^{13}C NMR Studies of Model Compounds for Bacteriorhodopsin: Factors Affecting the Retinal Chromophore Chemical Shifts and Absorption Maximum

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Abstract: Absorption maxima and ^{13}C NMR chemical shifts were measured for retinal iminium salts. A good correlation was found between the absorption maxima and the ^{13}C chemical shifts of the retinal polyene carbons within the same solvent system. The chemical shifts of the odd-membered carbons of the polyene are affected much more than the even carbons by π-electron delocalization caused by perturbations in the Schiff base linkage vicinity. The absorption maxima of bacteriorhodopsin (bR) (568 nm) is closely mimicked by protonated Schiff base chromophores bearing ring–chain s-trans planarity and weak hydrogen bonding between their positively charged Schiff base linkage and its counteranion. These chromophores exhibit a C_5 chemical shift similar to the unusual one found in bacteriorhodopsin. These results indicate that it is possible to closely mimic the absorption maximum of bR and its C_5 chemical shift without requiring a nonconjugated negative charge in the vicinity of the retinal ring. The effect of nonconjugated positive and negative charges on the ^{13}C chemical shifts of the retinal polyene is evaluated using synthetic retinal chromophores. The charges affect the chemical shift in an alternating fashion (namely, upfield and downfield shifts) and mainly affect the double bond located in the immediate vicinity of the charge. The influence of the charge is diminished as its distance is increased. The spatial arrangement of the charge, relative to the polyene, is crucial for its effect. A symmetric C=C/charge arrangement causes only a minor change in the chemical shift, still, however, affecting the absorption maximum of the retinal protonated Schiff base. The implications of these measurements for bacteriorhodopsin are discussed.

Introduction

The discovery of bacteriorhodopsin (bR), the protein pigment of the purple membrane of the halophilic microorganism *Halobacterium halobium*,[1] had a strong impact both in the general field of bioenergetics and in the study of visual photochemistry.[2] The role of bacteriorhodopsin is to convert light energy into a proton gradient across the membrane, which is subsequently used, via a chemiosmotic mechanism, to synthesize ATP. It was found that the absorption (568 nm) of bacteriorhodopsin as well as its photobiological activity is due to an all-trans retinal chromophore, which is covalently bound to a membrane apo-protein (bacterioopsin) via a protonated Schiff base linkage with a lysine residue.[3] A similar complex was found in visual pigments which consists of 11-*cis*-retinal instead of the all-trans isomer in bR.

One of the intriguing problems in bR, as well as in visual pigments chemistry, is associated with the mechanism through which the apo-proteins regulate the absorption maxima of the various pigments. The λ_{max} of retinal protonated Schiff base (RPSB) in methanol solution is 440 nm, while in bR it is 568 nm, and in visual pigments it ranges from 420 to 600 nm.

The red shifts found in the various pigments relative to the absorption of retinal protonated Schiff base in methanol solution were defined as the opsin shifts.[4] An external point charge model was proposed to explain the opsin shift (2700 cm^{-1}) found in bovine rhodopsin.[5a] The model, which was based on a series of dihydro retinal pigments, proposed an external negative charge introduced by the protein in the vicinity of carbons 12–14 of the retinal skeleton. This negative charge red shifts the spectrum and is responsible for the opsin shift. Recently it was suggested that one negative charge serves as the counteranion and interacts with carbons C_{12}–C_{14}.[5b]

In bacteriorhodopsin (bR), the model for wavelength regulation in the retinal binding site was based on absorption data of artificial bR pigments derived from dihydro retinals[6] and on a series of solid-state NMR measurements carried out by Harbison et al.[7] who succeeded in obtaining both ^{13}C and ^{15}N chemical shifts of the retinal chromophore of bR. Thus, the considerable red shift absorption (568 nm) found in light-adapted bacteriorhodopsin (bR) relative to a retinal protonated Schiff base in methanol solution (440 nm) was attributed to a combination of three factors: (1) interactions of the positively charged Schiff base linkage with its binding site environment different from those prevailing in methanol solution, leading to a weak interaction of the positively charged Schiff base linkage with its counterion[8] (the contribution of the Schiff base environment to the red shift was deduced from the ^{15}N chemical shift of the retinal chromophore in bR,[7b,9] dihydro retinals,[6] as well as aliphatic polyene[10] and short-chain aromatic based artificial pigments[11]); (2) a ring–chain s-trans planar conformation adopted by the retinal chromophore in the bR

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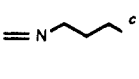
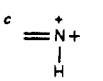
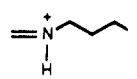
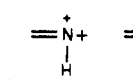
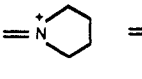
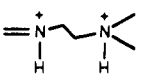
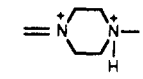
Table I. ^{13}C NMR Chemical Shifts (in ppm)[a] and Absorption Maxima of Retinal Derivatives (1) in Polar (Alcoholic) Solvents

	R	bR[b]	CHO	 [c]						
					Cl^-	Cl^-	Cl^-	ClO_4^-	Cl^-	ClO_4^-
	solvent		CD_3OD	CD_3OD	CD_3OD	CD_3OD	TFE	HFIP	TFE	HFIP
	λ_{max}(nm)	568	380	365	438	445	462	482	480	550
^{13}C-1			35.3		35.2	35.2	35.2	35.2	36.0	35.3
^{13}C-2			40.8		40.8	40.6	40.5	40.5	40.5	40.7
^{13}C-3			20.3		20.2	20.2	19.9	19.9	19.7	19.7
^{13}C-4			34.0		34.1	34.1	34.0	34.1	35.0	34.6
^{13}C-5		144.8	131.1	129.5	132.2	132.1	133.2	133.9	134.0	138.1[d]
^{13}C-6		135.4	139.0	138.7	138.9	138.7	138.7	138.7	138.6	139.1[d]
^{13}C-7		129.5	130.6[d]	128.4	132.7	132.4	134.4	135.3	135.5	140.2
^{13}C-8		132.7	138.7	138.4	138.4	138.5	138.1	138.0	137.6	137.7
^{13}C-9		146.4	142.4	138.4	146.5	146.3	148.9	150.5	151.3	158.3
^{13}C-10		133.0	130.8[d]	130.9	130.9	131.1	130.4	130.5	130.5	131.8
^{13}C-11		139.1	134.4	128.4	139.4	139.4	141.2	142.4	143.6	148.9
^{13}C-12		134.3	135.8	136.5	134.8	134.9	133.8	134.1	133.8	134.4
^{13}C-13		169.0	157.9	146.6	166.1	166.2	168.0	169.5	172.5	177.6
^{13}C-14		122.0	129.7	129.6	120.4	120.4	119.4	117.9	119.2	115.0
^{13}C-15		163.2	193.3	161.4	162.3	167.2	160.6	159.1	167.2	161.6
^{13}C-16			29.4		29.4	29.5	29.1	29.2	28.9	29.0
^{13}C-18		22.0	22.0		22.0	22.2	21.8	21.9	21.5	21.8
^{13}C-19		11.3	13.0		13.2	13.3	12.8	13.1	12.7	13.2
^{13}C-20		13.3	13.2		14.3	14.5	13.8	13.9	14.1	14.2

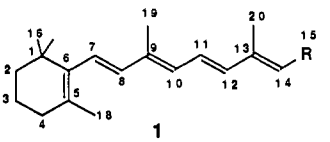
[a] Relative to solvent peak as an internal standard. [b] Taken from ref 7a. [c] Taken from ref 14b, ±0.1 ppm. [d] Assignment may be inverted. [e] TFE = trifluoroethanol; HFIP = hexafluoro-2-propanol.

binding site, rather than twisted s-cis conformation energetically favored in solution (the ring–chain planar conformation was suggested first by Schreckenbach et al.,[12a] and the s-trans conformation was deduced from ^{13}C NMR chemical shifts of bR as well as T_1 measurements[7a] and supported by studies with locked s-cis and s-trans retinals which were incorporated into bR[12b]); (3) an ion pair introduced by the protein which includes a negative charge in the vicinity of C_5 and a positive charge near C_7. The suggestion of a negative charge was based mainly on the unusual ^{13}C NMR chemical shift of C_5, which was shifted considerably downfield relative to retinal protonated Schiff base in solution or in the solid state.[7] The negative charge in the vicinity of C_5 further red shifts the absorption maximum of bR due to stabilization of the excited state. The presence of a positive charge in the vicinity of C_7 was deduced mainly from C_7 chemical shift,[7a] as well as from the absorption maxima data of dihydro-retinal-based pigments.[6] We note in this respect that two photon studies of the chromophore binding site of bacteriorhodopsin carried out recently are not consistent with charged species near the chromophore ring, but are consistent with polar species in the vicinity of the ring.[13]

The interpretation of the ^{13}C NMR data is clearly crucial for understanding the interactions of the retinal chromophore with its binding site environment. NMR measurements of retinal protonated Schiff bases in solution[14] and in the solid state,[15] as well as calculations of chemical shifts,[16] were used to study the effect of various factors on the chemical shifts. However, no experimental data, which take into account the various factors that influence bR absorption maximum, were available. In this study, we carried out series of ^{13}C NMR and absorption measurements of various retinal analogues, evaluating the effects of Schiff base environment alterations, ring–chain s-trans planarity, and nonconjugated positive and negative charges. Our results indicate that the C_5 chemical shift of retinal protonated Schiff base is influenced by the Schiff base environment and that the nonconjugated charge effect is dependent on the spatial arrangement between the latter and the double bond. The results support the possibility that the major factors regulating the absorption maximum of bR are weak interaction of the positively charged nitrogen with its counterion and ring–chain s-trans planarity.

Results

A. Schiff Base Environment Perturbations in Retinal Protonated Schiff Base. The effect of Schiff base perturbation on the ^{13}C NMR chemical shifts of retinal protonated Schiff base (RPSB, 1) was evaluated by carrying out a series of ^{13}C NMR measurements of RPSB with absorption maxima ranging from 440 nm to 550 nm. The alterations in the absorption maxima were achieved by using Schiff base environment perturbations. It was found that the absorption maximum of RPSB can be red shifted by counterion substitution[8a] or by introducing a positive charge in the vicinity of the Schiff base linkage.[8b] Alternatively, a significant shift is introduced by using fluorinated alcohols as solvents due to their weak hydrogen bonding with the positively charged Schiff base linkages.[17] The results are summarized in Table I for polar (alcoholic) solvents and Table II for a nonpolar solvent (e.g., chloroform).



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Most of the data were obtained using *tert*-butylamine (to form the Schiff base linkage) in order to prevent anti–syn isomerization around the C=N bond.[18] The RPSB with *tert*-butylamine is stable as the anti isomer, whereas *n*-butylamine leads to a mixture of syn and anti. As indicated in Table I, the Schiff base substitution affects mainly the chemical shift of carbon-15, which is shifted downfield by ca. 5 ppm in the case of *n*-butyl.

The results of the olefinic carbons can be analyzed in terms of a correlation between the absorption maxima and the ^{13}C NMR chemical shifts of the different chromophores. The chemical shifts of the even-numbered carbons 6, 8, 10, and 12 were found to be relatively insensitive to changes in the absorption maxima (the

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Table II. ^{13}C NMR Chemical Shifts (in ppm) and Absorption Maxima of Retinal Derivatives (1) in a Nonpolar (Chloroform) Solvent

	R	CHO	=N[b]	=N+H Cl⁻[c]	=N+H Cl⁻	=N+H TFA[d]	=N+H IO_4^-	=N+ ClO_4^-	=N+H TFA[d]
	λ_{max}(nm)	380	366	436	455	470	476	480	484
^{13}C-1		34.3	34.3	35.0	34.1	34.2	34.3	34.3	34.3
^{13}C-2		39.6	39.7	40.4	39.3	39.5	39.6	39.6	39.7
^{13}C-3		19.2	19.2	19.9	18.9	19.0	19.1	19.0	19.1
^{13}C-4		33.2	33.1	33.8	33.1	33.2	33.3	33.3	33.4
^{13}C-5		130.5	129.7	132.0	131.7	132.1	132.3	132.2	132.8
^{13}C-6		137.6	137.8	138.0	137.2	137.4	137.4	137.5	137.5
^{13}C-7		129.3	127.8	131.9	131.8	132.5[e]	132.7	132.8	132.9[e]
^{13}C-8		137.0	137.5	137.8	136.7	136.7	136.6	136.6	136.6
^{13}C-9		141.3	137.9	145.2	145.3	146.5	146.8	147.0	148.1
^{13}C-10		129.7	130.1	130.7	129.4	129.5	129.6	129.6	129.6
^{13}C-11		132.5	127.8	137.8	137.5	138.7	139.0	139.3	140.3
^{13}C-12		134.5	136.1	134.9	133.5	133.2[e]	133.3	133.7	133.5[e]
^{13}C-13		154.8	143.9	162.9	162.3	164.3	164.8	166.6	166.1
^{13}C-14		128.9	129.6	120.7	119.6	119.2	119.3	118.0	118.6
^{13}C-15		191.0	159.4	160.8	158.3	158.8	158.8	160.1	158.8
^{13}C-16		29.0	29.0	29.3	28.9	28.9	29.0	28.9	28.9
^{13}C-18		21.7	21.8	22.0	21.8	21.7	21.8	21.9	21.8
^{13}C-19		13.0	12.8	13.3	13.2	13.1	13.3	13.3	13.2
^{13}C-20		13.1	13.0	14.2	14.3	14.0	14.7	14.5	14.1

[a] Relative to solvent peak as an internal standard. [b] Taken from ref 13a. [c] In CD_3CN. [d] 1 M TFA solution (λ_{max} = 484, measured in CD_2Cl_2). There is chromophore concentration dependence of the UV and NMR. [e] Assignment may be inverted.

results are described in Figure 1). The measurements can clearly be divided into two groups. One includes model compounds measured in polar solvents (methanol and fluorinated alcohols) and the second, measurements in a nonpolar one (chloroform). The difference observed is due to the effect of the solvent, which causes a downfield shift of ca. 2 ppm in methanol in the even-numbered carbons.

The chemical shifts of the odd-numbered carbons are much more sensitive to alteration in the absorption maxima. A red shift, due to perturbation in the Schiff base region (weakening of hydrogen bonding due to counterion alteration, different solvation, or introduction of a positive charge in the vicinity of the Schiff base), is reflected in π-electron redistribution along the polyene. The effect is especially pronounced in the odd-numbered carbons which carry a partial positive charge. Thus, a longer wavelength is accompanied by a downfield shift of all the odd-numbered carbons (except C_{15}) in the ^{13}C NMR spectrum (Figure 2). A very good correlation was found between the absorption maxima and the chemical shifts of each carbon in the different RPSB analogues. The correlation was found in each group of solvents (chloroform as a nonpolar, nonprotic solvent and methanol and fluorinated alcohols representing polar solvents). The polar solvents shift the ^{13}C chemical shifts further downfield, due to their dipoles, and the effect is more pronounced in carbons bearing higher positive charge density.

It should be noted that the correlation between the absorption maxima and the chemical shifts exists in each solvent group, even though the absorption is altered using different factors in the Schiff base vicinity (counterions, solvent solvation capability, nonconjugated positive charges, and Schiff base linkage substitution). In fact, even most of the aldehyde and Schiff base carbons fit into the linear correlation.

Absorption alteration caused by Schiff base environment perturbations affects mainly the chemical shifts of the odd-numbered carbons that are closer to the Schiff base linkage, and less the odd-numbered carbons that are distant from the linkage. Thus, the ranges of the chemical shifts for carbons 13, 11, 9, 7, and 5 (in protonated Schiff bases) were 11.5, 9.5, 11.8, 7.5, and 5.9 ppm, respectively (absorption maximum changes from 440 to 550 nm in protic solvents). This trend is also clearly deduced from the line slopes summarized in Table III. The effect on the chemical shift of C_9 is exceptionally strong, probably due to its methyl substitution, which stabilizes the positive charge at C_9. The chemical shift of C_5 is less sensitive to absorption maximum alterations than the other carbons, but, still, a significant red shift

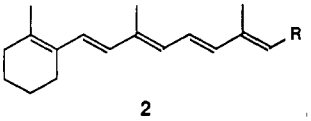
Table III. Slopes and Correlation Factors for Figure 2

carbon	polar solvents slope (correlation factor)	chloroform slope (correlation factor)
13	9.5 (0.97)	7.0 (0.96)
11	11.5 (0.99)	11.6 (0.97)
9	9.2 (0.99)	11.7 (0.96)
7	14.4 (0.99)	26.0 (0.99)
5	18.5 (0.99)	31.6 (0.90)

(up to 550 nm) is accompanied by a 5.9-ppm downfield shift.

Retinal protonated Schiff base adopts a ring–chain twisted s-cis conformation in methanol solution. It is reasonable to assume that the model compounds described in Tables I and II adopt a similar conformation. This assumption is supported by the similar chemical shifts observed for C_8, $C_{16,17}$, and C_{18} for all the derivatives. Furthermore, we have carried out ^1H NMR NOE measurements on two chromophores: RPSB with IO_4^- serving as a counterion in methylene chloride and a retinal protonated Schiff base bearing a second positive charge at the Schiff base vicinity in methylene chloride, absorbing at 476 nm and 520 nm, respectively. In both cases, the results indicated s-cis conformation. Irradiation of 16,17-H caused an increase (10–12%) of the 7-H intensity and only 2–5% of the 8-H intensity. 18-H irradiation led to an increase (ca. 7%) of 8-H intensity, but only a negligible (less than 2%) effect on the 7-H.

B. Schiff Base Environment Perturbation in Retinal Analogues Bearing a Ring–Chain s-Trans Planar Conformation. It was proposed that in bR the retinal chromophore adopts a ring–chain s-trans planar conformation.[7a] This coplanar conformation can affect π-electron distribution along the polyene chain, causing alterations in ^{13}C NMR chemical shifts, relative to twisted s-cis conformation prevailing in the retinal chromophore in solution. To check this possibility, we synthesized two chromophores, **2**[19] and **3**. Chromophore **2** serves as a very good model for bR



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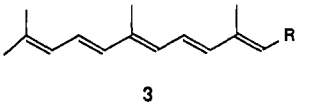


3

chromophore since it lacks the 1,1-dimethyl substitution, thus

(19) Friedman, N.; Sheves, M.; Ottolenghi, M. *J. Am. Chem. Soc.* **1989**, *111*, 3203.

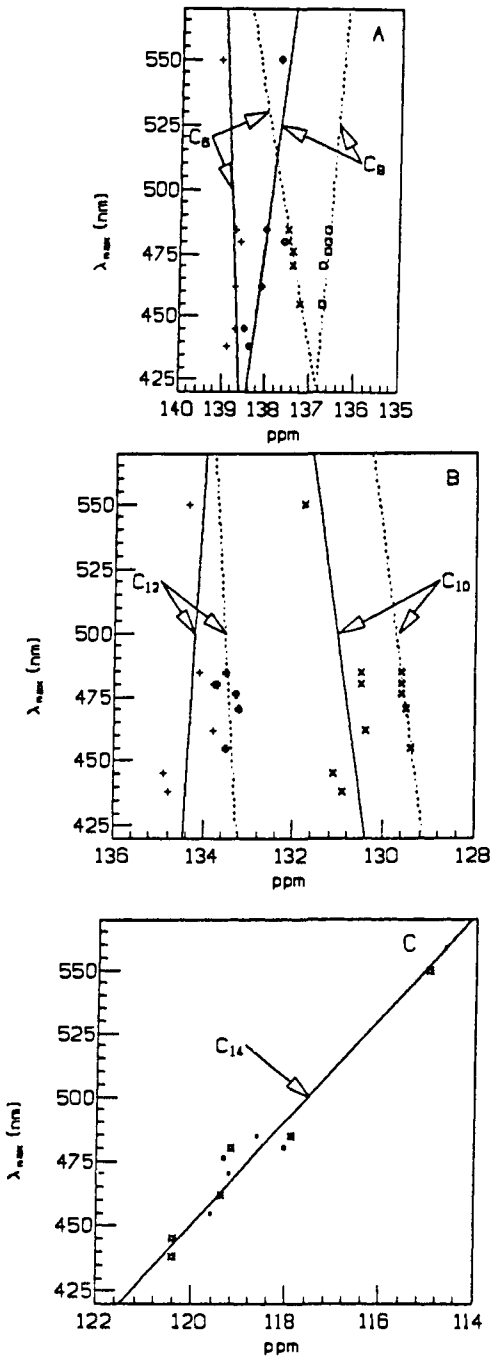


Figure 1. Correlation between the ^{13}C NMR chemical shifts of the even-numbered carbons and the absorption maxima of different retinal iminium salt derivatives: (—) polar (alcoholic) solvents system; (---) nonpolar and nonprotic (chloroform) solvent system.

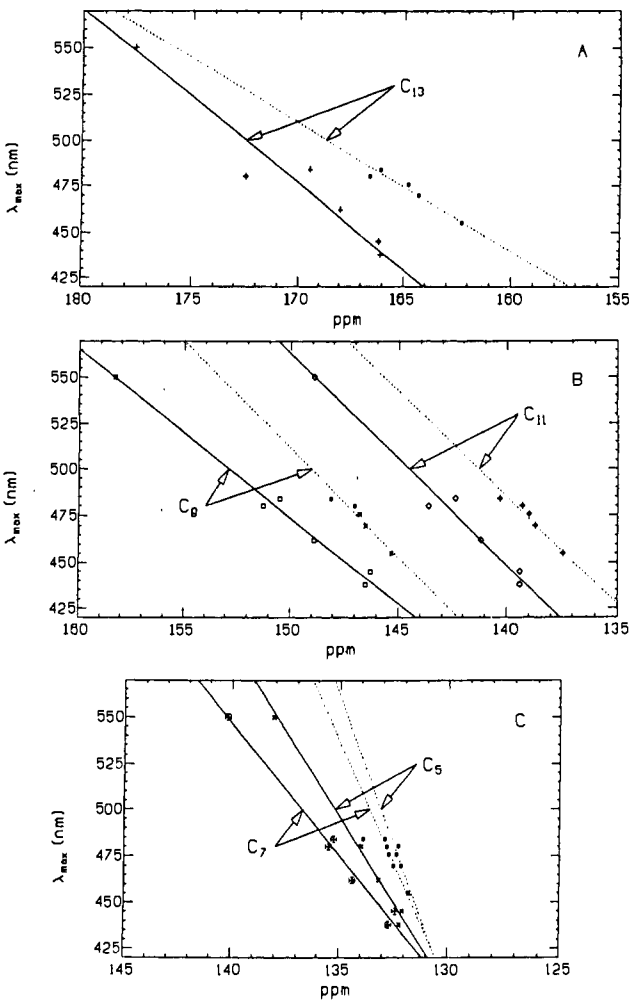


Figure 2. Correlation between the ^{13}C NMR chemical shifts of the odd-numbered carbons and the absorption maxima of different retinal iminium salt derivatives: (—) polar (alcoholic) solvents system; (---) nonpolar and nonprotic (chloroform) solvent system.

allowing the chromophore to adopt an s-trans conformation in solution, while its polyene skeleton is similar to the bR chromophore. The s-trans conformation was established by a ^1H NMR NOE experiment. Chromophore **3** lacks the retinal ring structure and adopts an s-trans conformation in solution as well.

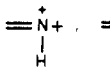
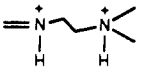
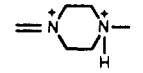
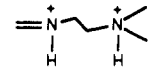
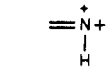
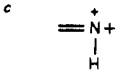
Perturbations in the Schiff base environment of the protonated Schiff bases of chromophores **2** and **3**, which weakened Schiff base–counterion interactions, and/or the presence of a positive charge in the vicinity of the Schiff base linkage led to significant red shifts in the absorption maxima. The absorption shifted up to 574 nm in chromophore **2** bearing a nonconjugated positive charge in the vicinity of the Schiff base in hexafluoroisopropanol (HFIP). Excess TFA in chloroform or methylene chloride red shifts the spectrum as well. The red shift depends on chromophore concentration, and 10^{-5} M *n*-butylamine Schiff base of **2** in 1 M TFA absorbs at 560 nm. The resonance Raman spectrum of this species reveals that the C=N stretching frequency appears at 1636 cm^{-1}. It has been proposed[20] that the C=N stretching frequency is sensitive to the degree of hydrogen bonding between the environment and the N–H bond. Therefore, the absorption maxima are correlated with the C=N stretching frequency in systems modified in their Schiff base–counterion interactions. Since the C=N stretching mode in bR appears at 1640 cm^{-1}, it is concluded that in chromophore **2**, which absorbs at 560 nm, the hydrogen bonding to N–H is somewhat weaker than that prevailing in bR.

The ^{13}C NMR chemical shifts of the derivatives of chromophores **2** and **3** are summarized in Table IV (for polar solvents) and in Table V (for nonpolar solvents). The aliphatic carbon chemical shifts are insensitive to the different perturbations in the Schiff base environment. The chemical shifts of the even-numbered carbons of the polyene are similar to those observed in the retinal chromophores, except for C_6 and C_8, which are upfield shifted in the s-trans planar chromophores, due to the different conformation and the modified ring structure. In addition, similarly to the results obtained for the retinal chromophore, the chemical shifts in the polar alcoholic solvents are 2–3 ppm downfield shifted, relative to chloroform solution.

Interesting data are obtained for the odd-numbered carbons of the polyene. Figure 3 represents the correlation between ^{13}C NMR chemical shifts of the odd-numbered carbons and the absorption maxima. The results clearly indicate high sensitivity of the odd-numbered carbons chemical shifts to π-electron delo-

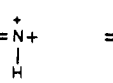
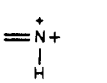
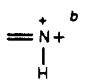
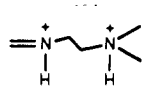
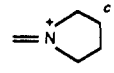
(20) Baasov, T.; Friedman, N.; Sheves, M. *Biochemistry* **1987**, *26*, 3210.

Table IV. ^{13}C NMR Chemical Shifts (in ppm)[a] and Absorption Maxima of Coplanar Retinal Derivatives (**2** and **3**) in Polar (Alcoholic) Solvents

	R	bR[b]	CHO					 [c]	 [c]
				Cl^-	Cl^-	ClO_4^-	Cl^-	TFA[d]	TFA[d]
	solvent		CD_3OD	CD_3OD	TFE	TFE	HFIP	CD_3OD	HFIP
	λ_{max}(nm)	568	400	471	526	548	574	470	500
^{13}C-1			26.6	26.5	26.0	26.0	26.2		
^{13}C-2			23.9	23.8	23.2	23.2	23.2		
^{13}C-3			24.9	23.9	23.4	23.4	23.4		
^{13}C-4			34.3	34.4	34.3	34.4	34.7	26.6	26.1
^{13}C-5		144.8	136.9	138.7	142.5	143.7	145.7	140.7	144.7
^{13}C-6		135.4	129.3	129.6	129.6	129.8	130.2	127.2	126.5
^{13}C-7		129.5	130.3	132.2	135.1	136.2	137.5	130.7	132.6
^{13}C-8		132.7	130.7[e]	130.5[e]	130.0[e]	130.1	130.2	135.2	134.9
^{13}C-9		146.4	143.4	147.6	153.2	155.3	157.3	147.2	151.0
^{13}C-10		133.0	131.1[e]	131.2[e]	131.2[e]	131.6	132.0	131.2	131.3
^{13}C-11		139.1	134.6	139.5	144.4	146.0	147.4	139.5	142.7
^{13}C-12		134.3	135.4	134.5	133.6	134.2	133.8	134.6	133.8
^{13}C-13		169.0	158.1	166.2	172.9	175.4	175.3	166.0	170.1
^{13}C-14		122.0	129.5	120.2	118.9	115.6	117.0	120.3	119.0
^{13}C-15		163.2	193.3	162.1	166.6	162.8	164.4	162.1	160.0
^{13}C-18		22.0	19.6	19.8	19.2	19.4	19.4	18.8	18.3
^{13}C-19		11.3	13.2	13.4	13.0	13.1	13.2	13.3	12.9
^{13}C-20		13.3	13.2	14.5	14.0	14.2	14.2	14.1	13.9

[a] Relative to solvent peak as an internal standard. [b] Taken from ref 7a. [c] Derived from aldehyde **3**. The rest are aldehyde **2** derivatives. [d] 1 M TFA solution. [e] Assignment may be inverted.

Table V. ^{13}C NMR Chemical Shifts (in ppm)[a] and Absorption Maxima of Coplanar Retinal Derivatives (**2** and **3**) in a Nonpolar Solvent (Chloroform)

	R	CHO			 [b]		CHO[c]	 [c]
			Cl^-	TFA[d]	TFA[d]	TFA[d]		ClO_4^-
	λ_{max}(nm)	400	470	512	530	540	400	494
^{13}C-1		25.4	25.2	25.3	25.8	27.1		
^{13}C-2		22.7	22.4	22.6	23.1	22.4		
^{13}C-3		22.7	22.5	22.7	23.2	22.5		
^{13}C-4		33.4	33.4	33.8	34.8	35.8	26.5	26.7
^{13}C-5		136.8	138.4	140.6	141.8	142.1	138.4	141.1
^{13}C-6		128.1	128.2	128.7	129.2	128.8	125.6	125.8
^{13}C-7		129.2	130.9	132.4	134.2	134.4	127.5	130.3
^{13}C-8		129.3[e]	129.1[e]	129.2[e]	129.5	129.1	134.0	133.8[e]
^{13}C-9		142.0	145.7	149.7	151.4	152.4	141.5	147.3
^{13}C-10		129.7[e]	129.8[e]	130.1[e]	130.5	130.4	129.7	130.0[e]
^{13}C-11		132.8	137.1	141.0	142.5	143.5	132.6	139.2
^{13}C-12		134.1	133.3	132.2	132.7	132.4	134.3	133.6
^{13}C-13		155.2	161.8	166.5	168.7	171.1	155.2	166.0
^{13}C-14		128.8	119.9	118.0	118.3	117.4	128.9	118.0
^{13}C-15		191.3	158.0	158.1	159.1	164.9	191.3	159.4
^{13}C-18		19.7	19.6	19.9	19.8	19.9	18.7	18.9
^{13}C-19		13.2	13.3	13.5	13.7	13.6	13.2	13.5
^{13}C-20		13.1	14.0	14.3	14.7	13.9	13.0	14.2

[a] Relative to solvent peak as an internal standard. [b] Spectrum was taken in methylene chloride. [c] Derived from aldehyde **3**. The rest are aldehyde **2** derivatives. [d] 1 M TFA solution. [e] Assignment may be inverted.

Table VI. Slopes and Correlation Factors for Figure 3

carbon	polar solvents slope (correlation factor)	chloroform slope (correlation factor)
13	10.2 (0.98)	8.3 (0.98)
11	12.9 (0.99)	10.9 (0.99)
9	10.3 (0.99)	10.1 (0.99)
7[a]	19.4 (0.99)	19.2 (0.97)
5[a]	14.9 (0.99)	18.4 (0.99)

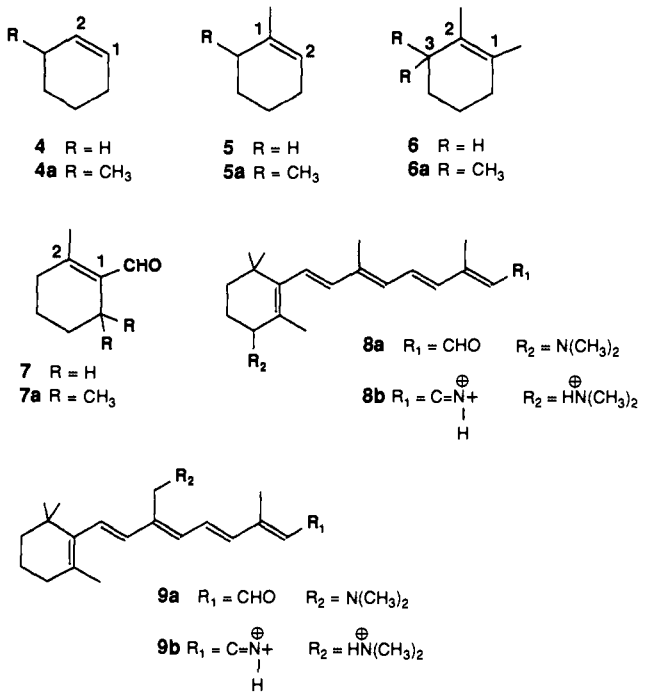
[a] Only derivatives of chromophore **2** were considered.

calization and a very good linear correlation with the absorption maxima.

Similarly to the retinal series, the correlation holds both in chloroform (nonpolar) and in protic polar solvents, where the former is slightly upfield shifted, due to solvent effect. The result for C_5 is significantly different from that obtained in the retinal series. Due to higher conjugation with the polyene in the planar chromophores, the chemical shift of C_5 is considerably downfield shifted. In addition, the slope of the graph representing the linear correlation (Table VI) is smaller than that observed for the retinal series, indicating a stronger effect of π-electron delocalization in the polyene on C_5 chemical shift, due to perturbations in the Schiff base vicinity.

It should be noted that the species absorbing at 548 nm and 574 nm exhibit C_5 chemical shifts at 143.7 and 145.7 ppm, respectively (Table IV), whereas in bR, a chemical shift of 144.8 ppm was observed. However, in contrast to the similar chemical shifts of C_5 in bR and the model compound, C_7, C_9, and C_{11} chemical shifts are considerably downfield shifted in the model compounds (absorbing around 550 nm) relative to bR.

In contrast to native bR chromophore, chromophore **2** lacks the 1,1-*gem*-dimethyl substitution. To evaluate the effect of the substitution on the chemical shifts of C_5 and C_6, we examined the chemical shifts of simple model compounds (**4**–**7**) bearing different substitutions. The results, summarized in Table VII,



clearly indicate that the allylic substitution of cyclohexene considerably affects (ca. 6.5 ppm downfield) the chemical shift of its closest olefinic carbon, but exhibits a negligible effect on the other olefinic carbon (C_6 and C_5 of retinal, respectively).

C. Effect of Nonconjugated Positive Charges along the Retinal Polyene. It has been proposed that nonconjugated charges introduced by the protein in the vicinity of the retinal skeleton affect the absorption maxima of retinal proteins. In bacteriorhodopsin, the presence of an ion pair in the vicinity of the retinal ring and C_7 of the polyene was postulated.[6,7]

To analyze the effects of nonconjugated positive charges on the ^{13}C NMR chemical shifts of protonated Schiff bases, we examined compounds **8** and **9**, bearing positive charges in the vicinity of C_4 and C_9, respectively.

It was demonstrated previously[8b] that the positive charges blue shift the absorption maximum of these compounds and that their influence increases as hydrogen bonding to the charges decreases. The chemical shifts of chromophore **8** and **9** and their protonated Schiff bases bearing nonconjugated positive charges are summarized in Table VIII. Comparison of the chemical shifts to the corresponding retinal derivatives (summarized in Tables I and II) clearly indicates that the positive charges influence the chemical shifts of the polyene in an alternate way. Namely, the odd-numbered carbons are upfield shifted, whereas the even-numbered carbons are shifted downfield. Figure 4, which graphically illustrates the influence of the positive charges, reveals that the nonconjugated charge in the vicinity of carbon 4 mainly influences the C_5=C_6 double bond and the effect diminishes as the distance between the charge and the double bond increases. The positive charge attached to carbon 19 influences significantly double bonds C_9=C_{10} and C_{11}=C_{12} and, to a lesser extent, the bonds C_5=C_6, C_7=C_8, and C_{13}=C_{14}.

Comparing the polyene bearing nonconjugated positive charges with native retinal, one should consider the substitution effect. The effect can be estimated by comparing native retinal chemical shifts with those of chromophores **8a** and **9a** bearing amino groups at C_4 and C_9.

In compound **8a** (Table VIII), C_5 chemical shift is 0.8 ppm, downfield, whereas C_6 chemical shift is 4.7 ppm upfield, relative to the corresponding chemical shifts in retinal (Table II) due to the substitution. Thus (taking into account the substitution influence), the actual effect of the nonconjugated positive charge (obtained by acidification of the amino group) on double bond C_5=C_6 is approximately 13 ppm upfield on C_5 and 9.4 ppm downfield on C_6. In chromophore **9a**, the substitution shifts carbons C_9 and C_{10} by 3.5 ppm upfield and by 3.2 ppm downfield, respectively, leading to the conclusion that in chromophore **9b** the positive charge affects carbons 9 and 10 by 7.2 upfield and 9.7 ppm downfield, respectively.

The experiments described above clearly indicate that a nonconjugated positive charge significantly influences the chemical shifts of retinal protonated Schiff base. The effect is much stronger than that observed for aliphatic carbons, probably due to the polarization effect on the double bonds. The observation raised the possibility that the spatial location of the charge, relative to

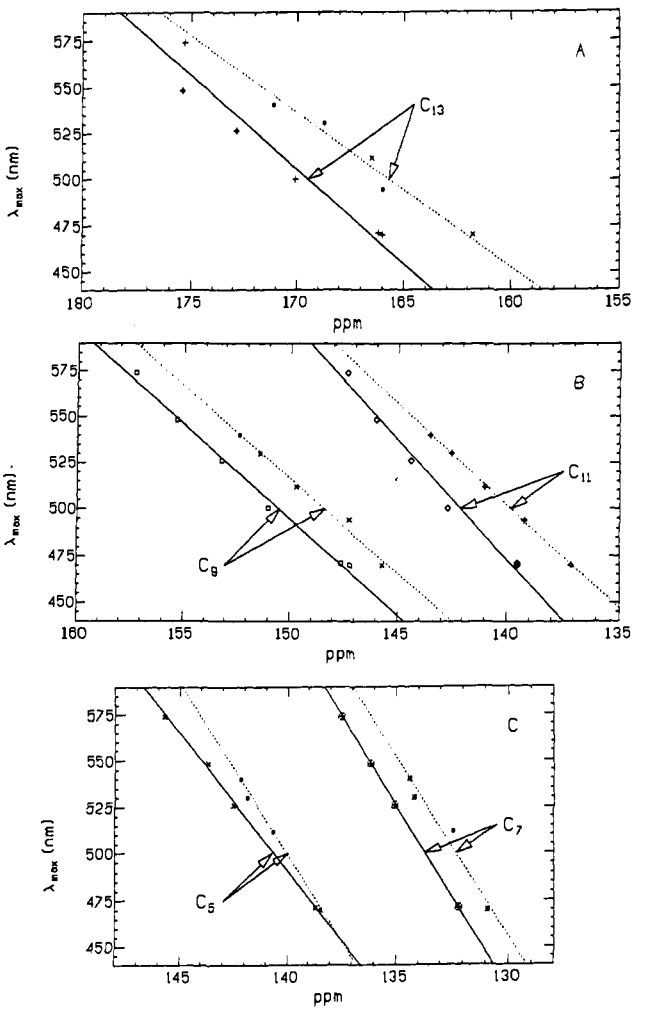


Figure 3. Correlation between the ^{13}C NMR chemical shifts of the odd-numbered carbons and the absorption maxima of different retinal iminium salt of derivatives retinal analogue **2**: (—) polar (alcoholic) solvents system; (---) nonpolar and nonprotic (chloroform) solvent system.

Table VII. ^{13}C Chemical Shifts of Substituted Cyclohexanes

compound	chemical shifts (ppm)[a] C-1	C-2
4[b]	127.3	127.3
4a[c]	126.6	134.0
5[b]	134.0	121.3
5a[b]	138.0	121.8
6[b]	125.6	125.6
6a[d]	125.0	132.0
7[d]	133.4	156.6
7a[e]	140.6	155.9

[a] In $CDCl_3$. [b] Taken from ref 21a. [c] Taken from ref 21b. [d] Taken from ref 21c. [e] This work.

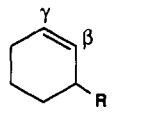
(21) (a) Senok, Y.; Suda, H.; Ishiyama, J.; Imaiyami, S. *Tetrahedron Lett.* **1976**, *23*, 1983. (b) Kalimowski, H.; Berger, S.; Braun, S. *^{13}C NMR Spectroskopie*; George Thiem Verlag: Stuttgart, 1984; p 123. (c) Mitchell, T. R. B. Private communication.

Table VIII. ^{13}C NMR Chemical Shifts for Amino Retinal Derivatives **8** and **9**

	4-Me_2N-retinal (**8**)			19-Me_2N-retinal (**9**)		
R_1	CHO	$=\overset{+}{N}+H$ Cl^-	$\overset{+}{N}+H$ Cl^-	CHO	$=\overset{+}{N}+H$ Cl^-	$=\overset{+}{N}+H$ Cl^-
R_2	$N(CH_3)_2$	$HN(CH_3)_2$	$HN(CH_3)_2$	$N(CH_3)_2$	$HN(CH_3)_2$	$HN(CH_3)_2$
solvent	$CDCl_3$	CD_3OD	TFE	$CDCl_3$	CD_3OD	$CDCl_3$
λ_{max}(nm)	382	420	430	389	416	423
^{13}C-1	34.4	35.9	35.6	34.3	35.3	34.3
^{13}C-2	37.1	36.3	35.5	39.6	40.8	39.2
^{13}C-3	15.0	18.0	17.6	19.2	20.1	18.9
^{13}C-4	64.3	67.6	68.9	33.2	34.3	33.1
^{13}C-5	131.3	122.8	121.0	130.9	133.7	132.5
^{13}C-6	142.3	151.6	152.8	137.5	138.8	137.0
^{13}C-7	129.6	129.9	130.6	130.5	133.5	131.6
^{13}C-8	137.7	141.1	141.0	135.4	135.5	135.2
^{13}C-9	141.0	144.9	146.1	137.8	135.8	135.2
^{13}C-10	129.8	132.8	132.3	132.9	137.4	135.6
^{13}C-11	132.4	138.6	139.8	131.7	136.3	134.5
^{13}C-12	134.7	136.1	135.3	136.9	139.7	137.9
^{13}C-13	155.0	165.6	167.5	154.4	164.9	160.1
^{13}C-14	129.1	121.0	120.2	129.8	122.5	122.0
^{13}C-15	191.3	162.5	160.9	191.1	163.0	158.0
^{13}C-16[a]	27.6	27.5	26.8	29.0	29.4	29.0
^{13}C-17[a]	29.5	29.4	28.6	29.0	29.4	29.0
^{13}C-18	19.2	19.0	18.5	21.8	22.3	22.0
^{13}C-19	13.1	13.1	12.5	54.3	52.4	51.7
^{13}C-20	13.1	14.3	13.7	13.2	14.8	15.0

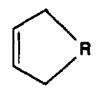
[a] Arbitrarily assigned.

the double bond, is crucial for its influence. To gain further insight into this effect, we studied chromophores **10–17** bearing nonconjugated positive charges in various locations relative to a double bond.



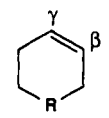
10a R = $N(CH_3)_2$

10b R = $\overset{\oplus}{H}N(CH_3)_2$



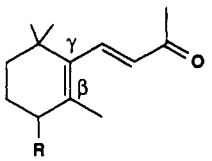
11a R = NH

11b R = $\overset{\oplus}{N}H_2$



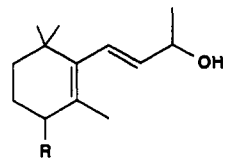
12a R = NH

12b R = $\overset{\oplus}{N}H_2$



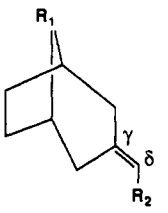
13a R = $N(CH_3)_2$

13b R = $\overset{\oplus}{H}N(CH_3)_2$



14a R = $N(CH_3)_2$

14b R = $\overset{\oplus}{H}N(CH_3)_2$

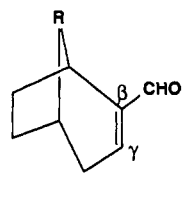


15a R_1 = N-CH_3 R_2 = CN

15b R_1 = $\overset{\oplus}{H}NCH_3$ R_2 = CN

16a R_1 = N-CH_3 R_2 = CHO

16b R_1 = $\overset{\oplus}{H}NCH_3$ R_2 = CHO



17a R = N-CH_3

17b R = $\overset{\oplus}{H}NCH_3$

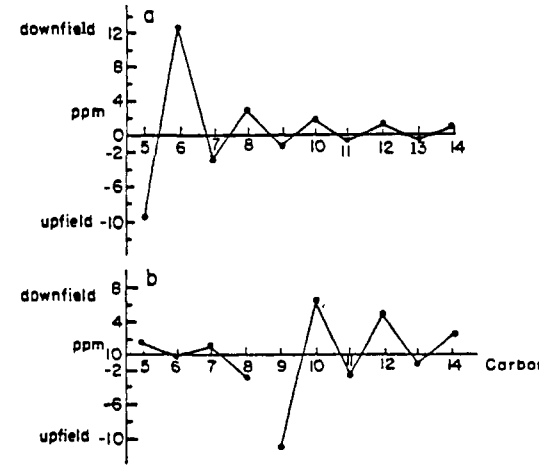


Figure 4. The effect (in methanol) of nonconjugated positive charges on ^{13}C NMR chemical shifts by comparison with retinal protonated Schiff base. The numbers presented are without substitution correction. Δ ppm versus carbon number for (a) 4-dimethylamino derivative **8b**; (b) 19-dimethylamino derivative **9b**.

In compound **10**, the amino group is aligned approximately parallel to the long axis of the double bond, enabling strong polarization of the π-electrons, due to the presence of the positive charge (following protonation of the amino group). Accordingly, a significant effect of 8.8 and 6.5 ppm upfield and downfield for β and γ carbons ^{13}C NMR chemical shifts was observed (see Table IX). In **11**, the positive charge is located symmetrically between the two vinylic carbons, thus upfield shifting the two carbons by 3.9 ppm without polarizing the double bond. Compound **12** represents an intermediate situation in which the positive charge is aligned closer to one vinylic carbon, causing shifts of −4.6 and 1.3 ppm (upfield and downfield, respectively).[22] The polarization effect is further demonstrated in longer polyenes, such as **13** and **14**. Similarly to **10**, a significant effect was observed (Table IX). The effect was especially large in **13**, probably due to initially

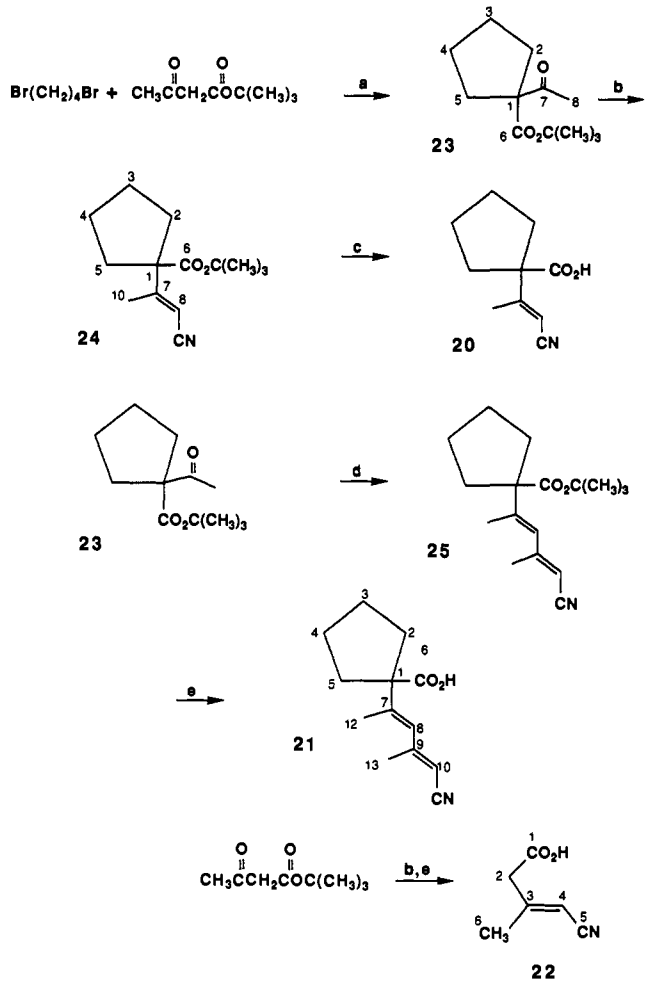
(22) Gottlieb, H.; Cheung, H. *J. Chem. Res.* (*S*) **1979**, 370.

Table IX. ^{13}C Chemical Shifts (ppm)[a] for Compounds **10–17**

compd	^{13}C-β[b]	^{13}C-γ[b]
10a	129.1	129.9
10b	120.3 (−8.8)	136.4 (6.5)
11a[c]	128.6	128.6
11b[c]	124.7 (−3.9)	124.7 (−3.9)
12a[c]	125.3	124.3
12b[c]	120.7 (−4.6)	125.6 (1.3)
13a	140.0	136.1
13b	123.5 (−16.5)	147.7 (11.6)
14a	130.2	141.4
14b	120.9 (−9.3)	149.5 (8.1)
15a	163.3	96.2
15b	154.3 (−9.0)	101.0 (4.8)
16a	162.0	129.5
16b	151.9 (−10.1)	131.4 (1.9)
17a	144.4	147.0
17b	141.4 (−3.0)	144.7 (−2.3)

[a] Spectra were taken in $CDCl_3$, solvent resonance as reference. The difference between protonated and nonprotonated species is in parentheses. [b] For convenience, C-β and C-γ are defined as the double bond carbons relative to the amine group. In **15–16**, β and γ are the γ and δ carbons, correspondingly. [c] Taken from ref 22.

Scheme I

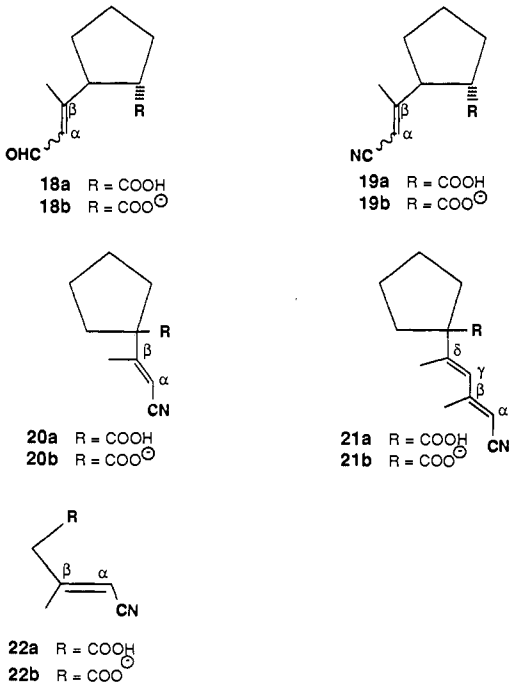


[a] NaOEt/EtOH, reflux, 12 h. [b] $(EtO)_2POCH_2CN$/NaH/THF, 25 °C, 12 h. [c] KOH/MeOH, 60 °C, 4 h. [d] $(EtO)_2POCH_2C(CH_3)$ = CHCN/THF, 25 °C, 12 h. [e] CF_3COOH/CH_2Cl_2, 25 °C, 30 min.

partial polarization of the double bond caused by the electron-withdrawing ketone group.

To gain further insight into the effect of the relative charge/double bond geometrical arrangement on the chemical shifts, we studied compounds **15–17**. In **15** and **16**, the amino group is aligned approximately parallel to the long axis of the chromophore, whereas in **17** it is aligned close to the middle of the double bond. It is clearly revealed that both **15a** and **16a** experience large shifts in the ^{13}C NMR chemical shifts, following protonation of the bridgehead nitrogen. The shifts are comparable to those obtained for compound **10** despite the γ nitrogen substitution to the double bond in **15** and **16** relative to the β position in **10**, establishing the through-space effect of the positive charge on the chemical shifts of a double bond. The similar effects exclude the possibility of a through-bond effect. The effect of the positive charge position, relative to the double bond, was further checked in compound **17**. In the latter, the nitrogen is located above the plane of the conjugated system almost symmetrically to the double bond. It is clearly evident in this case that the effect of the positive charge on the chemical shifts of the double bond carbons is significantly decreased, relative to compounds **15** and **16**, in keeping with the shifts observed in compound **11**.

D. Effect of a Nonconjugated Negative Charge. It has been suggested that a nonconjugated negative charge in the vicinity of the retinal ring moiety introduced by the protein affects the absorption maximum of bR, as well as the ^{13}C NMR chemical shift of C_5.[6,7a] To analyze the effect of a negative charge on the chemical shifts of polyenes, we synthesized chromophores **18–22**. Chromophores **18** and **19** were prepared as previously described.[23]



The syntheses of **20–22** are described in Scheme I. The nonconjugated negative charge was introduced into the corresponding compound by addition of triethylamine, and the effect of the charge on the chemical shifts was evaluated by comparison to the mother compound.

Compounds **18** and **19** bear a nonconjugated carboxyl group at a distance of ca. 3 Å from the double bond and arranged approximately symmetrically to the bond. In contrast, the negative charge in chromophores **20** and **21** is aligned closer to one edge of the double bond. The different arrangement of the charge relative to the polyene in these chromophores allows for evaluation of the charge influence on the ^{13}C NMR chemical shifts. The effect of the nonconjugated negative charge introduced into the system following addition of triethylamine can be evaluated from Table X.

It is clearly evident that the negative charge affects the chemical shifts of the polyenic carbons. The effect is characterized by an alternating pattern in which the olefinic β and δ carbons are shifted downfield, whereas α and γ carbons are shifted upfield. In addition, it appears that the effect of a carboxylate anion on the chemical shift is generally considerably smaller than that observed

(23) Sheves, M.; Nakanishi, K. *J. Am. Chem. Soc.* **1983**, *105*, 4033.

Table X. ^{13}C Chemical Shift (ppm) of Compounds **18–22** Bearing Nonconjugated Charges[a]

compound	^{13}C-β[b]	^{13}C-α[b]	^{13}CN[c]	^{13}C-γ	^{13}C-δ
18a	164.58; 164.41	126.99; 130.34	191.82; 190.83		
18b	167.64; 168.38	126.39; 129.69	191.66; 191.63		
	(2.94); (3.97)	(−0.6); (−0.65)	(−0.16); (0.8)		
19a	164.52; 161.28	96.29; 98.20	117.47; 116.67		
19b	167.80; 164.04	93.89; 95.78	118.78; 117.65		
	(3.28); (2.76)	(−2.40); (−2.42)	(1.31); (0.98)		
20a	163.60	96.87	116.79		
20b	168.97	93.30	118.26		
	(5.37)	(−3.57)	(1.47)		
21	158.36; 159.49	97.85; 97.38	117.50; 117.08	125.40; 123.50	143.90; 143.70
21b	159.39; 160.40	96.10; 95.80	118.37; 117.66	122.12; 120.62	149.49; 149.11
	(1.03); (0.91)	(−1.75); (−1.58)	(0.87); (0.58)	(−3.32); (−2.88)	(5.59); (5.41)
22a	156.32	99.63	116.06		
22b	161.47	96.18	117.26		
	(5.15)	(−3.55)	(1.20)		

[a] The effect of the negative charge (in comparison with the noncharged molecule) is in parentheses. [b] For convenience, C-α and C-β are defined as the carbons of the double bonds. Two values correspond to two isomers (cis and trans). [c] For compound **18**, the ^{13}C chemical shift is for the aldehyde group.

for iminium salts (ca. 5 ppm and above 10 ppm, respectively), although similar distances are assumed for both charges from the polyene in the various systems.

Discussion

A. Schiff Base Vicinity Perturbations. The chemical shifts of the even-numbered carbons of retinal protonated Schiff base polyenes are not sensitive to alterations in the absorption maxima originating from perturbations in the Schiff base linkage vicinity. However, a very good correlation was established between the ^{13}C NMR chemical shifts of the odd-numbered carbons and the absorption maxima altered by different Schiff base environments within a similar solvent system. Weak electrostatic interaction between the positively charged Schiff base linkage and its counteranion, introduction of a positive charge close to the Schiff base linkage, or weaker interaction with solvent dipoles (for example, fluorinated alcohols relative to methanol) all increase π-electron delocalization along the polyene. The π-electron delocalization is more pronounced in the odd-numbered carbons affecting their ^{13}C NMR chemical shifts. The effect diminishes with distance from the Schiff base in keeping with previously measured NMR data[14] and with recent X-ray structure of a retinal iminium salt.[24] It is interesting to note, in this respect, that the chemical shift of C_9 indicates an increased positive charge density, following π-electron delocalization along the chain, relative to carbon 11, even though it is located further down in the polyene chain. The effect can be attributed to C_9 methyl substitution, which stabilizes a positive charge at C_9. The C_5 chemical shift of the retinal polyene is affected by only ca. 1 ppm (131.7 to 132.8 ppm) by changing the absorption from 455 nm to 484 nm in chloroform. The effect is somewhat increased in polar solvents (2 ppm), probably due to positive charge stabilization by the solvent dipoles. The effect on C_5 of π-electron delocalization is smaller than that on C_7 despite methyl substitution in the former. This phenomenon originates from the ring/polyene chain nonplanarity which prevents effective conjugation with the $C_5{=}C_6$ double bond, reducing the effect on the C_5 chemical shift due to π-electron delocalization.

A significant effect on C_5 chemical shift was observed by introducing a positive charge in the vicinity of the Schiff base linkage in hexafluoroisopropanol, red shifting the absorption maximum up to 550 nm. Due to electrostatic interaction, the positive charge in the vicinity of the Schiff base linkage repulses the positive charge localized primarily on C_{13} and C_{15},[14c] leading to effective π-electron delocalization along the polyene chain. The effect is further enhanced by the fluorinated alcohol environment which allows for effective interaction between the two positive charges due to weak solvation in these solvents and efficient solvation of the counterions. The strong π-electron delocalization produces the usual red shifted absorption and shifts the C_5 chemical shift to 138 ppm (effect of ca. 5 ppm).

A significant difference was observed between nonpolar and polar (alcoholic) solvents. In the latter, all the vinylic carbons were shifted downfield probably due to interaction with solvent dipoles.[25a] In addition, an interesting effect was observed in carbons 7 and 5. The effect of the polar solvents was enhanced following red shifting the absorption maximum. The observation can be attributed to stabilization of the positive charge by the solvent dipoles as π-electron delocalization along the chain increases, inducing migration of the positive charge to carbons 7 and 5.

B. Influence of Ring–Chain s-Trans Planarity. Since it has been suggested that in bR the retinal chromophore adopts a ring–chain s-trans conformation,[7a] it is most interesting to compare the retinal chromophore to compounds **2** and **3**. As was shown by a ^1H NMR NOE experiment, **2** adopts, in solution, an s-trans planar conformation due to the lack of 1,1-dimethyl substitution, preventing steric interactions which prevail in the retinal chromophore. Similarly, s-trans conformation is adopted by compound **3**. The s-trans planar conformation red shifts the absorption maximum of the protonated Schiff base relative to the corresponding retinal chromophore. The magnitude of the red shift depends on the degree of π-electron delocalization along the polyene. For example, in methanol solution, the red shift is ca. 1500 cm^{-1} (absorptions of 440 nm and 471 nm for retinal protonated Schiff base and the protonated Schiff base of **2**, respectively), whereas in chloroform containing 1 M TFA a red shift of ca. 2000 cm^{-1} was detected (505 nm and 560 nm, respectively). The s-trans planar conformation induces red shifts mainly due to excited-state stabilization. The effect is enhanced in a more π-electron delocalized system, probably due to the increased migration of the positive charge toward the additional conjugated double bond. The additional conjugation does not significantly alter the positive charge density at carbons close to the Schiff base linkage in the ground state. The latter conclusion is deduced from comparison of the ^{13}C NMR chemical shifts of the retinal chromophore and chromophores **2** and **3** bearing an s-trans planar conformation. The chemical shifts of carbons close to the Schiff base linkage are similar even though the absorptions of **2** and **3** are red shifted relative to retinal protonated Schiff base. However, an important effect is observed at carbon 5. The chemical shift is downfield shifted significantly, due to increased positive charge density, especially in systems bearing increased π-electron delocalization. In addition, the slope of the graph (for C_5) representing the linear correlation between the absorption and the chemical shift is smaller in the planar chromophores, indicating a strong π-electron delocalization effect on C_5 chemical shift. It is interesting to compare C_5 and C_7 in the retinal and coplanar systems. In retinal, C_7 is affected more strongly by π-electron delocalization

(24) Santarsiero, B.; James, M.; Mahendran, M.; Childs, R. *J. Am. Chem. Soc.* **1990**, *112*, 9416.

(25) (a) Sakurai, M.; Ando, I.; Inone, Y.; Chujo, A. *Photochem. Photobiol.* **1981**, *34*, 367. (b) Nanasawa, H.; Kamogawa, H. *Chem. Lett.* **1983**, 1367.

than C_5. However, in the coplanar systems, the opposite effect is observed, due to the conjugation of the C_5=C_6 bond with the polyene system. We note that chromophore **2** lacks the 1,1-dimethyl substituents existing in the retinal chromophore. However, these substituents have a minor effect on the chemical shift of C_5 and a significant effect (about 6.5 ppm) on C_6. Therefore, it is legitimate to compare the effects on C_5 chemical shifts observed in the coplanar chromophore to those of bR.

C. Interactions with Nonconjugated Charges along the Polyene. Electrostatic interactions between nonconjugated charges in the vicinity of the ring moiety of the retinal chromophore and the retinal polyene affect the absorption maximum of protonated Schiff base. A positive charge causes a blue shift in the spectrum, due to excited-state destabilization. The present study indicates that the ground state is affected, as well, by a nonconjugated positive charge located in the vicinity of carbons 5 or 9. The effect is significant at close carbons and decreases rapidly along with increasing distance from the charge. In addition, it shows an alternating pattern; namely, the odd-numbered carbons shift upfield and the even-numbered carbons downfield. This observation is in keeping with a decrease in partial positive charge on the odd-numbered carbons and increasing positive charge on the even-numbered carbons. We note that the effect of the positive charge in chromophore **9b** is mainly on the C_9–N moiety of the polyene. This is probably due to the C_9 substituent conformation placing the nonconjugated charge closer to the C_9=C_{10} bond. The nonconjugated positive charge is more effective in solvents that do not solvate the charge effectively. Thus, in fluorinated alcohols, which are known to solvate negative charges, but only weakly positive charges,[17] the effect of nonconjugated positive charge on the ^{13}C NMR chemical shifts is stronger. This effect correlates with that observed on the absorption maximum.

The present results clearly demonstrate that the exact spatial location of the nonconjugated positive charge, relative to the double bond, is crucial for its influence on the ^{13}C NMR chemical shifts. A charge located along the axis of the double bond has a significant effect, due to polarization of the bond and repulsion of the positive charge from the closest carbon, due to electrostatic interaction. However, the effect is diminished if the charge is located symmetrically to both carbons of the double bond, since it interacts equally with both carbons preventing significant polarization of the bond.

A nonconjugated negative charge has an opposite effect on the chemical shifts. Namely, it induces a downfield shift at the odd-numbered carbons and an upfield shift at the even-numbered carbons. Similar effects were found previously[25b] by measuring the effect of a negative charge on a β-ionone skeleton. The exact location of the negative charge, relative to the double bond, is important for its influence. A nonsymmetrical arrangement around the double bond causes a stronger effect, due to an enhanced polarization effect.

It is interesting to note that a positive charge affects the chemical shift more strongly than a negative charge. Although the influence of a negative charge was not measured on protonated Schiff base or iminium salts (due to rapid decomposition), this conclusion can be derived from comparison of the influence of both charges on aldehydes, ketones, and nitriles. Clarification of this effect needs further study. Finally, it should be noted that the charge–double bond arrangement does not influence the absorption maxima which is only affected by the charge distance.

Implications for Bacteriorhodopsin. Accumulated evidence indicates that a significant part (ca. 3000 cm^{-1}) of the opsin shift observed in bR is attributed to a weak electrostatic interaction between the positively charged Schiff base linkage and its counterion.[6,7a,8,11] The C=N stretching frequency serves as a good probe for the hydrogen bonding strength of the N–H moiety with its counterion, or dipoles in its environment. Thus, the low (1640 cm^{-1}) C=N stretching frequency found in bacteriorhodopsin[26] indicates weak hydrogen bonding to the N–H moiety[20,27] and is in keeping with ^{15}N NMR data.[7c,28] Weak Schiff base/counterion electrostatic interaction can be produced by increased distance.[8a] However, strong hydrogen bonding of protein dipoles or residual water with the counterion can introduce a similar effect.[8b] In fact, weak hydrogen bonding with the positively charged Schiff base linkage and strong hydrogen bonding with the counterion were previously suggested[17] to account for bR protonated Schiff base stabilization and raising its pK_a[29] and to account for bR spectroscopic properties. This suggestion is supported by the recent bR electron density map based on electron diffraction by Henderson et al.,[30] indicating a strong hydrogen bonding network to Asp 212, which is probably part of the Schiff base environment. The negative charge of Asp 85, which is located in the vicinity of the protonated Schiff base as well, might also contribute to the high pK_a. This possibility is supported by studies with model systems in solution indicating that a positive charge in the vicinity of a retinal protonated Schiff base reduces its pK_a by ca. 5 units,[17b] suggesting that a negative charge will introduce an opposite effect. This possibility was recently supported by studies with model compounds carried out in our laboratory. In addition, it was proposed that electrostatic interaction of the Schiff base linkage with a positive charge is associated with the deprotonation process occurring following light absorption.[31a] In this respect, we note that replacement of Asp 85 by Gly significantly reduced the pK_a of the protonated Schiff base.[31b] This effect can be attributed both to elimination of the stabilizing effect of Asp 85 negative charge and/or to Schiff base exposure to the positively charged Arg 82, which was shielded by Asp 85 in the native system.

The present studies reveal a high resemblance between the absorption maximum of bR and a model compound in solution using a chromophore bearing a ring–chain s-trans planar conformation and weak Schiff base–counterion interaction enforced by strong hydrogen bonding to the counterion. The red shifted absorption maximum can be achieved also by just increasing the distance between the positively charged Schiff base linkage and its counterion. However, it is necessary to invoke stabilization of the counterion with hydrogen bonding or with additional positive charge in the vicinity to account for the high pK_a observed for bacteriorhodopsin protonated Schiff base. In addition, strong interaction of the Schiff base linkage with other dipoles will prevent a red shift in the absorption as it is observed, for example, in methanol. In this case, the Schiff base–solvent dipoles interaction stabilizes the ground state and prevents a red shift in the spectrum, although a weak Schiff base–counterion interaction takes place. Thus, the combination of the above-described effects in the model compound produces spectroscopic parameters which are very similar to bR: namely, absorption maximum of 550 nm, C=N stretching frequency of 1640 cm^{-1}, and C_5 chemical shift of ca. 143 ppm. These observations are in keeping with the conclusion that the ring–chain s-trans planar conformation red shifts the absorption maximum (relative to twisted s-cis conformation) more significantly in a weak Schiff base–counterion interaction system (ca. 2000 cm^{-1} relative to ca. 1500 cm^{-1} in a Schiff base–counterion system bearing a strong interaction). The above-described retinal analogue indicates that it is possible to closely mimic the absorption maximum of bR and its C_5 chemical shift without requiring a nonconjugated negative charge in the vicinity of the ring. Moreover, if such a charge is present in bR, it should be located

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(27) (a) Gilson, R. S.; Honig, B.; Croteau, A.; Zarrilli, G.; Nakanishi, K. *Biophys. J.* **1988**, *53*, 261. (b) Smith, S.; Pardoen, J.; Mulder, P.; Curry, B.; Lugtenburg, J.; Mathies, R. *Biochemistry* **1983**, *22*, 6141. (c) Ganter, U.; Gartner, W.; Siebert, F. *Biochemistry* **1988**, *27*, 7480. (d) Deng, H.; Callender, R. *Biochemistry* **1987**, *26*, 7418.

(28) De-Groot, H.; Harbison, G.; Herzfeld, J.; Griffin, R. *Biochemistry* **1989**, *28*, 3346.

(29) Sheves, M.; Albeck, A.; Friedman, N.; Ottolenghi, M. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3262.

(30) Henderson, R.; Baldwin, J.; Caska, T.; Zemlin, F.; Beckman, E.; Downing, K. *J. Mol. Biol.* **1990**, *213*, 899.

(31) (a) Hanamoto, J.; Dupuis, S.; El-Sayed, M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1083. (b) Otto, H.; Marti, T.; Holz, M.; Mogi, T.; Stern, L.; Engel, F.; Khorana, H.; Heyn, M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1018.

symmetrically above the C_5=C_6 bond to avoid a significant effect on the C_5 chemical shift. The absence of a shift at C_1 and C_4 in bR, relative to model compounds, does not support the presence of a negative charge either. In addition, FTIR studies indicate that Asp 115, the closest (ca. 10 Å) potential negative charge to the retinal ring according to the recent bR structure,[30] is protonated in the bR ground state.[32] In this respect, we note that studies with an artificial pigment bearing a fluorescent probe substituting the β-ionone ring did not detect a negative charge in its vicinity and indicated a low value of electric field at the probe site.[33] Two proton studies of bacteriorhodopsin support this conclusion as well.[13] Our studies indicated that ^{13}C NMR chemical shifts of the retinal polyene in solution are affected by solvent dipoles. Therefore, the presence of a protein dipole in the retinal ring vicinity that would have a small effect on the chemical shift of C_5 and on the absorption maximum cannot be completely ruled out at present.

The chemical shifts of the C_7–C_{11} moiety of the polyene skeleton of the protonated Schiff base of chromophore **2**, absorbing around 550 nm, are quite different from those observed in bR. The odd-numbered carbons are significantly downfield shifted (especially carbons 9 and 11), whereas the even-numbered carbons are shifted upfield. The effect is even more pronounced when bR chemical shifts are compared to model systems in polar solvents. One possibility to account for this difference is to assume an interaction of the polyene with a nonconjugated positive charge located in the vicinity of C_7–C_9 as suggested previously.[6,7a] The positive charge should affect the ^{13}C NMR but should have a negligible influence on the absorption maximum to maintain the bR red shifted absorption. The present studies reveal that a positive charge, located ca. 3 Å from the polyene, will significantly affect the ^{13}C NMR chemical shift (more than 10 ppm in the closest carbon), in an alternating fashion, but it will blue shift the absorption maximum as well.[8b] To prevent this influence, the nonconjugated positive charge should be located at a larger distance from the polyene. Further studies should be performed to check this point. In addition, in the structure suggested for bR,[30] there is no obvious potential positive charge in the immediate vicinity of C_7–C_9 of the polyene. Another possibility to account for the protein effect on the chemical shift is to invoke an interaction between the polyene and a tryptophan residue. It was suggested that at least two tryptophan residues[30,34] are in close contact with the retinal polyene. Such an interaction with the aromatic core might influence the chemical shift. Finally, since it was observed that a significant solvent effect is present, it is possible that a very nonpolar environment in the vicinity of carbons C_9–C_{11} induces upfield shifts in the chemical shifts. At present it is impossible to distinguish between these possibilities.

The chemical shifts of the carbons close to the Schiff base linkage are influenced significantly by other factors besides π-electron delocalization. The chemical shifts of C_{15} and C_{14} are affected by ca. 2 and 1 ppm, downfield and upfield, respectively, following a red shift of ca. 50 nm in the absorption. However, they are more sensitive to the type of group attached to the Schiff base nitrogen. For example, the *n*-butyl group shifts the chemical shift of C_{15} downfield by 5 ppm, relative to the *tert*-butyl group. This effect might be attributed to the exact location of the counterion relative to the C=N bond or to a γ effect introduced by the *tert*-butyl group. In addition, it was shown that the C_{14} chemical shift is sensitive to C=N bond configuration (syn or anti).[35] Thus, the changes in C_{14} observed in our model compounds may be originated from the type of group attached to Schiff base linkage. The chemical shift of C_{13} is affected strongly by π-electron delocalization (as is evident from the model systems studies), and also by dipoles located around it. Quite a different C_{13} chemical shift was observed for light adapted bR (568) and for its 13-cis dark adapted component (bR_{548}).[7a,b] This difference (164.8 versus 168.7 ppm) cannot be explained by their absorption maxima. Since different C_{13} chemical shifts were observed by us in chloroform and polar solvents in species that absorb similarly, we suggest that different dipole arrangements around C_{13} prevailing in the dark and light adapted forms are responsible for the corresponding chemical shift change. In this respect, we note that tryptophans experience different environment in the light and dark adapted forms,[36a] in addition to different protonation or hydrogen bonding degree found in a tyrosine residue[36b-e] located in the vicinity of the Schiff base linkage.

Acidic Form of bR. Acidification of bR suspension leads to reversible spectroscopic change associated with acid modification absorbing at 605 nm (bR_{605}). This transition exhibits an apparent pK_a value of 2.9.[1,37] It was later demonstrated that the transition to bR_{605} is markedly affected by deionization of the membrane suspensions.[38] Removal of bR-bound divalent cations causes an equilibrium shift to bR_{605}. The spectroscopic transition attracted considerable attention since it may bear on the chromophore–protein interactions. It was proposed[37,39-41] that the formation of bR_{605} is associated with protonation of the negative counterion of the Schiff base, or by a protein conformational change inducing an increased Schiff base counterion separation.[37,41,42]

More recently, it was demonstrated, using artificial bR pigments, that the bR_{605} transition is due to changes in polyene–opsin interactions in the vicinity of the Schiff base.[43] Bacteriorhodopsin mutants in which Asp 85 was replaced by alanine or asparagine have red shifted chromophores at pH 6 with λ_{max} at 610 and 590 nm, indicating the importance of the Schiff base environment in probing red shifted absorptions.[44]

^{13}C NMR experiments of bR_{605}[9] indicated that in this species the C_5 chemical shift is further downfield shifted from 144.8 ppm in bR_{568} to 148.8 ppm in bR_{605}. Our present studies with model compounds demonstrate that weakening Schiff base/counterion interaction in a chromophore bearing a ring–chain s-trans planarity shifts downfield the chemical shift of C_5. These results support the suggestion that the red shift observed in bR_{605} relative to bR_{568} is due to weakening electrostatic interactions of the Schiff base linkage with its environment.[9]

Conclusions

The present studies indicate that ^{13}C NMR chemical shifts of the odd-numbered carbons of retinal protonated Schiff base are highly sensitive to charge delocalization along the retinal polyene and that a linear correlation exists between such shifts and the absorption maxima within the same solvent system. Weakening

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the Schiff base linkage interaction with the counterion does not substantially affect the $^{13}C_5$ NMR chemical shift in retinal protonated Schiff base. However, in a chromophore bearing ring–chain s-trans planarity, the $^{13}C_5$ NMR chemical shift is affected significantly following weakening of Schiff base/counterion interaction. Weakening of Schiff base/counterion interaction either by increasing the distance between the two charges and/or by strong hydrogen bonding to the counterion leads to a significant π-electron delocalization along the polyene. Since the C_5=C_6 double bond is conjugated to the system in the s-trans planar chromophore, the C_5 chemical shift is shifted downfield. We note that in order to obtain the strong π-electron delocalization, it is necessary (in addition to diminished Schiff base/counterion interaction) to prevent strong electrostatic interaction of the positively charged Schiff base linkage with dipoles in its vicinity. Therefore, in a highly polar leveling environment (like methanol, for example) that solvates the counterion, enhanced π-electron delocalization is not observed due to strong interaction of the Schiff base linkage with solvent dipoles. Thus, the present studies indicate that it is possible to closely mimic the spectroscopic properties of bR (absorption, C_5 NMR chemical shift, and C=N stretching frequency) in solution without requiring a nonconjugated negative charge in the vicinity of the retinal ring. It is concluded that the dominant factors contributing to the red shift observed in bR are the weak Schiff base/counterion interaction as well as weak electrostatic interactions of the Schiff base linkage with dipoles in its vicinity, and ring–chain s-trans planarity. The latter conformation contributes to the red shift more than was previously assumed for protonated retinal Schiff base, if π-electron delocalization is enhanced by weak electrostatic interaction between the positively charged Schiff base linkage and its counterion. The present studies also analyze the influence of nonconjugated positive and negative charges on the chemical shifts of the polyene carbons, and reveal that the influence of such a charge depends on the spatial arrangement of the charge relative to the double bond. A symmetric C=C/charge conformation causes only a minor change in the chemical shift, but still substantially affects the absorption maximum of the chromophore.

The model systems studies support the previous suggestion that a perturbation in the vicinity of carbons 7–9 is necessary to explain the ^{13}C data of bR. A positive charge is a possibility, but its distance from the polyene should be more than 3 Å. Other possibilities like an interaction with a tryptophan residue or a very nonpolar environment in the vicinity of these carbons cannot be excluded.

Experimental Section

Absorption spectra were carried out using an 8452A Hewlett-Packard diode array spectrophotometer. NMR spectra were recorded on a Bruker AM-300 instrument, at 300.12 (^1H) and 75.47 (^{13}C) MHz, respectively. Chemical shifts, all in the δ scale were referenced either to internal TMS or to the carbon, or the residual protons, of the solvent. The assignment of the proton spectra was established by decoupling experiments. Following that, protonated carbons could be correlated to the proton shifts via a series of off-resonance decoupled spectra (also known as Birdsall plots[46]). Finally, nonprotonated carbons were identified by comparisons with closely related compounds. Chromatographies were performed using a flash column technique with Merck silica gel 60 (230–400 mesh ASTM) with solvents mentioned. Chromophores **2**,[19] **3**,[29], **7**,[19] **7a**,[45] **8**,[18] **9**,[18] **13–17**,[18] and **18–19**[23] were prepared as previously described.

Carboxycyclopentane Ester 23. *tert*-Butyl acetoacetate (24.5 ml, 0.148 mol) was added dropwise at 25 °C to a solution of sodium ethoxide in ethanol (prepared from 3.5 g of sodium and 80 mL of absolute ethanol). 1,4-Dibromobutane (10.5 mL, 0.088 mol) was added dropwise to the stirred solution and the mixture was refluxed for 12 h, followed by extraction with water and ether. The product (13.3 g, 0.052 mol) was distilled at 75 °C (0.5 mmHg). ^1H NMR ($CDCl_3$) δ 1.45 (s, 9, *t*-Bu), 1.62 (m, 8, methylenes 2–5), 2.15 (s, 3, 8-H).

Carboxycyclopentane Nitrile 24. Ester **23** (500 mg, 2.36 mmol) was reacted with the sodium salt of diethyl phosphonoacetonitrile (830 mg, 4.7 mmol) in 10 mL of dry THF under argon atmosphere at 25 °C for 12 h. Extraction with water and ether, followed by chromatography with ether–hexane (3:7), gave 438 mg (2.11 mmol) of **24**. ^1H NMR ($CDCl_3$) δ 1.42 (s, 9, *t*-Bu), 1.66 (m, 8, methylenes 2–5), 2.06 (d, J = 0.8, 3, 10-H), 5.27 (q, J = 0.8, 1, 8-H).

Carboxycyclopentane Nitrile 20. Nitrile **24** (100 mg, 0.48 mmol) was dissolved in a solution of 5% KOH in methanol and stirred at 60 °C for 4 h. The reaction mixture was extracted with water and ether. The water layer was acidified with hydrochloric acid. Extraction with ether gave 32 mg (0.179 mmol) of **20**: UV (CH_2Cl_2) λ_{max} 234 (ϵ 14000). ^1H NMR ($CDCl_3$) δ 1.72 (m, 8, methylenes 2–5), 2.06 (d, J = 0.8, 3, 10-H), 5.34 (q, J = 0.8, 1, 8-H).

Carboxycyclopentane Nitrile 21. Ester **23** (500 mg, 2.9 mmol) was reacted with the sodium salt of triethyl-3-methyl-4-phosphonocrotononitrile (3.5 mmol) in 15 mL of dry THF at 25 °C under argon atmosphere. After 12 h, water was added and the mixture extracted twice with ether. Usual workup gave 120 mg of crude **25**, which was dissolved in a mixture of 10 ml of CH_2Cl_2 and 50 mg of trifluoroacetic acid. Solvent evaporation and chromatography with ether–hexane (1:1) gave 100 mg (0.456 mmol) of **21** as a mixture of two isomers (all-trans and 9-cis): UV (CH_2Cl_2) λ_{max} 270 (ϵ 17000). ^1H NMR ($CDCl_3$) δ (all-trans) 1.70 (m, 8, methylenes 2–5), 1.91 (d, J = 2, 3, 12-H), 2.17 (s, 3, 13-H), 5.16 (s, 1, 10-H), 5.93 (s, 1, 8-H); (9-cis) 1.50 (m, 8, methylenes 2–5), 1.87 (d, J = 2, 3, 12-H), 2.03 (s, 3, 13-H), 5.08 (s, 1, 8-H), 5.24 (s, 1, 10-H).

3-Methyl-4-cyano-3-butenoic Acid 22. *tert*-Butyl acetoacetate (1 g, 6.3 mmol) was reacted with the sodium salt of methyl phosphono acetonitrile (9 mmol) in 10 mL of dry THF under argon atmosphere at 25 °C for 12 h. Extraction with water and ether, followed by usual workup and chromatography using ether–hexane (3:7) gave 750 mg (3.7 mmol) of the *tert*-butyl ester of **22**. The compound was dissolved in 5 mL of CH_2Cl_2 and treated at 25 °C with 0.2 mL of trifluoroacetic acid for 1 h. Solvent evaporation, followed by chromatography with ether, gave 525 mg (3 mmol) of acid **22**: UV λ_{max} 236. ^1H NMR ($CDCl_3$) δ 2.18 (d, J = 1.8, 3, 6-H), 3.28 (q, J = 1.8, 2, 2-H), 5.35 (q, J = 1.8, 2, 4-H).

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Registry No. 1 (R = CHO), 116-31-4; **1** (R = CH=NCH$_3$), 51424-44-3; **1** (R = CH=NC(CH$_3$)$_3$H$^+$Cl$^-$), 111535-83-2; **1** (R = CH=NCH$_3$H$^+$Cl$^-$), 138837-44-2; **1** (R = CH=N$^+$(CH$_2$)$_4$ClO$_4^-$), 23369-82-6; **1** (R = CH=N$^+$H(CH$_2$)$_2$N(CH$_3$)$_2$H$^+$·2TFA$^-$), 138857-06-4; **1** (R = CH=N$^+$(CH$_2$CH$_2$)$_2$NCH$_3$H$^+$·2ClO$_4^-$), 99213-50-0; **1** (R = CH=NC(CH$_3$)$_3$H$^+$TFA$^-$), 92216-33-6; **1** (C = CH=NC(CH$_3$)$_3$H$^+$IO$_4^-$), 138837-46-4; **2** (R = CHO), 113767-68-3; **2** (R = CH=NC(CH$_3$)H$^+$Cl$^-$), 120525-00-0; **2** (R = CH=NC(CH$_3$)$_3$H$^+$TFA$^-$), 138837-43-1; **2** (R = CH=NH$^+$(CH$_2$)$_2$N(CH$_3$)$_2$H$^+$·2TFA$^-$), 138857-05-3; **2** (R = CH=NH$^+$(CH$_2$)$_2$N(CH$_3$)$_2$H$^+$·2Cl$^-$), 138837-45-3; **2** (R = CH=N$^+$(CH$_2$CH$_2$)$_2$NCH$_3$H$^+$·2ClO$_4^-$), 138837-28-2; **3** (R = CHO), 80172-51-6; **3** (R = CH=NC(CH$_3$)$_3$H$^+$TFA$^-$), 138837-30-6; **3** (R = CH=N$^+$(CH$_2$)$_5$ClO$_4^-$), 85406-00-4; **4**, 110-83-8; **4a**, 56688-75-6; **5**, 591-49-1; **5a**, 98540-04-6; **6**, 1674-10-8; **6a**, 3949-35-7; **7**, 54625-15-9; **7a**, 432-25-7; **8a**, 138837-14-6; **8b**, 138921-08-1; **9a**, 94773-19-0; **9b**, 138837-31-7; **10a**, 138837-15-7; **10b**, 138837-32-8; **11a**, 109-96-6; **11b**, 138837-33-9; **12a**, 694-05-3; **12b**, 138837-34-0; **13a**, 138837-16-8; **13b**, 138837-35-1; **14a**, 138837-17-9; **14b**, 138837-36-2; **15a**, 91817-61-7; **15b**, 138837-37-3; **16a**, 99213-88-4; **16b**, 138837-38-4; **17a**, 138921-06-9; **17b**, 138921-09-2; (*E*)-**18a**, 138921-07-0; (*Z*)-**18a**, 138921-11-6; (*E*)-**18b**, 139012-18-3; (*Z*)-**18b**, 139012-19-4; (*E*)-**19a**, 138837-18-0; (*Z*)-**19a**, 138921-12-7; (*E*)-**19b**, 138921-10-5; (*Z*)-**19b**, 139012-20-7; **20a**, 138837-19-1; **20b**, 138837-39-5; **21a**, 138837-20-4; 9-*cis*-**21a**, 138837-25-9; **21b**, 138837-40-8; 9-*cis*-**21b**, 138837-47-5; **22a**, 25341-95-1; **22a** (*tert*-butyl ester), 138837-24-8; **22b**, 138837-41-9; **23**, 138837-21-5; **24**, 138837-22-6; **25**, 138837-23-7; CH$_3$COCH$_2$COOC(CH$_3$)$_3$, 1694-31-1; (*E*)-(EtO)$_2$P(O)-CH$_2$C(CH$_3$)=CHCN, 82648-70-2; (EtO)$_2$P(O)CH$_2$CN, 2537-48-6; Br(CH$_2$)$_4$Br, 110-52-1.

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