The Structure of Visual Pigments. I. Carbon-13 Nuclear Magnetic Resonance Spectroscopy of *N-all-trans*-Retinylidenepropylimine and Its Protonated Species

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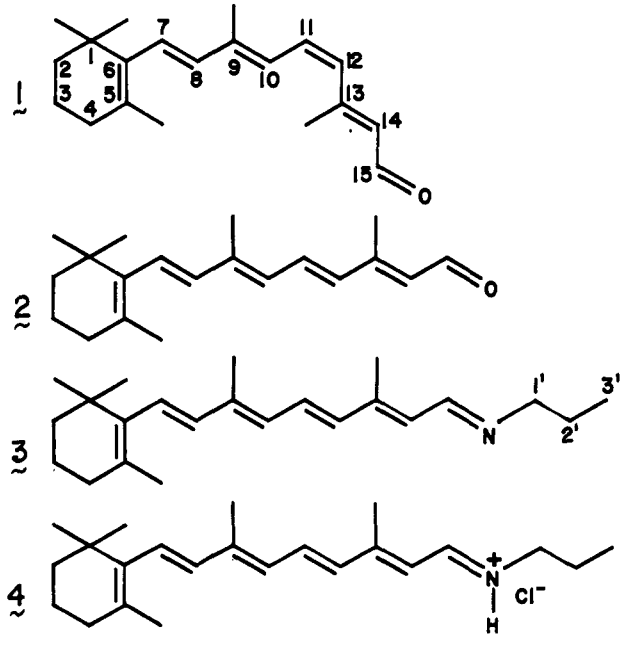
Abstract: Carbon-13 NMR spectra of *all-trans*-retinal, the propylamine Schiff base of *all-trans*-retinal, and the protonated propylamine Schiff base were measured and interpreted in terms of a charge density–chemical shift correlation by means of the Pople–Karplus equation. A key resonance line (C-14) of the protonated Schiff base was found in a spectral domain normally free from intense protein carbon resonances. The results substantiate the feasibility of a ^{13}C labeling study of the rhodopsin chromophore.

It is presently believed that in rhodopsin (the vertebrate visual pigment) the nitrogen atom of the Schiff base linkage between the 11-*cis*-retinylidene chromophore and a lysine residue of opsin is protonated.[2] This has been suggested by a large bathochromic shift (about 122 nm) in the absorption of rhodopsin as compared to that of 11-*cis*-retinal and by resonance Raman spectroscopy which reveals the presence of a C–N linkage similar to that found in model protonated Schiff bases.[3] To better substantiate this contention it would be useful to probe directly several sites of the chromophore in rhodopsin. Since carbon-13 nuclear magnetic resonance spectroscopy provides a sensitive probe of charge distribution and steric interactions in aromatic and conjugated alkene systems,[4] its use is expected to be particularly appropriate for this purpose.

We present here the results of the first stage of a ^{13}C labeling study of the prosthetic chromophore of rhodopsin in which we have measured and interpreted the ^{13}C-NMR spectra of *all-trans*-retinal (**2**), a model imine of *all-trans*-retinal (**3**), and its protonated species (**4**). The goal of this



stage was to establish the feasibility of the ^{13}C labeling experiment, i.e., to determine if there are key carbon atoms of the chromophore which would resonate in a frequency domain particularly free from typical protein carbon resonances. We also show that ^{13}C NMR is an excellent indicator of changes in electron density in this system.

Experimental Section

The chemicals used for synthesis were of commercial origin. The imine and its salts were prepared according to methods similar to those reported in the literature.[5] The chloride salt was prepared by adding an ether–HCl solution directly to the imine in ether under nitrogen. The resulting precipitate was collected under nitrogen and dried in vacuo. This procedure resulted in a one-to-one proportion of acid to base which was stable at room temperature for at least 12 h.

Visible and uv spectra were taken on a Cary 14 spectrometer for identification purposes. The absorption maxima were identical with those reported in the literature.[5] The ^{13}C NMR spectra were recorded at approximately 27 °C on a Varian Fourier transform XL-100-15 spectrometer (12-mm sample tubes). The solvent was $CDCl_3$ with TMS as an internal standard. Typical conditions were 30° pulses, 0.8 s acquisition time, no pulse delay, 8192 data points, and approximately 10 000 transients per spectrum. Shift reagent experiments were run with 0.1 M solutions in $CDCl_3$; the ratios between $Eu(fod)_3$ and the sample were varied between 0.0 and 0.2.

Results

NMR assignments were made by comparison to previously published data, decoupling experiments, comparison of relative T_1 relaxation times from high pulse rate experiments, application of the shift reagent $Eu(fod)_3$, and titration of the Schiff base with trifluoroacetic acid (to aid in the assignments of the protonated Schiff base). $Eu(fod)_3$ initiated shifts primarily through a contact interaction that extended to C5 and C6, except in those incidences mentioned below.

Chemical shifts for *all-trans*-retinal (**2**), *N-all-trans*-retinylidenepropylimine (**3**), and *N-all-trans*-retinylidenepropyliminium chloride (**4**) in $CDCl_3$ are given in Table I. Figure 1 illustrates the changes in the alkene carbon chemical shifts which take place upon transformation of retinal into a Schiff base and upon protonation of the Schiff base. The signals of even-numbered carbon atoms shift upfield and those of odd-numbered carbon atoms shift downfield upon protonation. Note also the inverse relationship between shift magnitude and distance from the protonation site. The most interesting result is the dramatic upfield shift of C-14 into a region of the spectrum where alkene absorptions are not normally seen. Only one resonance was noted for Cl′ of the imine and C-15, indicating that only one isomer with respect to the C–N bond was present. A $Eu(fod)_3$ induced shift attributed to a pseudocontact mechanism at C-14 implies that the isomer is trans since no pseudocontact

Table I. Assignments of ^{13}C NMR Resonances of *all-trans*-Retinal (**2**), *N-all-trans*-Retinylidenepropylimine (**3**), and *N-all-trans*-Retinylidenepropyliminium Chloride (**4**)

Carbon[a]	2[b]	3[b]	4[b]	Δδ[c]
1	34.24	34.30	34.36	0.06
2	39.63	39.68	39.67	−0.01
3	19.22	19.28	19.17	−0.11
4	33.13	33.13	33.30	0.17
5	130.31	129.73	131.77	2.04
6	137.64	137.82	137.42	−0.40
7	129.57	127.79	132.07	4.28
8	137.12	137.52	136.89	−0.63
9	141.06	137.94	145.33	7.39
10	129.49	130.05	129.55	−0.50
11	132.41	127.79	137.42	9.63
12	134.55	136.09	133.64	−2.45
13	154.46	143.91	162.33	18.42
14	128.99	129.55	120.14	−9.41
15	190.66	159.37	163.65	4.28
1,1-CH_3	28.96	29.00	29.04	0.04
5-CH_3	21.73	21.77	21.88	0.11
9-CH_3	12.98	12.84	13.25	0.41
13-CH_3	12.98	13.02	14.28	1.26
1′		63.99	53.93	−10.06
2′		24.28	22.84	−1.44
3′		11.92	11.19	−0.73

[a] See Formulas **1** and **3** for numbering scheme. [b] In $CDCl_3$ at room temperature, approximately 0.1 M. Spectra were taken on a Varian XL-100 Spectrometer in Fourier transform mode with a thousand 30° pulses, 0.666 s acquisition time for **2**, and 0.80 s for **3** and **4**. Resonances are reported in ppm relative to TMS. [c] $\Delta\delta = \delta(\mathbf{4}) - \delta(\mathbf{3})$.

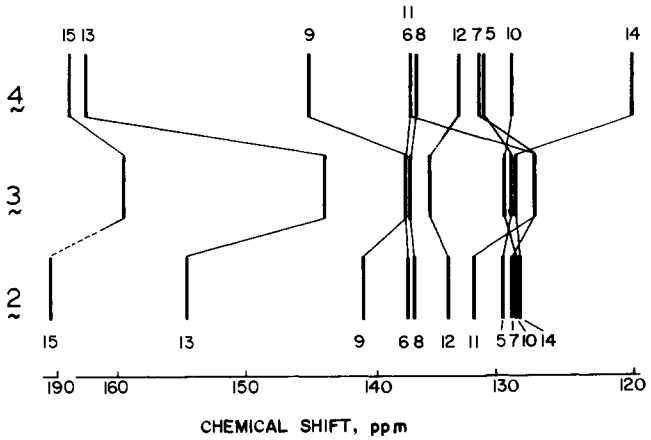


Figure 1. Schematic presentation of the 25.16 MHz Fourier transform ^{13}C NMR spectra (alkene carbon region) of **2**, **3**, and **4** in $CDCl_3$ (approximately 0.1 M).

but rather a contact shift has been observed in *all-trans*-retinal.

Discussion

The pattern which emerges from this series is clearly the one expected for a conjugated polyenic system: an upfield shift of the resonance signals of those carbon atoms expected to experience increased electron densities and an opposite shift for those expected to experience reduced electron densities. On going from the imine **3** to *all-trans*-retinal (**2**), the nitrogen atom is substituted by the more electronegative oxygen which therefore effectively diminishes the electron density in the chain. A comparatively stronger effect is observed upon protonation of the imine (**3** → **4**). In both cases the odd-numbered carbon lines shift downfield and the even-numbered carbon lines shift upfield, with the downfield shifts much more pronounced. The pattern can be interpreted in terms of the possible resonance structures for the retinal and protonated imine in which a positive charge may be placed on each of the odd-numbered alkene carbons, with decreased importance of those resonance structures having a positive charge far from the heteroatom.

Table II. Calculated and Experimental Relative Carbon Chemical Shifts upon Going from Imine **3** to Its Protonated Species **4**

Carbon	Calcd Δδ[a]	Exptl Δδ[a]
5	0.45	2.04
6	−0.27	−0.40
7	3.46	4.28
8	−1.44	−0.63
9	4.05	7.39
10	−1.89	−0.50
11	9.63	9.63
12	−2.88	−2.45
13	16.29	18.42
14	2.43	−9.41
15	34.24	4.28

[a] $\Delta\delta = \delta(\mathbf{4}) - \delta(\mathbf{3})$. Calculated values were normalized for C-11.

Using the Pople-Karplus relation[8] for the ^{13}C screening constant for planar conjugated alkene systems, we made calculations which justify the charge-shift correlation in our system. Carbon chemical shifts in conjugated molecules are a complicated function of charge densities, bond orders, and the electronic excitation energies. The mean value of the shielding tensor for an sp^2 carbon atom in a planar conjugated system is given approximately by the Pople-Karplus relation for the local paramagnetic contribution (diamagnetic and neighboring atom circulation terms are ignored here):

$$\sigma_P^{AA} = [e^2\hbar^2/48m^2c^2a_0^3(\Delta E)][3.25 - 0.35(\rho_A - 1)]^3 \times [2 + \tfrac{4}{9}\lambda_H(1 - P_{ZAZA}) + \tfrac{4}{9}(P_{ZAZB} + P_{ZAZC})]$$

where ΔE is the average electronic excitation energy, ρ_A is the local π-electron density of atom A, λ_H is the polarity parameter for a C_A-H bond, and $P_{\mu\nu}$ are elements of the charge density and bond order matrix. The matrix elements used were those obtained by Cross and Abrahamson[9] from a Pariser-Parr-Pople type calculation. The calculated chemical shifts are given in Table II. Our results show that the contribution of the charge density parameter is the most important and the bond order parameter contribution is small and generally parallels that of the charge density. Due to the neglect of many small factors, the calculated order of chemical shifts did not agree with that found experimentally for carbons with very similar shifts. However, except for C-15, the significant changes induced by protonation in the shifts of the odd-numbered alkene carbons were correctly predicted (see Table II). The calculations also describe properly the lesser changes of the even-numbered carbon lines (both in sign and magnitude).

The results obtained for C-14 and C-15 should be particularly noted. It is obvious that they do not agree with experiment because this section of the molecule departs from the type of structure for which the Pople-Karplus theory was designed. The trend of the C-14 shift can be accounted for if the polarity factor λ_H is appropriately adjusted. On the other hand, the observed small change in shift of C-15 upon protonation is most likely due to both polarity and bond order changes (C-15 experiences an increased tendency toward sp^3 hybridization). Even in the absence of a more advanced theoretical calculation the assignments of C-14

and C-15 lines are straightforward and they can be readily used in our investigation.

It is evident that the mobility of the π electrons allows for extensive delocalization of the positive charge. This mobility proved equally efficient in the case of complexation of the imine with SO_2 where we observed similar shifts as upon protonation. This is to be expected since SO_2 is known to act as a Lewis acid with amines.

For a nuclear magnetic resonance signal to be an acceptable indicator of protonation and perturbation by the protein, it must be in a region relatively free from protein signals. C-14 is likely to be the most informative since it absorbs at about 120 ppm downfield from TMS in the protonated *all-trans*-imine. C-14 in the 11-cis protonated imine should not differ by more than one or two parts per million from that of the all-trans isomer.[10] The protein is expected to contribute 32 to 47 ^{13}C resonances in the region 115 to 125 ppm.[11] Recent work with ribonuclease-S indicates that the line broadening of protonated carbons in macromolecules will not be prohibitive for ^{13}C NMR studies.[12] If C-14 is enriched to 90% ^{13}C and we consider the expected spread of protein signals, we foresee no difficulty in obtaining useful spectra.

On the basis of the results reported here we are synthesizing 11-*cis*-retinal ^{13}C-labeled at C-14. This will be complexed with natural opsin obtained by bleaching bovine rhodopsin. Further labeling of C-13 and C-15 is also being undertaken.

In the ^{13}C-labeled rhodopsin we expect to establish that the Schiff base linkage is indeed protonated. In addition we expect to obtain useful data for the characterization of the effect of the protein environment in the visual chromophore.

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References and Notes

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Organometallic Chemistry of the Transition Metals. XXXII. Some Reactions of Iron Carbonyls with Dimethylenecyclobutane Derivatives[1,2]

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Abstract: 1,2-Dimethylenecyclobutane reacts with $Fe_3(CO)_{12}$ in boiling benzene to give both yellow-orange liquid $C_6H_8Fe(CO)_3$ in which the 1,2-dimethylenecyclobutane functions as a 1,3-diene ligand in the usual manner and yellow crystalline $C_{12}H_{16}Fe(CO)_2$ in which two 1,2-dimethylenecyclobutane units couple to form a symmetrical bis(η^3-allylic) $C_{12}H_{16}$ ligand. 3,4-Dimethylenecyclobutene reacts with $Fe_2(CO)_9$ in tetrahydrofuran at room temperature to give an unstable yellow liquid $C_6H_6Fe(CO)_4$ in which the cyclobutene double bond is bonded to the $Fe(CO)_4$ unit. Mild warming of this complex results in conversion to the shift isomer $C_6H_6Fe(CO)_4$ in which one of the exocyclic double bonds is bonded to the $Fe(CO)_4$ unit. Bicyclo[3.2.0]hepta-1,4,6-triene reacts with $Fe_3(CO)_{12}$ in boiling benzene to give a yellow-orange liquid $C_7H_6Fe(CO)_3$ containing a novel bicyclic cyclobutadiene ligand formed by hydrogen migration.

The thermal dimerization of allene is well known[4] to give a mixture of ~85% 1,2-dimethylenecyclobutane (I) and ~15% 1,3-dimethylenecyclobutane (II). In 1962 one of us (R.B.K.) first observed the reaction of this allene dimer with $Fe_3(CO)_{12}$ to give yellow crystals of stoichiometry $C_{12}H_{16}Fe(CO)_2$. This apparent $(diene)_2Fe(CO)_2$ stoichiometry was totally different from both the known $(diene)Fe(CO)_3$ stoichiometry of iron carbonyl complexes of other 1,3-dienes[5-7] and the $(diene)_2FeCO$ stoichiometry predictable in 1962 from effective atomic number considerations and subsequently demonstrated for butadiene[8] and 1,3-cyclohexadiene[9] complexes. However, the available spectroscopic data on this $C_{12}H_{16}Fe(CO)_2$ complex, including even the proton NMR spectrum, gave no clue at that time concerning possible structures. Even though this $C_{12}H_{16}Fe(CO)_2$ complex was subsequently also characterized mass spectrometrically[10,11] its structure remained a mystery until the start of this work in 1974.

The availability of pulsed Fourier transform carbon-13 NMR spectroscopy provided a new technique of potential value for elucidation of the structure of this apparently anomalous $C_{12}H_{16}Fe(CO)_2$ complex. Accordingly, we repeated the reaction between allene dimer and $Fe_3(CO)_{12}$ in order to obtain some $C_{12}H_{16}Fe(CO)_2$ for carbon-13 NMR