question as well as others when a functional purified transporter is available. The techniques described in this communication provide a fast and useful assay for the above described purification.

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A Proton and Carbon-13 Nuclear Magnetic Resonance Spectroscopy Study of the Conformation of a Protonated 11-*cis*-Retinal Schiff Base†

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ABSTRACT: Model visual pigment chromophores, *N*-(11-*cis*-retinylidene)propylimine (I) and the trifluoroacetate and chloride salts of I, have been prepared in highly purified form and their proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra analyzed. The ^{1}H NMR chemical shifts and coupling constants for *N*-(11-*cis*-retinylidene)-propyliminium trifluoroacetate indicate that the polyene chain is planar from C(7) to C(12) and twisted about the C(12)–C(13) bond, forming a mixture of distorted 12-s-cis and distorted 12-s-trans conformations. Therefore, a protonated 11-*cis*-retinal Schiff base relieves steric strain due to interactions of 10-H with 13-CH_3 and 14-H in a manner similar to 11-*cis*-retinal. Significant differences are seen between the behavior of the respective ^{13}C chemical shifts of the 11-cis and all-trans isomers upon going from retinal to the imine to the protonated imine. A comparative analysis of the ^{13}C NMR chemical shifts of the 11-cis and all-trans isomers of these chromophores, using the steric interaction hypothesis to explain the γ shift, indicates that protonation of I in $CDCl_3$ at −65 °C results in a change of the more stable conformation from distorted 12-s-cis to distorted 12-s-trans. Determination of the ^{1}H nuclear Overhauser enhancement of 10-H upon irradiating 13-CH_3 of the protonated retinylidenimine in acetone-d_6 also supports this result. A consideration of the effects of solvent and temperature on the solution conformation of the retinylidene chain in a protonated Schiff base leads to conclusions in agreement with the NMR studies. It is proposed from these results and resonance Raman experiments that the conformation of the chromophore of rhodopsin is distorted 11-cis-12-s-trans.

The chromophore of the vertebrate visual pigment rhodopsin is postulated to be a specifically perturbed 11-*cis*-retinylidene group that is bound through a protonated Schiff base linkage to an amino group of the apoprotein opsin (Morton & Pitt, 1955; Kropf & Hubbard, 1958; Hubbard, 1969; Rimai et al., 1970; Lewis et al., 1973; Oseroff & Callender, 1974; Abrahamson & Fager, 1973; Mathies & Stryer, 1976; Callender et al., 1976). The hypothesis of a protonated Schiff base was stated by Morton & Pitt (1955) to explain the properties of "indicator yellow" and by Kropf & Hubbard (1958) to explain the large difference between the visible absorptions of retinal and rhodopsin. Further evidence was obtained by Bownds (1967) and Fager et al. (1972), who demonstrated that the chromophore could be covalently attached to opsin through an amino linkage by reacting the pigment with $NaBH_4$ or

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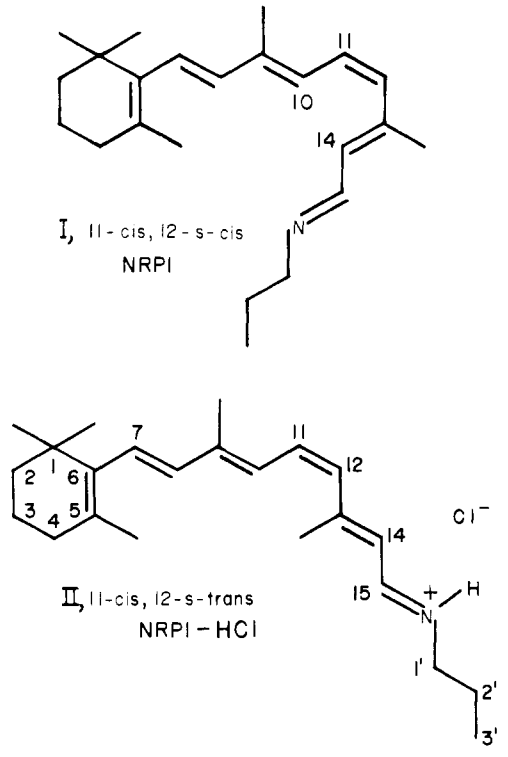
0006-2960/79/0418-4785$01.00/0

$NaCNBH_3$, thereby providing evidence for a C=N linkage in the native protein. Oseroff & Callender (1974), Lewis et al. (1973), Mathies & Stryer (1976), and Callender et al. (1976) have obtained resonance Raman spectra of rhodopsin which contain peaks of nearly the same frequency as that due to the C=N stretching mode of model protonated Schiff bases.

A ^{13}C NMR study of model chromophores has been initiated to aid in the interpretation of the ^{13}C NMR spectra of enriched visual pigments and to indicate those positions which would make the most informative probes (Shriver et al., 1976, 1977). Previous experimental work on the most appropriate model of the chromophore of rhodopsin, i.e., a protonated 11-cis Schiff base, has been limited (Erickson & Blatz, 1968; Johnston & Zand, 1973; Poincelot et al., 1970; Alchalel et al., 1975; Menger & Kliger, 1976; Mathies et al., 1977). Other model systems have included retinal isomers (Patel, 1969; Rowan & Sykes, 1974; Rowan et al., 1974; Becker et al., 1974; Inoue et al., 1974), various all-trans (Blatz et al., 1972; Rosenfeld et al., 1974; Schaffer et al., 1974), 9-cis, 11-cis, and 13-cis (Schaffer et al., 1974; Rosenfeld et al., 1974) Schiff bases of retinal, and protonated all-trans (Waddell et al., 1973; Fisher & Weiss, 1974; Adams et al., 1974; Blatz et al., 1972; Mathies & Stryer, 1976; Tokito et al., 1975), 9-cis, and 13-cis (Adams et al., 1970) Schiff bases of retinal. We report here the preparation and a ^1H and ^{13}C NMR study of *N*-(11-*cis*-retinylidene)propylimine (NRPI,[1] arbitrarily shown in the 12-s-cis conformation) and its trifluoroacetate and chloride salts (NRPI-HCl, II, shown in the 12-s-trans conformation).



Experimental Methods

Materials. *all-trans*-Retinal (Eastman Kodak) and 11-*cis*-retinal (a generous gift of Hoffmann-La Roche) were used without further purification. Isooctane was reagent grade, and the ethanol was freshly opened absolute. Spectrograde methylene chloride was purified by washing with an aqueous Na_2CO_3 solution and 3 times with distilled water and then dried over K_2CO_3. It was then refluxed and distilled from P_2O_5 under nitrogen. The resulting distillate was stored under nitrogen with 4-Å molecular sieves.

Preparation of Imines. The imines were prepared according to a procedure modified from those reported in the literature (Erickson & Blatz, 1968; Waddell et al., 1973). The appropriate crystalline retinal isomer was dissolved in isooctane and reacted with an excess of *n*-propylamine in the dark at 0 °C under N_2 with 4-Å molecular sieves for 3 h. After this time, excess amine and solvent were removed on a rotary evaporator without heating. Remaining traces of solvent and amine were removed in vacuo. No further purification was generally required; however, crystallization from acetonitrile could be performed at −20 °C. If the imine was not used immediately, it was stored under liquid nitrogen.

An alternative method for preparing the imine was used for UV–vis spectroscopy experiments. Approximately 5×10^{-8} mol of 11-*cis*-retinal in isooctane was added to a 5-mL volumetric flask with 100 μL of isooctane. Approximately 100 μL of *n*-propylamine was added and the reaction allowed to proceed under N_2 at 0 °C for 10 min, after which the excess amine and isooctane were driven off with N_2 and completely removed under vacuum. The NRPI was dissolved in the desired solvent and immediately transferred to a cuvette at −77 °C.

Preparation of Protonated Imines. The chloride salt of the imine was prepared by adding a small amount of ether acidified with HCl gas to an ether solution of the imine in a liquid nitrogen bath under N_2. The resulting precipitate was collected immediately by filtration and briefly dried in vacuo. This procedure resulted in a salt with no noticeable isomerization as revealed by ^{13}C NMR of various isomer preparations (Shriver, 1977).

Protonation with trifluoroacetic acid (F_3AcOH) was accomplished by adding F_3AcOH directly to the Schiff base in nitrogen-saturated solvent in a dry ice–acetone bath.

UV–Vis Spectroscopy. UV–vis spectra were taken on a Cary 14 spectrometer. The temperature was controlled either with cooled N_2 gas flowed through a jacketed cell or with a Dewar with flat-faced Pyrex windows and a metal cell holder cooled with a dry ice–acetone bath. Temperatures were measured with a copper–constantan thermocouple.

NMR Spectroscopy. ^1H NMR spectra were taken on a Varian HA-100 in continuous wave mode. ^1H NMR spectra at 300 MHz were taken at the University of Akron. Me_4Si served as a field frequency lock and internal standard.

^{13}C NMR spectra were taken in the pulse Fourier transform mode on a Varian XL-100 spectrometer interfaced to a Varian 620i computer. In general, a rapid pulse scheme was used, i.e., a 30° pulse angle and a 0.8-s cycle time.

Approximately 0.1 M solutions of the model chromophores were used in NMR experiments in $CDCl_3$ or acetone-d_6, with the exception of 11-*cis*-NRPI-HCl which was ~0.03 M. Me_4Si was used as an internal standard throughout. Temperatures were maintained with a Varian temperature control unit. ^1H NMR spectra were taken by using 5-mm tubes and ^{13}C NMR spectra by using 12-mm tubes.

^1H NMR nuclear Overhauser enhancement experiments were performed on samples in 5-mm tubes. Selective saturation was achieved with a Varian 200 CD decoupler with a power level resulting in maximal enhancement. Enhancements were measured relative to spectra obtained either with the decoupler off or with the decoupling frequency off-resonance.

[1] Abbreviations used: F_3AcOH, trifluoroacetic acid; NRPI, *N*-retinylidenepropylimine; NRPI-HCl, *N*-retinylidenepropyliminium chloride; NOE, nuclear Overhauser effect; $f_a(b)$, percent change in intensity of resonance "a" upon saturating the "b" resonance.

Table I: ^{1}H NMR Chemical Shifts of Protonated Propylamine Schiff Bases of Retinal Isomers[a]

^{1}H	11-cis SB[b,c] + F_3AcOH	all-trans SB[c] + F_3AcOH
1,1-CH_3	1.05	1.04
5-CH_3	1.73	1.74
9-CH_3	2.14	2.12
13-CH_3	2.57	2.42
7-H	6.55	6.52
8-H	6.36	6.24
10-H	6.98	6.38
11-H	7.12	7.64
12-H	6.31	6.67
14-H	6.71	6.63
15-H	9.19	9.15

[a] Chemical shifts (in parts per million) are measured relative to internal Me_4Si in acetone-d_6 at −55 °C. [b] SB = Schiff base. [c] The Schiff bases were protonated with a fivefold excess of F_3AcOH.

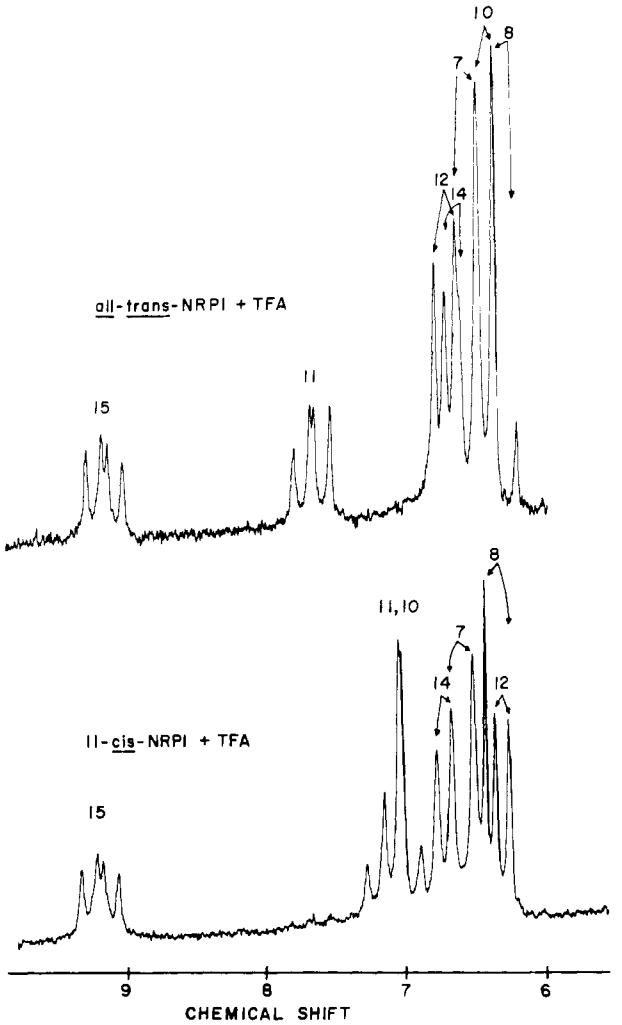


FIGURE 1: Low-field region of the 100-MHz ^{1}H NMR spectra of *N*-(*all-trans*- and *N*-11-*cis*-retinylidene)propylimine acidified with a fivefold excess of trifluoroacetic acid at −55 °C in acetone-d_6. Chemical shifts are in parts per million relative to internal Me_4Si.

Peak areas were measured by using planimetry on spectra expanded to 250 Hz/50 cm. All solutions were deoxygenated by N_2 saturation.

Results

^{1}H NMR chemical shifts of *all-trans*-NRPI + F_3AcOH and 11-*cis*-NRPI + F_3AcOH are given in Table I, and the alkene regions of the spectra are shown in Figure 1. Assignments were made by using conventional methods, including selectively decoupling 15-H, 9-CH_3, and 13-CH_3 and titration of the Schiff base with F_3AcOH, which aided in the assignment of overlapping resonances. The chain methyls, 9-CH_3 and 13-CH_3, and the ring protons could be assigned by comparison with retinal assignments (Patel, 1969; Rowan et al., 1974). Due to a number of closely lying resonances which are coupled, the ^{1}H spectra of the model chromophores are not first order in the alkene region. The chemical shifts of 7-H and 8-H were calculated by assuming an AB spectrum using the procedure outlined by Bernstein et al. (1957). In 11-*cis*-NRPI + F_3AcOH, $J_{10,11}/\nu_0\delta_{10,11}$ is nearly equal to 1, so a second-order spectrum results. Very good agreement between experimental and calculated spectra was obtained if the 10-H, 11-H, 12-H spin system was treated as an ABX system (see Figure 2). Of the fifteen transitions predicted, one has zero intensity and two, ν_{14} and ν_{15}, have such low intensities that they were not detected. The calculated (Bernstein et al., 1957; Abraham & Bernstein, 1961) chemical shifts are given in Table I. These assignments were confirmed with 300-MHz spectra. The coupling constants for the chain protons are given in Table II.

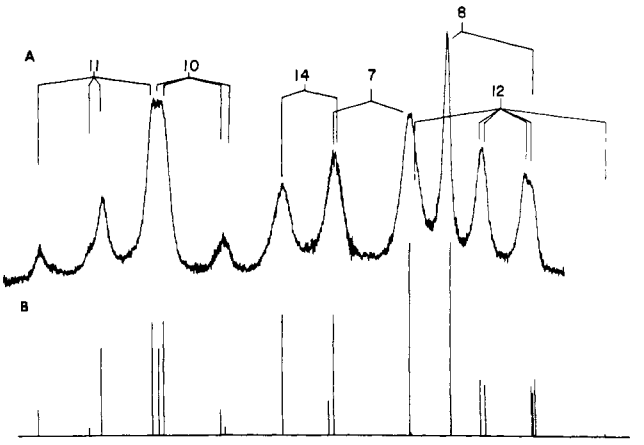


FIGURE 2: (A) Expanded alkene proton resonance region of the 100-MHz ^{1}H NMR spectrum of *N*-(11-*cis*-retinylidene)propylimine acidified with a fivefold excess of trifluoroacetic acid at −55 °C in acetone-d_6. Numbers refer to the proton position. (B) Calculated alkene carbon region assuming that 10-H, 11-H, 12-H can be represented as an ABX spin system. Numbers refer to transitions [see Abraham & Bernstein (1961)].

Table II: ^{1}H Coupling Constants of the All-Trans and 11-Cis Isomers of *N*-Retinylidenepropyliminium Trifluoroacetate[a]

H_i,H_j	$J_{i,j}$ (Hz) all-trans	11-cis
7,8	15.5	15.5
10,11	11.6	13.0
11,12	14.7	12.3
10,12		−0.8
14,15	10.8	10.8

[a] Spectra were taken of samples in acetone-d_6 at −55 °C. The imine was protonated by adding a fivefold excess of trifluoroacetic acid under nitrogen at −65 °C.

Table III gives the ^{13}C NMR chemical shifts of 11-*cis*-retinal, *N*-(11-*cis*-retinylidene)propylimine, and its protonated species. Shift assignments were made by using the conventional methods, titration of the imine with F_3AcOH and, in the case of retinal and the imine, application of the shift reagent Eu(fod)$_3$. Eu(fod)$_3$ led to shifts primarily due to a Fermi contact interaction as indicated by the alternating sign of the induced shift. A pseudocontact interaction was implied

Table III: ^{13}C NMR Chemical Shifts of 11-*cis*-Retinal, *N*-(11-*cis*-Retinylidene)propylimine, *N*-(11-*cis*-Retinylidene)propyliminium Chloride, and *N*-(11-*cis*-Retinylidene)propyliminium Trifluoroacetate[a]

carbon	11-*cis*-retinal	NRPI	NRPI-HCl	NRPI + F_3Ac-OH[b]	NRPI + 5F_3Ac-OH[b]
1	34.08	34.07	34.13	34.13	34.14
2	38.92	38.87	38.86	38.90	38.92
3	18.93	18.94	18.80	18.79	18.81
4	32.84	32.76	33.00	33.01	33.05
5	130.35	129.66	131.72	132.06	132.18
6	137.28	137.48	137.22	137.21	137.22
7	129.59	127.85	132.26	132.96	133.17
8	137.58	138.00	137.22	137.21	137.22
9	142.07	139.26	146.64	147.76	148.12
10	125.88	126.26	126.40	126.39	126.44
11	132.04	127.67	137.48	138.68	139.08
12	129.97	131.70	129.05	128.70	128.62
13	157.08	145.02	162.65	165.83	166.55
14	129.59	129.96	121.28	120.53	120.28
15	192.00	159.62	163.87	163.31	
1,1-CH_3	28.87	28.83	28.85	28.85	28.84
5-CH_3	22.01	21.99	22.10	22.05	22.04
9-CH_3	12.46	12.24	12.49	12.56	12.56
13-CH_3	18.03	17.72	18.80	18.79	18.81
1′		63.93	54.33	54.35	54.27
2′		24.08	22.78	22.51	22.51
3′		12.03	11.25	10.85	10.76

[a] Spectra were taken in $CDCl_3$ at −65 °C with concentrations of ~0.1 M on a Varian XL-100 spectrometer in the Fourier transform mode. Chemical shifts are reported in parts per million relative to internal Me_4Si. A rapid pulse scheme was generally used, i.e., a 30° pulse angle and a 0.8-s cycle time. [b] NRPI + F_3AcOH = 1:1 ratio of F_3AcOH to NRPI; NRPI + 5F_3AcOH = 5:1 ratio of F_3AcOH to NRPI.

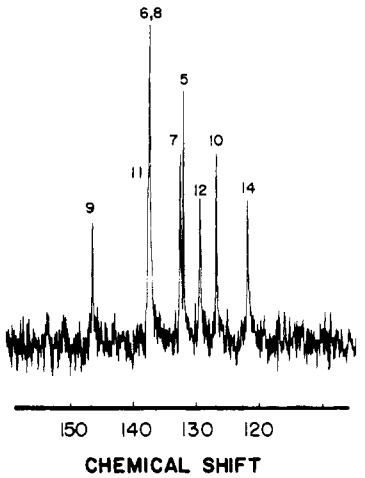


FIGURE 3: Alkene carbon resonance region of the 25.16-MHz ^{13}C NMR spectrum of *N*-(11-*cis*-retinylidene)propyliminium chloride in $CDCl_3$ at −65 °C. Chemical shifts in parts per million are relative to internal Me_4Si. Numbers refer to carbon positions.

in the imines at C(14) since the C(14) resonance did not shift upfield as did the other even-numbered resonances. The assignment of C(5), C(6), and C(11) of the protonated 11-cis imine should be specifically discussed due to their proximity (Figure 3). C(5) and C(6) were assigned from the proton-coupled spectrum, and C(11) was assigned by selectively decoupling the C(11)–11-H interaction. Assignments for the all-trans isomers at −65 °C were made as described for the room-temperature spectra (Shriver et al., 1976) and are given in Table IV.

The ^1H and ^{13}C NMR shifts of NRPI and protonated NRPI isomers (Shriver et al., 1976; Shriver 1977) indicate that no isomerization about the alkene chain double bonds resulted

Table IV: ^{13}C NMR Chemical Shifts of *all-trans*-Retinal, *N*-(*all-trans*-Retinylidene)propylimine, and *N*-(*all-trans*-Retinylidene)propyliminium Chloride[a] and Shifts of the 11-Cis Isomer Relative to All-Trans

	retinal[b]	Δ[c]	t-NRPI[d]	Δ[e]	t-NRPI-HCl[f]	Δ[g]
1	34.08	0.00	33.98	0.09	34.10	0.03
2	38.91	0.01	38.86	0.01	38.94	−0.08
3	18.87	0.06	18.82	0.12	18.82	−0.02
4	32.92	−0.08	32.80	−0.04	33.08	−0.08
5	130.71	−0.36	129.86	−0.20	131.93	−0.21
6	137.20	0.08	137.39	0.09	137.00	0.22
7	129.51	0.08	127.43	0.42	131.93	0.33
8	137.11	0.47	137.29	0.71	136.89	0.33
9	141.58	0.49	138.03	1.23	145.48	1.16
10	129.38	−3.50	129.80	−3.54	129.52	−3.12
11	132.86	−0.82	127.75	−0.08	137.64	−0.16
12	134.30	−4.33	135.79	−4.09	133.32	−4.27
13	155.85	1.23	144.38	0.64	162.38	0.27
14	128.99	0.60	129.01	0.95	119.72	1.56
15	191.94	0.06	159.68	−0.06	162.92	−0.95
1,1-CH_3	28.84	0.03	28.76	0.07	28.87	−0.02
5-CH_3	21.97	0.04	21.86	0.13	22.11	−0.01
9-CH_3	13.14	−0.68	12.90	−0.66	13.39	−0.90
13-CH_3	13.14	4.89	13.06	4.66	14.19	4.61
1′			63.85	0.08	54.02	0.31
2′			24.00	0.08	22.79	−0.01
3′			11.94	0.09	11.25	0.00

[a] All solutions were ~0.1 M in $CDCl_3$ at −65 °C except for 11-*cis*-NRPI-HCl, which was ~0.03 M. Chemical shifts (in parts per million) are relative to internal Me_4Si. [b] *all-trans*-Retinal. [c] δ(11-*cis*-retinal) − δ(*all-trans*-retinal). [d] *N*-(*all-trans*-Retinylidene)propylimine. [e] δ[*N*-(11-*cis*-retinylidene)propylimine] − δ[*N*-(*all-trans*-retinylidene)propylimine]. [f] *N*-(*all-trans*-Retinylidene)propyliminium chloride. [g] δ(11-*cis*-NRPI-HCl) − δ(*all-trans*-NRPI-HCl).

Table V: Changes in ^{13}C NMR Chemical Shifts upon Shiff Base Formation and Protonation of the Schiff Base[a]

	formation of SB,[b] δ(NRPI) − δ(retinal)		protonation of SB,[b] δ(NRPI-HCl) − δ(NRPI)	
carbon	trans	11-cis	trans	11-cis
1	−0.10	−0.01	0.12	0.06
2	−0.05	−0.05	0.08	−0.01
3	−0.05	0.01	0.00	−0.14
4	−0.12	0.08	0.28	0.24
5	−0.85	−0.69	2.07	2.06
6	0.19	0.20	−0.39	−0.26
7	−2.08	−1.74	4.5	4.41
8	0.18	0.42	−0.40	−0.78
9	−3.55	−2.81	7.45	7.38
10	0.42	0.38	−0.28	0.14
11	−5.11	−4.37	9.89	9.81
12	1.49	1.73	−2.47	−2.65
13	−11.47	−12.06	17.63	18.85
14	0.02	0.37	−9.29	−8.68
15	−32.26	−32.38	3.25	3.03
1,1-CH_3	−0.08	−0.04	0.11	0.02
5-CH_3	−0.11	−0.02	0.25	0.11
9-CH_3	−0.24	−0.22	0.49	0.25
13-CH_3	−0.08	−0.31	1.13	1.08

[a] All solutions were ~0.1 M in $CDCl_3$ at −65 °C. Chemical shifts (in parts per million) are measured relative to internal Me_4Si. See footnotes to Tables III and IV for further details. [b] SB = Schiff base.

from imine formation or protonation if these were synthesized and handled as described under Experimental Methods. It should be noted that the chemical shift differences due to isomerization in the NRPI isomers and the protonated NRPI isomers are nearly identical with the differences seen in the retinal isomers (see Table V). Discrepancies can be inter-

Table VI: UV-Vis Spectral Parameters for All-Trans and 11-Cis Isomers of NRPI and NRPI + F_3AcOH

compd	temp (°C)	solvent	λ_{max} (nm)	ϵ_{max} (cm^{-1} mol^{-1})
all-trans-NRPI	RT[b]	$CHCl_3$	371	48 600
	RT	isooctane	358	52 600
	−55	isooctane	361	52 800
	−77	isooctane	366	51 800
	−77	ethanol	370	
	−77	CH_2Cl_2	371	53 200
all-trans-NRPI + F_3AcOH[a]	−77	CH_2Cl_2	482	49 400
11-*cis*-NRPI	RT	$CHCl_3$	364	26 000
	RT	isooctane	348	29 800
	−55	isooctane	357	34 100
	−77	isooctane	361	37 700
	−77	ethanol	363.5	
	−77	CH_2Cl_2	367	38 100
11-*cis*-NRPI + F_3AcOH[a]	−77	CH_2Cl_2	479	38 000

[a] NRPI was protonated with the addition of a 100-fold excess of F_3AcOH in CH_2Cl_2 at −77 °C. [b] All imines were synthesized at 0 °C as described under Experimental Methods. RT = room temperature.

preted in terms of conformation differences and will be discussed below.

Only one isomer with respect to the imine linkage was present in appreciable concentration since one ^{13}C resonance was noted for C(15) and C(1′). A pseudocontact interaction at C(14) in the Schiff base with Eu(fod)$_3$ was indicated by a downfield shift of C(14), while other even-numbered carbon resonances shifted upfield. We interpret this to imply that the imine linkage is trans since this would allow close approach of the coordinated metal and C(14). One resonance was observed for C(15) and C(1′) in the protonated Schiff bases (both the chloride and trifluoroacetate salts), indicating only one isomer, which was assumed to be trans.

UV–vis spectral values are reported in Table VI. Both the *all-trans*- and 11-*cis*-NRPI absorptions at ~360 nm have a broad maximum in isooctane; however, a narrow peak is seen in $CHCl_3$. Decreasing temperature led to the characteristic increase (by a factor of 1.27) in extinction of 11-*cis*-NRPI in isooctane, which in 11-*cis*-retinal has been attributed to an altered population of the distorted 12-s-cis and 12-s-trans conformations (Honig & Ebrey, 1974; Honig & Karplus, 1971; Schaffer et al., 1974). A relatively intense absorption near 250 nm exists for both 11-*cis*-NRPI and its protonated form.

A discussion of the lability and ease of isomerization of protonated NRPI isomers seems warranted as judged by previous work on these compounds. *all-trans*-NRPI could be successfully synthesized and handled in an inert atmosphere at room temperature for ~12 h without the appearance of significant impurities or other isomers in the ^{13}C NMR spectrum. However, in general the UV maximum of *all-trans*-NRPI synthesized at room temperature was shifted to shorter wavelengths by ~5 nm from preparations synthesized at 0 °C. This is attributed to a small amount of isomerization. Attempting to synthesize 11-*cis*-NRPI at room temperature instead of 0 °C resulted in significant isomerization, which was indicated again by the UV–vis spectrum; when synthesized at room temperature, the λ_{max} was 367.5 nm in $CHCl_3$ and 356 nm in isooctane. The room-temperature preparations had a significant absorption at 250 nm; however, the bandwidth was greater and the intensity less than what was seen if 11-*cis*-NRPI was synthesized at 0 °C. When an isooctane solution of impure 11-*cis*-NRPI synthesized at room temperature was acidified, a 250-nm absorption was seen as shown by Erickson & Blatz (1968). If 11-*cis*-NRPI that had been synthesized at 0 °C was protonated at −55 °C, briefly warmed to room temperature, and taken again to −55 °C, the intensity of the primary visible transition increased relative to that observed before warming and the 250-nm absorption band intensity decreased, indicating that isomerization had occurred. Protonation of 11-*cis*-NRPI with F_3AcOH at room temperature in $CDCl_3$ under nitrogen resulted in extensive isomerization and side reactions as evidenced by greater than 30 lines in the alkene region of the ^{13}C NMR spectrum (compare with Figure 3 and see below).

Discussion

Previous preparations of 11-*cis*-NRPI and its protonated form have been performed at room temperature, and the products have been identified with UV–vis spectroscopy. Our NMR results indicate that there is appreciable isomerization when the cis isomers of NRPI are synthesized and handled at room temperature for even short periods, possibly due to trace Lewis acid impurities in the solvents. NRPI readily isomerizes at room temperature in the presence of acid to an equilibrium mixture of isomers. This may explain the recent observation (Waddell et al., 1976) that bleaching of rhodopsin results in isomers other than *all-trans*-retinal being released from opsin. The bleaching of rhodopsin results in a number of intermediates which are protonated Schiff bases, presumably all the intermediates leading to the intermediate meta II. The lifetime of these intermediates would be expected to vary with the environment of the protein, i.e., whether it is in the native membrane or solubilized by detergent (Abrahamson, 1975). In addition, the kinetics of bleaching vary with detergent. As a result, the different relative concentrations of isomers resulting from bleaching in various detergents are not necessarily due to different photochemistries, but may be due to different rates for the thermal reactions following the initial absorption of light.

We have found that the isomerization of protonated 11-*cis*-NRPI may be slowed sufficiently for the purposes of NMR by protonating at decreased temperatures, e.g., −65 °C. The precautions outlined here ensure a preparation of 11-cis isomers with purity more than adequate for NMR experiments due to the narrow NMR line width relative to chemical shift. However, due to the broadness and proximity of the UV–vis absorptions of the isomers, small impurities can lead to errors in the UV–vis spectra. In addition, the λ_{max} of protonated Schiff bases is dependent on the ratio of acid to base. For these reasons, the λ_{max} and ϵ_{max} have an estimated accuracy of ±2 nm and ±5%, respectively.

A comparison of ^1H chemical shifts in Table I and the coupling constants given in Table II for the two isomers indicates that the protonated 11-*cis*-retinal Schiff base is essentially planar from C(7) to C(12) if analyzed in a manner similar to that given for the retinals (Patel, 1969; Rowan et al., 1974). The coupling constants of the all-trans and 11-cis protonated imine are essentially the same as those observed for *all-trans*- and 11-*cis*-retinal, respectively. In addition, the chemical shifts of protonated 11-*cis*-NRPI relative to *all-trans*-NRPI are nearly the same as those of 11-*cis*-retinal relative to *all-trans*-retinal. Minor differences between the retinal and protonated imine coupling constants may be attributed to differences in bond orders resulting from protonation. $J_{10,11}$ is larger in 11-cis than all-trans for both the protonated imine and retinal, and this has been attributed to a smaller 11-H–C(11)–C(10) bond angle in the 11-cis isomer due to steric interactions of 13-CH_3 and 14-H with 10-H. The difference in $J_{11,12}$ for the two isomers of the protonated imine

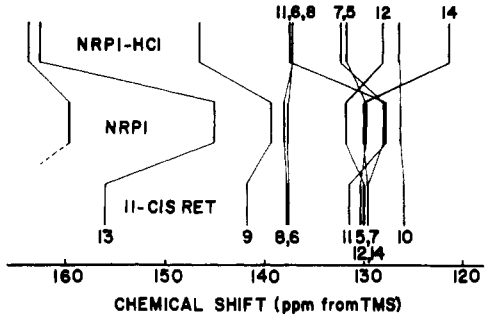


FIGURE 4: Schematic presentation of alkene carbon resonance changes in the 25.16-MHz NMR spectra of 11-*cis*-retinal (11-*cis*-RET), *N*-(11-*cis*-retinylidene)propylimine (NRPI), and *N*-(11-*cis*-retinylidene)propyliminium chloride (NRPI-HCl) in $CDCl_3$ at −65 °C. Numbers refer to carbon position. Note that upon formation of the imine the even-numbered carbons shift downfield and the odd shift upfield with the magnitude proportional to the distance from the end of the chain. A similar effect with the direction of the shift reversed is seen upon protonation. However, C-10 noticeably deviates from this pattern upon protonation of the imine.

is exactly that expected for a cis–trans isomerization about an ethylenic linkage (Karplus, 1959). The upfield shift of 12-H of 11-cis relative to all-trans (−0.36 ppm) in the protonated imine is similar to that observed in retinal and is attributed to a twisting about C(12)–C(13) to relieve steric strain and thereby making 12-H more like a terminal polyene proton. In addition, 11-H shifts upfield (−0.52 ppm) just as in retinal, presumably due to the prevention of a resonance structure with a positive charge on C(11) due to twisting about C(12)–C(13). In summary, the protonated 11-cis Schiff base removes steric strain due to interactions of 13-CH_3 and 14-H with 10-H similar to 11-*cis*-retinal, i.e., by twisting about the C(12)–C(13) bond primarily. Twisting about other bonds, particularly C(10)–C(11) and C(11)–C(12), does not appear to be significant. The ^1H NMR spectra do not give any insight into the magnitude of twisting about C(12)–C(13), i.e., whether the conformation is distorted 12-s-cis or 12-s-trans.

As expected, little change in the ^{13}C NMR chemical shifts of the aliphatic ring carbons results from conversion of the carbonyl group to an imine or protonation of the imine (see Table V). However, formation of the imine leads to a downfield shift of the even-numbered alkene carbon resonances and an upfield shift of the odd-numbered alkene resonances, as would be expected from the effective release of electron density into the polyene chain by substituting oxygen with the less electronegative nitrogen (see Figure 4). The opposite effect is seen upon protonation, and the magnitude of the shift is inversely proportional to the distance from the site of protonation. The changes in chemical shifts can be predicted by using the Karplus–Pople relation (Karplus & Pople, 1963) for the paramagnetic contribution to the chemical shift tensor for conjugated polyenes. It has been shown that chemical shift changes in this system are primarily due to modifications of electron densities and bond orders at each specific carbon atom (Shriver et al., 1976). The success of this theory implies that results from quantum mechanical calculations in conjunction with ^{13}C NMR data from the protein may be used in probing the structure and environment of the chromophore in opsin (Shriver et al., 1977).

The ^{13}C NMR results presented here may be used to determine the effect of protonation on the conformation of the alkene chain of 11-*cis*-NRPI. Calculations on 11-*cis*-retinal (Rowan et al., 1974) have shown that the two conformations distorted 12-s-trans and distorted 12-s-cis differ by only 1.5 kcal/mol, with the distorted 12-s-cis being the more stable. 11-*cis*-Retinal has been shown to exist in a distorted 12-s-cis conformation in the crystal by Gilardi et al. (1972). Recent spectroscopic studies of 14-methylretinals indicate that the preferred conformation of 11-*cis*-retinal at room temperature in hexane is distorted 12-s-cis (Chan et al., 1974; Ebrey et al., 1975). However, the work of Rowan et al. (1974) and Birge et al. (1976) shows that since the difference in energy between the two conformers is so small, the relative stability is solvent- and temperature-dependent due to different dipole moments and cavity radii for the two conformers. The distorted 12-s-trans conformation is expected to become increasingly more populated with increasing solvent polarity and decreasing temperature. Consistent with this work, Rowan et al. (1974) have shown that in acetone-d_6 at −47 °C the distorted 12-s-trans conformer is the more populated. Although Birge et al. (1976) did not investigate the protonated Schiff base of 11-*cis*-retinal, it might be expected that protonation would increase the population of the 12-s-trans conformation in polar solvents if the dipole moment of a 11-*cis*-NRPI salt is greater than retinal since the difference between the dipole moments of the two distorted conformers would be larger in the salt than in retinal. The dipole moment of *all-trans*-retinylidenebutylimine hydrochloride has been measured, and the moment of the salt is nearly twice that of the aldehyde (approximately 6.2 and 3.5 D, respectively) (Mathies & Stryer, 1976). These considerations, i.e., solvent polarity, decreased temperature, and creation of a charged molecule, indicate that the preferred conformer of 11-*cis*-NRPI-HCl would be distorted 12-s-trans in polar solvents at low temperatures. It should be noted that although we refer to the distorted "12-s-trans" and "12-s-cis" conformers of the protonated imine, they are not expected to be identical with those of 11-*cis*-retinal.

The ^{13}C NMR chemical shift differences upon going from retinal to NRPI to NRPI-H^+ are in general similar for the all-trans and 11-cis isomers. A notable exception is C(10) in 11-cis (Figure 4). It is instructive to ask if the lack of similarity in some instances could be due to altered conformations and therefore altered steric interactions upon going from retinal to NRPI to NRPI-H^+. If this is the case, the ^{13}C NMR may provide evidence in support of the above argument for a 12-s-trans conformation for 11-*cis*-NRPI-H^+ in polar solvents.

The steric interaction of two protons presumably results in a polarization of the respective C–H bonds with a resultant increase in electron density on the carbons and an upfield shift in the ^{13}C NMR spectral line and is the explanation given for the commonly observed γ effect (Grant & Cheney, 1967; Martin et al., 1975). While this is qualitative reasoning and neglects other important contributions to ^{13}C chemical shifts besides electron densities, the observation that steric interactions can explain quite well the differences in chemical shifts of the retinals (Rowan & Sykes, 1974) is very encouraging and is used as a basis for the analysis given here. For example, in *all-trans*-retinal 9-CH_3 and 13-CH_3 interact with neighboring protons, 7-H and 11-H in the case of 9-CH_3 and 11-H and 15-H in the case of 13-CH_3. In 9-*cis*-retinal the steric interaction of 9-CH_3 and 11-H is removed and 9-CH_3 is shifted downfield 7.95 ppm relative to that in all-trans (Rowan & Sykes, 1974). The 13-CH_3 resonance shifts negligibly since 13-CH_3 still interacts with 11-H and 15-H. In 13-*cis*-retinal the 13-CH_3 resonance shifts downfield 7.94 ppm relative to that in all-trans since the 11-H–13-CH_3 interaction is removed. In 11-*cis*-retinal the 11-H–C(11)–C(10) angle is slightly smaller than that in *all-trans*-retinal, so the 11-H–9-CH_3 interaction is increased and 9-CH_3 is shifted slightly upfield (0.6 ppm) in 11-cis relative to all-trans. The 13-CH_3–11-H

interaction is removed in 11-*cis*-retinal, and 13-CH_3 is shifted downfield, but not as much as in 13-*cis*-retinal, because 13-CH_3 still interacts with 10-H. Similar arguments can explain the olefinic carbon resonances of the retinylidene chain (Rowan & Sykes, 1974). It should be emphasized that the success of the steric interaction hypothesis in analyzing retinylidene chain spectra is most likely due to the ability of the retinylidene chain to remove interactions partially by twisting about formal single bonds without requiring large perturbations of C–C–C and C–C–H bond angles and therefore perturbations in the orbital hybridizations.

If the ^{13}C NMR spectra of 11-*cis*-retinal may be understood in terms of that of *all-trans*-retinal and altered steric interactions, it would be expected that differences between shifts for equivalent positions in retinal, the imine, and the protonated imine for all-trans and 11-cis may be due to different conformations of the 11-cis isomer for the three model chromophores. Consider first the changes resulting from formation of the imine from retinal. On comparison of the first and second columns of Table V, the largest differences upon imine formation are seen for C(9), C(11), and C(13) (viz., −3.55, −5.11, and −11.47 ppm for trans and −2.81, −4.37, and −12.06 ppm for 11-cis). C(13) moves upfield farther in 11-cis (−12.06 ppm) than in all-trans (−11.47 ppm), and C(11) and C(9) move upfield farther in the trans isomer. This can be attributed to a decreased π-electron mobility in the 11-*cis*-retinylidene chain compared to that in the trans; i.e., electron density is not as easily withdrawn from the 11-cis chain as from the all-trans due to twisting about C(12)–C(13). There appears to be a greater change in electron density from C(12) to C(15) in the 11-cis isomer apparently due to an effectively shorter polyene chain in conjugation with the nitrogen. The differences seen for 13-CH_3 and also for C(12) and C(14) are in line with this reasoning. There appears to be no reason to expect a greatly altered conformation or a different equilibrium between various distorted 12-s-cis and 12-s-trans structures in the retinylidene chain upon formation of the imine, and the preferred 11-*cis*-NRPI conformation in $CDCl_3$ is expected to be 12-s-cis from considerations of solvent, the imine dipole moment (roughly 1.3 D for all-trans; Mathies & Stryer, 1976), and previous work on retinal.

On consideration next of the changes in shifts resulting from protonating the Schiff base, the same effect appears to be present in general. However, there are two notable exceptions: C(10) and C(14). C(10) would be expected to shift upfield upon protonation with a magnitude close to that of C(8) [compare with the all-trans isomer, where C(8) shifts upfield 0.4 ppm and C(10) 0.3 ppm]. It is especially obvious from Figure 4 that C(10) does not behave as expected. A possible explanation is a decreased steric interaction at C(10) upon protonation of 11-*cis*-NRPI. As a result, C(10) would shift upfield relative to C(10) in the imine to a lesser extent in the cis isomer than in the trans and possibly even downfield. Without invoking a steric interaction hypothesis, C(10) would be expected to shift upfield as a result of an inductive mechanism, but to a lesser extent than the trans due to broken conjugation. Since the observed effect is not only a decreased upfield shift but actually a downfield shift, it is proposed that there is a decreased interaction at C(10) with C(14) in 11-*cis*-NRPI-HCl compared to 11-*cis*-NRPI. The shift of C(14) is consistent with this proposal. C(14) would be expected to move upfield farther in the 11-cis isomer than in the trans upon protonation (Table V). That it does not can be explained by a decreased steric interaction at that point also. A decreased interaction at C(14) results in a downfield shift, making the difference with NRPI smaller than expected from simply decreased conjugation. By use of similar reasoning, the small changes seen at 13-CH_3 are consistent with an interaction between 13-CH_3 and 10-H. If a greater change in chemical shift is expected in the 11-*cis*-retinylidene chain from C(12) to C(15) upon protonation, 13-CH_3 should move farther downfield and a value larger than 1.13 ppm observed for the trans isomer would be expected (Table V). The downfield shift is lessened by an upfield shift due to a steric interaction with C(10). The decreased steric interaction at C(10) may be due to a difference in distortion from planarity in the two conformers and does not contradict an interaction of 13-CH_3 with 10-H. These results indicate an increased preference for a distorted 12-s-trans conformation as a result of protonation of 11-*cis*-NRPI in $CDCl_3$ at −65 °C.

This analysis represents an attempt to obtain a consistent explanation for the significant differences in chemical shifts [primarily C(10) and C(14)] and is justified by the success of the theory of steric interactions in explaining differences in the retinal isomer chemical shifts. Other possible explanations for these differences exist; chemical modification may result in differences in intermolecular interactions of retinylidene chains or solvent interactions which could cause altered chemical shifts. However, the internal consistency and the fact that these results are consistent with those expected from the arguments based on the work of Birge et al. (1976) given above lead us to believe that other contributions are insignificant.

An increased interaction between 13-CH_3 and 10-H should be reflected in an increased nuclear Overhauser enhancement of 10-H upon irradiating 13-CH_3. NOE experiments could not be performed with $CDCl_3$ solutions due to prohibitive line widths at low temperatures. Therefore, NOE experiments were performed in acetone-d_6 at −55 °C. Rowan & Sykes (1974) have made an extensive study of NOE's in 11-*cis*-retinal in acetone-d_6 at 32 and −47 °C. If we assume that the potential energy surfaces for rotation about single bonds in the polyene chain of 11-*cis*-retinal and the 11-*cis*-retinylideniminimum ion are qualitatively similar, they would be expected to relieve steric strain due to interaction of 13-CH_3 with 10-H and 12-H similarly, i.e., by twisting about the C(12)–C(13) bond. Evidence for this comes from the ^1H NMR spectra. Large differences in the nuclear Overhauser enhancement of 10-H upon irradiating 13-$CH_3$2, $f_{10\text{-}H}$(13-CH_3), should be indicative of different conformations about C(12)–C(13).

The $f_{10\text{-}H}$(13-CH_3) increases from 11 in 11-*cis*-retinal at −55 °C (also measured both at 32 and −47 °C; Rowan & Sykes (1974) to 19 in *N*-(11-*cis*-retinylidene)propyliminium trifluoroacetate at −55 °C. This value is probably small due to the inclusion of an 11-H transition[2] in the measured resonance at 7.0 ppm (see Figure 2). Correction for this results in an NOE of 23. These considerations indicate that $f_{10\text{-}H}$(13-CH_3) is approximately double that of 11-*cis*-retinal. It is assumed here that any effects on the NOE due to tight coupling of 11-H to 10-H are similar in both molecules since the extent of tight coupling, i.e., J_{AB}/δ_{AB}, is nearly the same in both systems. The large increase in $f_{10\text{-}H}$(13-CH_3) for 11-*cis*-NRPI + F_3AcOH over that for 11-*cis*-retinal indicates that the distorted 12-s-trans conformer is even more preferred in the protonated imine than in retinal in acetone-d_6. The enhancement $f_{12\text{-}H}$(13-CH_3) was found to be 2. Unfortunately, $f_{10\text{-}H}$(14-H)

[2] A transition in the second-order spectrum is defined as being due to either 10-H or 11-H according to whether the change in the wave function is primarily at 10-H or 11-H.

could not be measured due to the proximity of the 10-H and 14-H resonances (see Figure 2). These results are consistent with the above discussion and provide further evidence for a preferred 12-s-trans conformation in a polar solvent at low temperature for protonated 11-*cis*-NRPI.

These NMR results in conjunction with resonance Raman data may be used to determine the conformation of the chromophore of rhodopsin. Mathies et al. (1977) have presented the resonance Raman spectrum of a protonated Schiff base of 11-*cis*-retinal in ethanol at ~0 °C, and there are many similarities with the resonance Raman spectrum of rhodopsin, particularly in the "fingerprint" region. This is given as strong evidence for the retinylidene chromophore in rhodopsin having the same conformation as the protonated imine in solution. From the discussion above, we would expect the preferred conformation in ethanol at 0 °C to be distorted 12-s-trans, and this in conjunction with the work of Mathies et al. (1977) implies that the conformation of the retinylidene chain in rhodopsin is distorted 12-s-trans. These results are consistent with the observation by Chan et al. (1974) that 11-*cis*-14-methylretinal, which cannot assume a 12-s-cis conformation similar to 11-*cis*-retinal, combines with opsin to form a pigment similar to rhodopsin, while in solution it has a UV spectrum significantly shifted from 11-*cis*-retinal.

Callender et al. (1976) have also concluded that the chromophore of rhodopsin is 12-s-trans from their studies of the resonance Raman spectrum of thodopsin. Cookingham & Lewis (1978) have recently argued that a 1271-cm^{-1} vibration in the resonance Raman spectrum of 11-*cis*-retinal may be used as an indication of the 11-cis, 12-s-trans conformation. The observation of an intense 1271-cm^{-1} vibration in rhodopsin is given as evidence that the conformation of the chromophore within rhodopsin is 12-s-trans. Our results would support these conclusions.

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