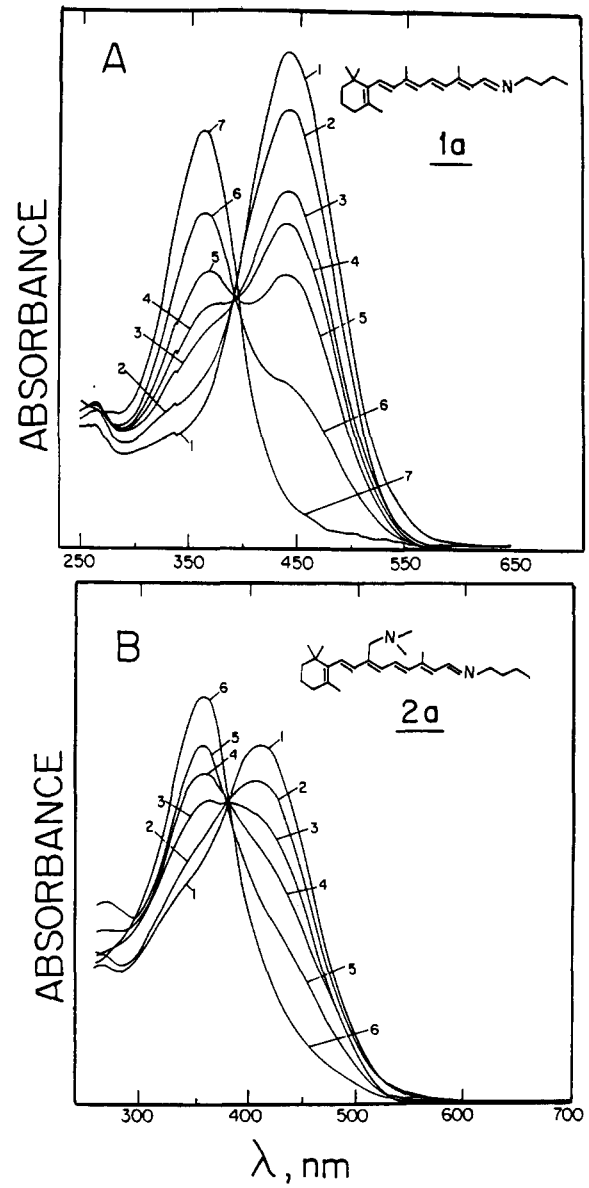


FIGURE 4: Absorption spectra of retinal Schiff base **1a** (A) and Schiff base **2a** (B) at 0 °C in a 50% H_2O–MeOH solution at different pH. The red-shifted band corresponds to the protonated species. (A) pH 5.7, 6.6, 7.2, 7.4, 7.6, 8.0, and 8.65 for 1–7 correspondingly. (B) pH 5.1, 6.02, 6.4, 6.7, 7.04, and 7.84 for 1–6 correspondingly.

Further insight into the influence of nonconjugated positive charges on the pK_a values of polyene protonated Schiff bases was gained by studying chromophores **4–6** where the polyene chains are significantly shorter. Table I lists the apparent pK_a values of the chromophores in 50% MeOH–H_2O, clearly demonstrating a reduction in pK_a due to a nonconjugated positive charge. Chromophore **6d** is markedly affected by ca. 2.5 units (relative to **4b**), whereas **5d** changes by only 0.5 unit (Figure 8), due to the larger distance between the positive

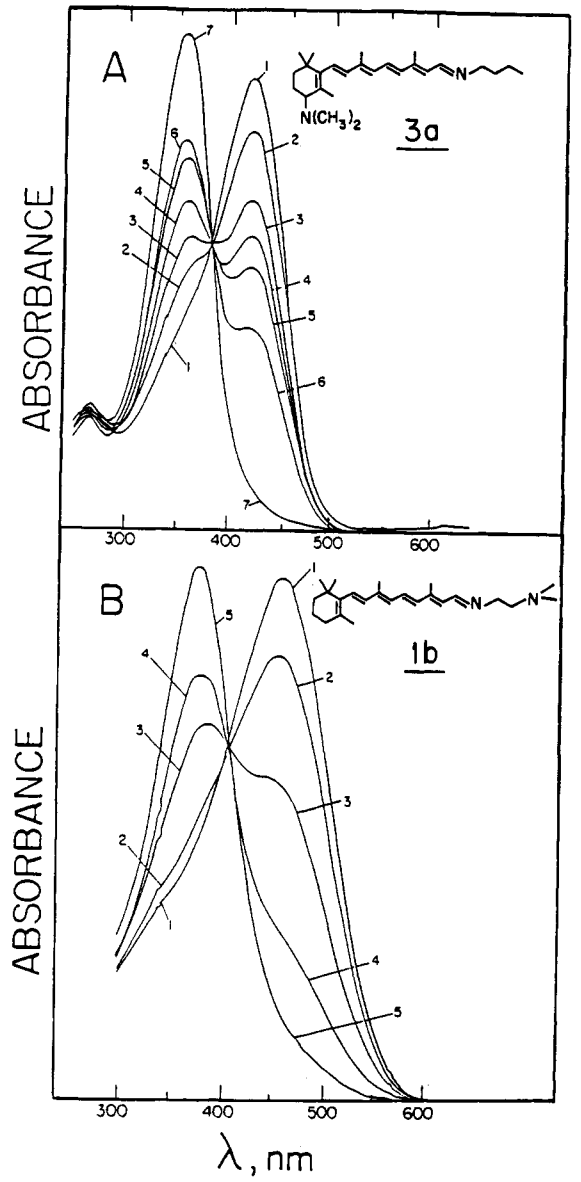


FIGURE 5: Absorption spectra of Schiff bases **3a** (A) and **1b** (B) at 0 and −20 °C correspondingly in a 50% H_2O–MeOH solution at different pH. The red-shifted band corresponds to the protonated species. (A) pH 5.25, 6.65, 7.1, 7.2, 7.4, 7.6, and 8.3 for 1–7 correspondingly. (B) pH 4.1, 4.8, 5.5, 6.12, and 6.8 for 1–5 correspondingly.

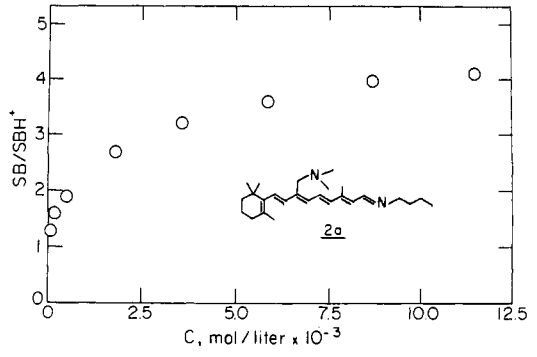


FIGURE 6: Absorbance ratio of Schiff base and its protonated species bands in different concentrations of retinal Schiff base **2a** in trifluoroethanol.

charge and the Schiff base linkage in the latter.

In TFE the protonation of **4a** was less effective than that of **1a** (RSB). Only ca. 70% of **4a** was protonated at 10^{-3} M, whereas almost complete protonation occurred in **1a** at a similar concentration. This difference can be explained by stabilization by a conjugation effect, due to the longer polyene chain in the protonated Schiff base derived from **1a**. The Schiff base of **4d** could not be protonated by TFE due to the presence of a positive charge in the vicinity of the Schiff-base linkage, but almost complete protonation did occur in HFIP. Even poorer protonation was found in **6b**, in which less than 5% protonation was achieved in either TFE or HFIP. We note that, contrary to **6b**, the Schiff base of 13-CF_3 retinal was efficiently protonated in HFIP. We have recently shown (Sheves et al., 1986) that the apparent pK_a of the protonated Schiff base derived from this retinal derivative is reduced by ca. 5 units in 50% H_2O–MeOH relative to $RSBH^+$. Since $RSBH^+$ and the short chromophore **4b** do not differ significantly in their pK_a values (see Table I), we can conclude that the nonconjugated positive charge in **6d** causes a reduction of the protonated Schiff base apparent pK_a value in HFIP by more than 5 units.

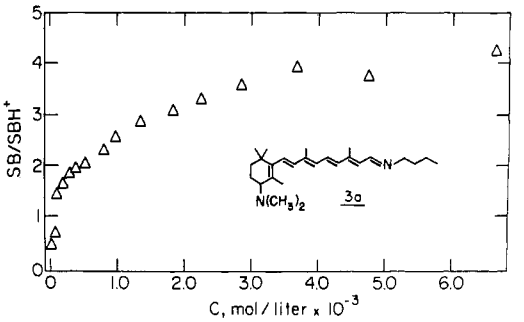


FIGURE 7: Absorbance ratio of Schiff base and its protonated species bands in different concentrations of retinal Schiff base **3a** in trifluoroethanol.

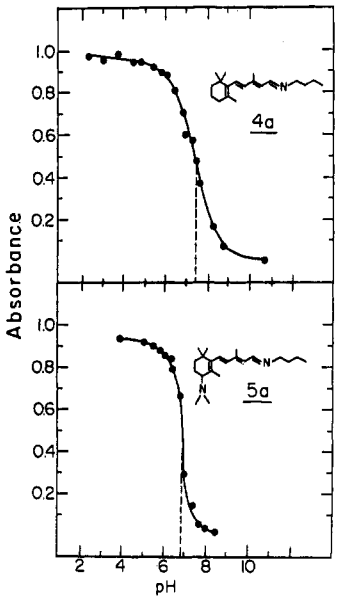


FIGURE 8: Titration curves of Schiff bases **4a** and **5a** in 50% MeOH–H_2O at 0 °C. Absorbance of protonated species.

In **5a**, with a dimethylamino group located further from the Schiff base relative to **6a**, only ca. 5% protonation was observed in a dilute solution (3×10^{-5} M) in TFE, but full protonation was achieved in HFIP. The experiments clearly demonstrate the importance of the location of nonconjugated positive charge along the polyene in influencing pK_a values.

Several research groups have suggested that tyrosine can serve as a counteranion for the protonated Schiff base in bR and visual pigments [Khristoforov et al., 1974; Rastogi & Zundel, 1981; recently, Rothschild et al. (1986)]. However,

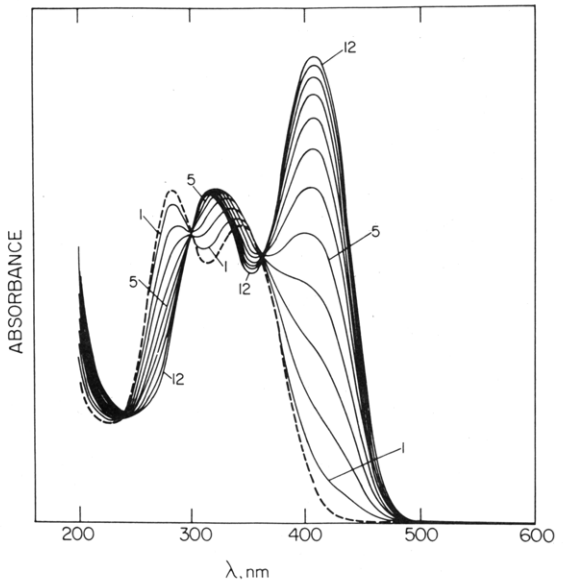


FIGURE 9: Condensation between the aldehyde derived from Schiff base **4a** and glycine in trifluoroethanol. 1, 5, and 12: after 0.7-, 3.5-, and 8.4-h reaction time, correspondingly (0.7 h between each measurement). (---) Aldehyde absorption.

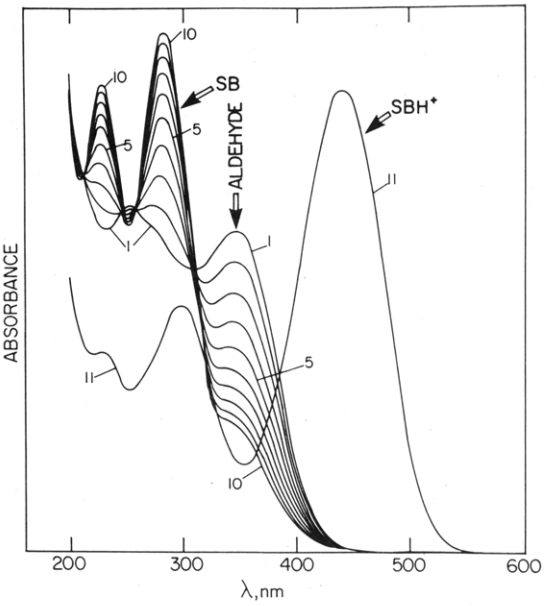


FIGURE 10: Condensation between the aldehyde derived from Schiff base **6a** and glycine in trifluoroethanol. 5 and 10: after 5- and 10-min reaction time, correspondingly. SB: Schiff base (condensation product **6c**). SBH^+: protonated Schiff base (obtained following addition of HCl to **6c**, curve 11).

the strongest possible proton donor in bR and visual pigments is a protein carboxyl group. Thus, we have studied the proton transfer from a carboxyl group to the retinal Schiff base using the condensation product of *trans*-retinal and glycine (**1f**), having an internal carboxylic acid group. This species serves as a better model for bR and visual pigments than **1e**. In TFE **1f** exhibited complete protonation of the Schiff-base bridge, which resulted in an absorption maximum of 467 nm. FT-IR spectrum showed a carboxylate band at 1630 cm^{-1}, demonstrating that the carboxyl group was responsible for Schiff-base protonation. Replacing TFE by chloroform caused deprotonation, as was evident from the absorption maximum. However, complete protonation of the Schiff base could be achieved with the strong acid HCl. In MeOH only partial protonation by the carboxyl group was observed.

The influence of nonconjugated positive charges on Schiff base protonation by a carboxyl group was studied by using the condensation products of the corresponding aldehydes with glycine (**2d**, **3c**, **4f**, **5c**, and **6c**). In TFE the Schiff base of chromophore **3c** is completely protonated by the carboxyl group of the glycine moiety at a concentration of 2×10^{-5} M despite the presence of a nonconjugated positive charge, as evident from the absorption maximum of the protonated species (436 nm). In a chromophore bearing a dimethylamino group in the vicinity of carbon 9, a mixture of protonated and nonprotonated species (**2e** and **2d**) was observed in a ratio of 6:4 in 7×10^{-4} M and 7:3 in 1.5×10^{-5} M. This concentration dependence points to ionic hydrogen-bonding stabilization by the solvent. We note that the protonated Schiff base fraction (**2e**) consists of a mixture of two species (as was deduced from the 440-nm absorption maximum), the protonated and nonprotonated dimethylamino group. The absorption maximum shifted to 423 nm upon dilution (1×10^{-5} M) and did not change further following addition of HCl. Since ionic stabilization increases with dilution, the protonated fraction also increases, leading to a larger blue shift. The mixture of species points to the mutual influence of both positively charged groups (the protonated Schiff base bridge and that of the dimethylamino group) on their pK_a values.

Almost complete protonation of Schiff base **2d** (to give **2e**) was observed in HFIP. Effective protonation of the Schiff-base bridge by a carboxyl group of chromophores **4** and **5** affording **4f** (Figure 9) and **5c** was also noted both in TFE and in HFIP. Moreover, a most remarkable effect of nonconjugated positive charge was found in **6b**, in which Schiff base protonation was absent in both TFE and HFIP. Condensation of the aldehyde derived from **6a** with glycine in TFE afforded only Schiff base **6c** alone (Figure 10) without formation of any protonated Schiff base. Protonation in this case could be achieved with addition of HCl. Thus, in contrast to **4f** in which effective Schiff-base protonation occurs in TFE, in **6c** protonation by the carboxylic acid is not observed even in the effective ion stabilizing solvent HFIP (due to the nonconjugated positive charge in the vicinity of the Schiff base linkage).

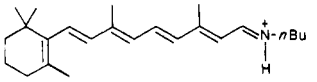
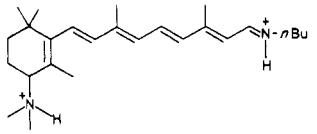
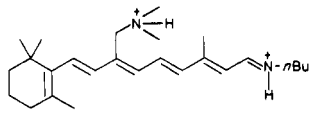
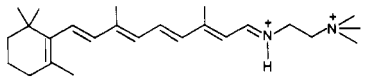
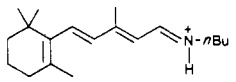
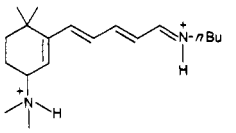
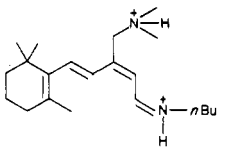
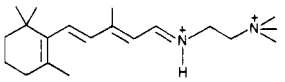
Absorption Maxima. $RSBH^+$ absorption in fluorinated alcohols is considerably red-shifted relative to that in EtOH. In TFE it absorbs at 467 nm whereas in EtOH, at 440 nm; this is despite the similar dielectric constants of the two solvents. An even greater red shift was observed in HFIP (492 nm). In all the solvents, the nature of the counterion had no effect on the absorption peak.

Nonconjugated positive charges, however, do influence the absorption maxima of protonated retinal Schiff base. We have recently demonstrated (Baasov & Sheves, 1985), that these effects are enhanced using excesss trifluoroacetic acid (TFA) (in CH_2Cl_2) as the protonating agent, probably due to a homoconjugation effect that weakens the interaction of the nonconjugated charge with its counterion and allow for strong interaction between the latter and the polyene. As outlined in Table II, large shifts in absorption maxima were observed in the protonated forms of chromophores **1**–**6** in TFE, due to nonconjugated positive charges, and further enhancement was observed with HFIP. The effects of nonconjugated positive charges on the absorption are quite comparable to those obtained with excess TFA in methylene chloride, which was stronger in magnitude than those observed in EtOH.

Discussion

Protonated Schiff Base pK_a. Warshel (1981) has shown that ion stabilization by polar groups in proteins can be of the same magnitude as solvation by water. For example, aspartate

Table II: Absorption Maxima Values[a]

	chromophore	λ_{max} (nm) EtOH[b]	TFE[b]	HFIP[b]	CH_2Cl_2 (1 equiv of TFA)[c]	CH_2Cl_2 (1 M TFA)[c]
1e		440	467	492	448	513
3b		423 (810)	431 (1800)	442 (2300)	426 (1150)	461 (2200)
2c		419 (1035)	419 (2450)	428 (3050)	423 (1320)	455 (2500)
1d		455 (−850)	508 (−1750)	536 (−1700)	468 (−950)	538 (−900)
4b		382	404	426	395	433
5d		335 (3670)	237 (5900)	331 (6740)	331 (4900)	342 (6150)
6d		391 (−600)	422 (−1050)	442 (−850)	406 (−700)	436
4e		396 (−920)	430 (−1500)	458 (−1650)	410 (−920)	450 (−870)

[a] Values in parentheses indicate difference in energy (cm^{-1}) between the corresponding chromophore and its mother compound (without the nonconjugated charge). [b] Protonation was carried out with HCl(g) (in cases in which the chromophore was not protonated by the solvent itself). [c] Protonation was carried out with 1 equiv (or 1 M concentration) of TFA; correspondingly, Schiff-base concentration was 0.5×10^{-5} M.

ion in water is stabilized by ~70 kcal/mol, while aspartate ions in proteins are stabilized by a similar amount where about 30 kcal/mol can be due to hydrogen bonding with protein groups (Russell & Warshel, 1985). In fact, Hildebrant and Stockburger (1984) suggested that ionic stabilization in bR was the result of trapped water. Similarly Rafferty and Shichi (1981) suggested that this phenomenon occurs in visual pigments. Kollman and Hayes (1981) calculated a stabilization of 29 kcal/mol of formate ion, relative to formic acid (in vacuo) by two hydrogen bonds of two molecules of water. A survey of known protein structures suggests that adjacent hydrogen donors and acceptors in the interior of proteins can form their own hydrogen bonds (Rashin & Honig, 1984). Another possible mechanism for the altered protonated Schiff base pK_a in bR was suggested by Scheiner and Hillenbrand (1985), who proposed that the angle between the acceptor and the proton donor groups are crucial for proton transfer, and changes in this angle (due to protein conformational changes) could have profound effects on such transfers. Influence of an electric field on a transfer of proton from a donor to an acceptor was proposed by Merz and Zundel (1983).

Our observations on the protonation of retinal Schiff base in TFE clearly demonstrate that the effective pK_a of the protonated Schiff base is strongly altered due to strong hydrogen bonding. The importance of hydrogen bonding is further demonstrated by the protonation of the Schiff-base nitrogen by carboxylic acid, which is very effective in trifluoroethanol and considerably weaker in EtOH and chloroform. We note that though TFE and EtOH have very similar dielectric constants (Mukherjee & Grunwald, 1958), TFE is much more effective at anion solvation. Another way of altering effective pK_a's is by introducing charges close to the Schiff-base bridge. Chromophores **1**–**6** clearly demonstrate that introducing nonconjugated positive charges in the vicinity of the retinal chromophore alters the protonated Schiff base pK_a. We note that there is no direct correlation between the influence of the positive charge on absorption maxima and on pK_a. Positively charged groups in the vicinity of carbon 9 or the ring moiety of the retinal Schiff base blue-shift the absorption maximum relative to the protonated mother compound, whereas a positive charge adjacent to the Schiff-base bridge produces a red shift. However, all these positive charges reduce the pK_a value relative to $RSBH^+$. The lack of correlation between the pK_a values and the absorption maxima arises from the fact that the latter is determined solely by the difference between the excited and ground states, whereas the former is determined by the ground-state energy difference between protonated and nonprotonated species. The positive charges destabilize the protonated species, resulting in a pK_a reduction. The distance between the charges is also crucial for the pK_a reduction. The effect is stronger as the distance is shorter, as expected for charge interactions. Thus, the

weakest interaction was observed in chromophore **3b**, whereas in **1d**, **4e**, and **6d** interaction was considerable. It is interesting to compare **4e** and **6d**. The distance of the nonconjugated positive charge from the Schiff-base nitrogen is similar in both cases. However, the influence of the positive charge in **6d** on the pK_a is stronger, contrary to its weaker influence on the absorption maxima (Tables I and II). This difference probably lies in a charge delocalization of the protonated Schiff base positive charge along the polyene that takes place in the ground state. In **6d** the nonconjugated positive charge interacts both with the positive charge delocalized along the polyene and that localized on the Schiff-base nitrogen. In **4e** the nonconjugated positive charge interacts mainly with the positive charge located on the Schiff-base nitrogen.

The effect of additional positive charge is much stronger in fluorinated alcohols than in MeOH–H_2O. The apparent pK_a of the protonated Schiff base **6d** is reduced relative to **4b** by ca. 2.5 units in MeOH–H_2O. However, in trifluoroethanol as well as in hexafluoro-2-propanol the nonconjugated positive charge has a striking effect on pK_a, and the Schiff base of **6c** cannot be protonated (Figure 10) even by a carboxylic acid. The same chromophore lacking the nonconjugated positive charge (**4a**) is protonated in trifluoroethanol alone (pK_a = 12.5). In fluorinated alcohols the counterions are solvated efficiently but not the positive charges (Evans et al., 1971; the nonconjugated, as well as the positive-charged Schiff-base nitrogen), creating "naked" positively charges which interact with each other very effectively. Thus, comparison of **6d** with 13-CF_3 protonated retinal Schiff base reveals that the nonconjugated positive charge in **6d** reduces the protonated Schiff base pK_a in HFIP by more than 5 units.

The nonconjugated dimethylamino groups are protonated by fluorinated alcohols. However, the pK_a values of their protonated species are affected by the positively charged Schiff-base nitrogen. In cases in which the intramolecular distance between the two groups is relatively large (as in **3a**), both of them are protonated (affording **3b**). In other cases such as **2c** or **5d**, the protonated Schiff base chromophore consists of a mixture of two species containing charged and noncharged dimethylamino groups as deduced from the absorption maxima.

The apparent pK_a of the protonated Schiff base of bR is 13.3 ± 0.3. We demonstrated that it is possible to raise substantially the effective pK_a's of retinal protonated Schiff bases by using solvents capable of strong hydrogen bonding. Our results strongly support the suggestion that the protonated Schiff base and its counterion in bR and visual pigments are stabilized by protein dipoles (Warshel & Barboy, 1982; Warshel et al., 1984). Stabilization by bound water molecules is also possible (Hildebrandt & Stockburger, 1984).

During the photocycle of bR_{570} and the bleaching of visual pigments deprotonation is known to take place. It was proposed that during the photocycle of bR_{570} the pK_a is shifted to a value that is below 5 (Kalisky et al., 1981). Recently we have shown in a study in which CF_3 is substituted at C_{13} (Sheves et al., 1986) that pK_a of bR is shifted by 5 units. However, the rate of formation of M_{412} (the deprotonated species in the photocycle of bR) was not changed. If proton transfer from the Schiff base was the determining factor in the overall deprotonation process, a modification in M_{412} production rate could be expected following pK_a changes. Thus, the formation of M_{412} must be controlled by another process involving changes in the protein. Our present results demonstrate that it is possible to reduce the pK_a value of bR either by eliminating hydrogen bonding, which stabilizes the pigment ions, or by introducing a positive charge in the vicinity of the Schiff-base bridge. Reduction of pK_a value can occur at an early stage of the photocycle following dislocation of the positively charged Schiff-base nitrogen from a stabilizing (hydrogen bonding) protein environment. In this case, the rate-determining step for deprotonation will involve an approaching proton acceptor group. Alternatively, the rate-determining step might involve an approaching positive charge to the vicinity of the Schiff-base linkage, which reduces the pK_a value. The possibility of reducing the pK_a by a positive charge, suggested recently by Hanamato et al. (1984) and Dupuis et al. (1985), is strongly supported by our results.

Absorption Maxima. The absorption maximum of light-adapted bR is considerably red-shifted (570 nm) relative to a protonated retinal Schiff base in EtOH (440 nm). This difference was termed the "opsin shift" (Nakanishi et al., 1980) and was attributed to a combination of factors, as described earlier. Studies on artificial pigments derived from dihydroretinals (Spudich et al., 1986), short-chain aromatic (Sheves et al., 1985), and aliphatic polyenes (Muradin-Szweykowska et al., 1984) led to the suggestion that part of the opsin shift in bR (ca. 2700 cm^{-1}) can be attributed to interaction of the charged Schiff-base nitrogen with its surrounding. Such a red shift was suggested to be introduced by separating the positively charged nitrogen from its counterion. However, our results with fluorinated alcohols demonstrate another interesting possibility. These alcohols represent a special situation due to their strong solvation of anions but relatively weak solvation of cations. This behavior differs from that in other hydrogen-bonding solvents such as ethanol in which solvation of both cation and anion occurs with equal efficiency. Thus, despite the similar (and relatively high) dielectric constant in EtOH and TFE, the positively charged Schiff-base nitrogen is less effectively solvated in TFE. This leads to a red shift due to a weaker ground-state stabilization in TFE relative to EtOH. Thus, $RSBH^+$ absorbs in TFE at 467 nm and at 492 nm in HFIP, whereas in EtOH, it absorbs at 440 nm (a shift of ca. 2400 cm^{-1} in HFIP relative to EtOH). We note that the red shift in $RSBH^+$ resulting from relatively weak hydrogen bonding with the Schiff-base nitrogen proton as in fluorinated alcohols may also apply to bR photochemically induced intermediates. The red shifts observed for the K_{610} or O_{640} intermediates in bR_{570} photocycle might be explained not only by charge separation but also by weakening of stabilizing hydrogen bonding on the positively charged nitrogen as originally suggested by Warshel & Barboy (1982). Continued counterion stabilization could also occur, thus keeping the high pK_a value for the protonated retinal Schiff base.

The fraction of the opsin shift in bR (ca. 2200 cm^{-1}) still unaccounted for probably arises from the protein environment in the vicinity of ring moiety. Two factors may contribute here: (a) a planar s-trans ring-chain conformation and (b) an ion pair in the vicinity of the β-ionone ring (Harbison et al., 1985).

We have shown that the absorption maximum of the protonated retinal Schiff base in solution is affected by interaction with nonconjugated positive charges, an effect that is strongly influenced by the interaction of these nonconjugated charges with their counterions. In nonprotic solvents such as methylene chloride, in which a bound ion pair exists, the effect of the nonconjugated positive charge located close to carbon 5 (chromophore **3b**) is of the order of 1150 cm^{-1}, whereas it is 1320 cm^{-1} in **2c** in where the positive charge is located close to carbon 9. The effect of the nonconjugated positive charge is significantly enhanced in instances where the interaction between the operating charge and its counterion is weakened

or where positive charge solvation is lowered relative to the situation in ethanol. Weakening of positive charge counterion interaction was previously demonstrated by us (Baasov & Sheves, 1985) using the homoconjugation effect of excess TFA in CH_2Cl_2. In the present studies, using fluorinated alcohols, we achieved strong anion solvation and weak solvation of both the nonconjugated positive charge and the Schiff-base linkage, resulting in a remarkable shift of the absorption maxima (2300 cm^{-1} in **3b** and 3050 cm^{-1} in **2c** in HFIP). Shifts obtained in HFIP were comparable to those observed in CH_2Cl_2 with excess TFA (Table II). It should be noted that the effect of a nonconjugated positive charge should be similar in magnitude (only the direction of influence is opposite) to a nonconjugated negative charge (Baasov & Sheves, 1985).

If one assumes that s-trans ring-chain planarity also contributes significantly to the opsin shift, one must conclude that the situation of the ion pair in the vicinity of the ring of bR is probably closer to the situation in CH_2Cl_2 by using 1 equiv of TFA (closely tied ion pair), rather than that in fluorinated alcohols or excess TFA in CH_2Cl_2. This conclusion is supported by a recent NMR evidence pointing to an ionically bound ion pair in the vicinity of the ring moiety (Harbison et al., 1985). Clarification of the relative contributions of the ion pair in the vicinity of the ring and the s-trans ring-chain planarity to the opsin shift needs further investigation.

Conclusions. (a) The high pK_a of the bR protonated Schiff base can be explained from factors operating within the Schiff-base environment alone and cannot be attributed to factors located in the vicinity of the ionone ring. This was demonstrated by studying the pK_a of an artificial pigment substituted at C_4 position of the retinal and by the raising of the effective pK_a of $RSBH^+$ by strong hydrogen bonding with the counteraion and relatively weak solvation of the positively charged Schiff-base nitrogen in TFE solution.

(b) A positive charge in the vicinity of the protonated Schiff base linkage significantly reduces the effective pK_a. The influence of the nonconjugated positive charge on the positively charged Schiff base is stronger in the case wheree both charges are weakly solvated such as in fluorinated alcohols and when the distance between the charges is smaller. A positive charge located ca. 3 Å from the Schiff-base bridge as in **6d** reduces the effective pK_a in more than 5 units in HFIP.

Thus, we have examined and supported the possibilities that the drastic pK_a reduction taking place in bR_{570} following light absorption may originate either from elimination of hydrogen-bonding stabilization or from a cation or a positive charge on a protein residue approaching the Schiff-base environment.

(c) Strong hydrogen bonding with the counterion, combined with weak solvation of the positively charged Schiff-base nitrogen relative to EtOH solution, leads to a red shift as was found in HFIP (2400 cm^{-1} relative to EtOH), despite the high dielectric constant of the solvent. Thus, part of the red shift found in bR_{570}, as well as that found in the bR photocycle intermediates, might arise from this mechanism.

Registry No. **1a**, 36076-04-7; **1b**, 103383-45-5; **1c**, 103383-46-6; **1d**, 103383-47-7; **1e**, 32798-55-3; **1f**, 103475-78-1; **2a**, 103475-79-2; **2b**, 103383-48-8; **2c**, 103383-49-9; **2d**, 103421-93-8; **3a**, 97885-65-9; **3b**, 103383-50-2; **3c**, 103383-51-3; **4a**, 73432-27-6; **4b**, 73432-17-4; **4c**, 103383-52-4; **4d**, 103421-94-9; **4e**, 103383-53-5; **4f**, 103383-54-6; **5a**, 103383-55-7; **5b**, 103383-56-8; **5c**, 103383-57-9; **5d**, 103383-58-0; **6a**, 103383-59-1; **6b**, 103383-60-4; **6c**, 103421-95-0; **6d**, 103383-61-5; HFIP, 920-66-1; TFE, 75-89-8; EtOH, 64-17-5; MeOH, 67-56-1.

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Effect of Trypsin Treatment on the Heparin- and Receptor-Binding Properties of Human Plasma Low-Density Lipoproteins†

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ABSTRACT: The effect of trypsin treatment on the heparin- and receptor-binding properties of human plasma low-density lipoproteins (LDL) was examined. LDL were treated with trypsin (2% by weight) for 16 h at 37 °C, and the trypsinized core particles (T-LDL) were isolated by gel permeation chromatography on Sepharose CL-4B. Trypsin degraded the apolipoprotein B moiety (M_r = 550 000) of LDL into numerous peptides of M_r <110 000, resulting in the release of 25% ± 5% (n = 6) of its surface-associated protein. Relative to LDL, T-LDL had an increased phospholipid/protein ratio, decreased flotation density and α-helical structure, and increased fluidity of the surface and core constituents. Compared to LDL, T-LDL showed a 60% decreased capacity to suppress [1-^{14}C]acetate incorporation into cellular sterols consistent with decreased binding to the LDL receptor. In contrast, T-LDL showed an enhanced capacity to form soluble complexes with heparin in the absence and presence of 2 mM Ca^{2+}. Between 5 and 25 mM Ca^{2+}, both LDL and T-LDL were maximally precipitated by heparin; the stoichiometry of the insoluble complexes (uronic acid/phospholipid, w/w) was 0.054 ± 0.004 and 0.055 ± 0.005 (n = 18) for LDL and T-LDL, respectively. Thus, trypsin treatment significantly diminished the lipoprotein's interaction with cells but not with heparin. This finding suggests that proteolysis may decrease receptor-mediated uptake of LDL without diminishing the lipoprotein's reactivity with acellular components of the arterial wall.

Human plasma low-density lipoproteins (LDL)[1] are spherical micellar structures containing an outer monolayer of phospholipid and protein and an inner core of neutral lipids, primarily cholesteryl esters [see Morrisett et al. (1977) for a review]. There is great interest in understanding the structure and metabolism of LDL since their plasma levels are positively correlated with risk of coronary artery disease (The Lipid Research Clinics Coronary Primary Prevention Trial Results, 1984). The cellular catabolism of LDL occurs by their binding to specific high-affinity membrane receptors followed by the internalization and degradation of both protein and lipid (Brown & Goldstein, 1986); LDL–cholesterol is a major source of sterol for cell growth, maintenance, and steroid hormone production. LDL also bind glycosaminoglycans (GAG) of the extracellular matrix (Camejo, 1982). The interaction of LDL and GAG may play an important role in cholesterol deposition in the arterial wall and, thus, in the development of atherosclerosis (Hollander, 1976; Camejo, 1982).

It is generally accepted that apolipoprotein B (apoB), the major polypeptide of LDL, mediates the binding of the lipoprotein to membrane receptors and GAG; however, limited information is available on the details of these interactions. Although certain GAG, like heparin, release LDL from their cellular receptors (Goldstein et al., 1976), the structural relationship between the regions in apoB which mediate heparin binding and receptor binding is unknown.

The purpose of the present study was to compare the heparin- and receptor-binding properties of LDL and trypsin-treated LDL in order to define a possible relationship between

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[1] Abbreviations: LDL, low-density lipoprotein(s); GAG, glycosaminoglycan(s); apoB, apolipoprotein B; PMSF, phenylmethanesulfonyl fluoride; HRH, high reactive heparin; PBS, phosphate-buffered saline; DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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