RESONANCE ENHANCED RAMAN SPECTRUM OF ALL-*trans* ANHYDROVITAMIN A

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ABSTRACT The Raman spectrum of all-*trans* anhydrovitamin A in hexane at 77°K is presented. The similarity of the Raman spectra of anhydrovitamin A and the protonated Schiff base of retinal is striking. The implications of this for visual pigment studies and bacteriorhodopsin are discussed. Tentative assignments of geometry for four *cis-trans* isomers of anhydrovitamin A are made on the basis of the observed room-temperature absorption spectra.

INTRODUCTION

It is now accepted that the chromophore of rhodopsin, the vertebrate visual pigment, is the protonated lysyl Schiff base of 11-*cis* retinal (1). Similarly, the purple membrane of the bacterium *Halobacterium halobium* contains as its chromophore a protonated Schiff base of all-*trans* retinal (2). In modeling the possible mechanisms by which these Schiff bases can gain or lose protons, intermediates where the polyene has a retroelectronic configuration are often invoked (3,4). Accordingly, it is of interest to characterize these compounds spectroscopically. We report here the low resolution absorption spectra of four isomers of anhydrovitamin A and the enhanced Raman spectrum for the isomer most likely to be all-*trans*. While the structural assignments for these four compounds are not yet determined with absolute certainty, the spectra and provisional assignments should prove helpful in interpreting the rapidly expanding collection of spectra of retinal based pigments.

ANHYDROVITAMIN A ISOMERS

Preparation

The synthesis of anhydrovitamin A by dehydration of vitamin A using various forms of acid catalysis has been described by several workers (5–8). This reaction was first thought to be one of cyclization, but it is now accepted to be water elimination followed by double bond migrations and finally loss or abstraction of a proton from the β-ionone ring (5,9,10), as shown in Fig. 1. In this work all-*trans* retinol (Eastman-5159, Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.) was subjected to the synthetic scheme described by Zechmeister (6, 11). All chemistry was carried out under red light (no emission 200–500 nm).

Separation of the Isomers

Very little has been said about *cis-trans* isomers of anhydrovitamin A. The assumption has always been that the crystalline material of Shantz (7) was a pure isomer (11). Some caution here

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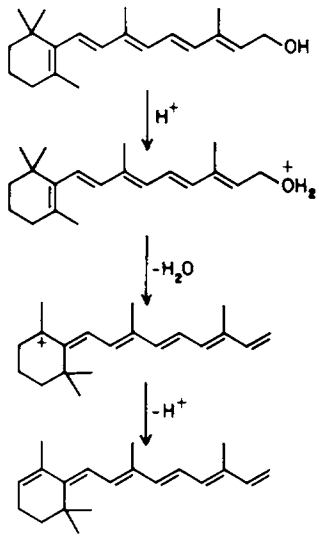

FIGURE 1 Dehydration of vitamin A to give anhydrovitamin A. In our hands the reaction has about a 10% overall yield.

is reasonable, given the fact that carotenoids and vitamin A-related compounds are notorious for their ability to co-crystallize. *Cis-trans* isomers must be expected from such dehydration reactions since different isomers of vitamin A seem to give the same product distribution (8,12).

Column chromatography of anhydrovitamin A on Woelm neutral alumina (ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, Ohio), as described by Christensen and Kohler (11), yields three fractions. Analysis by high pressure liquid chromatography (HPLC) shows that the second fraction, previously presumed to be all-*trans*, contains two major components.

In this work we used a Waters Associates model 201 liquid chromatograph with model 6000A solvent delivery system (Waters Associates Inc., Milford, Mass.). The refractive index detector was bypassed in favor of an ultraviolet-visible (UV-VIS) absorption detector, model SF-770 (Schoeffel Instruments Div., Kratos Inc., Westwood, N.J.). Two 3.9 mm × 30 cm μPorasil (Waters Associates) columns were connected in series and hexane (Burdick & Jackson Laboratories Inc., Muskegon, Mich.) was used as the solvent. Fig. 2 shows a typical chromatogram. Peak A corresponds to the "fast" *cis* isomer found by Christensen and Kohler and peaks B and C together constitute their "all-*trans*" isomer. A few other isomers and impurities can also be seen. The possibility of chemistry occurring on the columns or photochemistry occurring in the detector was ruled out by the observation that a fraction of a peak could be recycled through the apparatus without degradation.

Slightly larger-scale separations can be done by the traditional method of column chromatography. Basic materials such as calcium carbonate and magnesium oxide have been very successfully used for carotenoid separations. We found that four fractions (A–D) could be separated with Woelm basic alumina, activity I, if the alumina was freshly activated by baking at 450°C for 8 h. The second and third fractions (B and C) tend to run together unless a very large amount of alumina is used. A 2.2 × 30 cm column can hold no more than 2.5 mg without losing its ability to separate B and C. Isomer A (10%) comes off the column first with a 5% vol/vol ether-hexane mixture. Isomer B (10%) follows with 7½% ether, isomer C (60%) with 10% ether, and isomer D (20%) with 20% ether. HPLC analysis shows that B and C obtained in this way are pure while A and D contain small amounts of other isomers. A few separations done in this fashion were combined and concentrated by evaporation to yield approximately 1 ml of a hexane stock solution of isomer C.

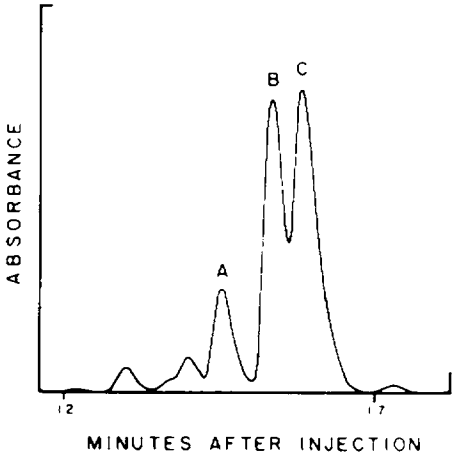

FIGURE 2

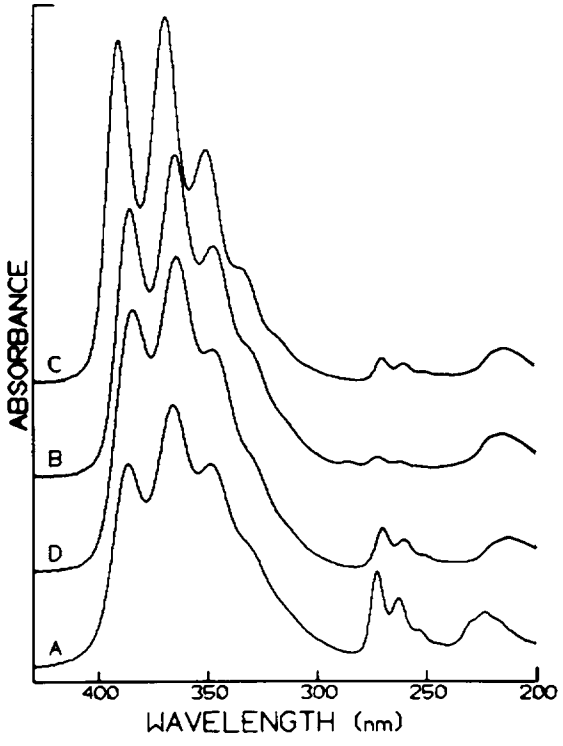

FIGURE 3

FIGURE 2 HPLC of a thermalized anhydrovitamin A sample. The conditions were: 2 μPorasil columns (3.9 mm × 30 cm) in series, hexane solvent, 2 ml/min flow (900 psi), detect absorbance at 368 nm.
FIGURE 3 Room temperature absorption spectra of anhydrovitamin A isomers in hexane. The extinctions are not known except for the ϵ_{max} of isomer C, which is approximately 100,000 l/mol-cm. The spectra are plotted to the same scale and have been normalized to equal areas.

Shantz (5) has commented that anhydrovitamin A is very labile. We found that all the isomers of anhydrovitamin A in solution will convert to nonnegligible amounts of the other isomers within 48 h, even if put in a freezer at −20°C. For this reason, the purified samples were stored under liquid nitrogen and removed only long enough to be mounted in sample holders. All sample preparation was done under red light.

ABSORPTION SPECTRA AND TENTATIVE IDENTIFICATION OF THE ISOMERS

Fig. 3 shows the UV-VIS absorption spectra of fractions A–D in hexane. Since these isomers have not been obtained in crystalline form, the exact extinction coefficients are not known. From Shantz's work (5,7), however, the ϵ_{max} of isomer C is approximately 100,000 l/mol-cm. Three common features can be distinguished in the spectra. The strong absorption from 300 to 400 nm can be assigned to the $1^1A_g^- \rightarrow 1^1B_u$ transition.[1] The "*cis*-peak" from 240 to 280 nm is the $1^1A_g^- \rightarrow 1^1A_g^+$ transition, and the broad high energy band

[1] We are using electronic state notation corresponding to the ideal polyene of 2 mm symmetry as discussed in reference 13.

can be consistently assigned to $1^1A_g^- \rightarrow 2^1B_u$ absorption. The similarity of these spectra contrasts sharply to the "degraded" spectra of *cis* isomers presented by Oroshnik and Mebane (14).

The unambiguous identification of these isomers remains a major problem. To reduce the number of theoretically possible isomers, the concept of stereochemically hindered double bonds is usually invoked (15). If we consider only the all-*trans* and mono-*cis* forms, the five isomers shown in Fig. 4 are possible. Of these, the all-*trans* form is expected to be the least, and the 6-*cis* the most, hindered. The C_{19}—H_{12} interaction in the 10-*cis* isomer is the same form as that which makes 11-*cis* retinal an unfavorable isomer. The C_{18}—H_8 interaction in the 6-*cis* compound is almost certainly stronger than the C_{19}—H_{12} interaction in the 10-*cis* compound. We would expect the relative order of stability to be all-*trans* > 12-*cis* ≃ 8-*cis* > 10-*cis* ≃ 6-*cis*.

Traditionally, *cis-trans* isomers of polyenes have been identified by their absorption spectra. *Cis*-bonds shift the absorption to higher energies and tend to increase the size of the *cis*-peak (15). The size of the *cis*-peak is greatest for central *cis*-bonds. Steric hindrance tends to degrade the vibronic structure of the spectrum and to increase the size of the *cis*-peak (15).

Based on the above considerations, fraction C is most probably the all-*trans* isomer. It has the most resolved vibronic structure, the reddest $1^1A_g^- \rightarrow 1^1B_u$ absorption, and a relatively small *cis*-peak. Fraction A has the largest *cis*-peak and is most logically assigned the 10-*cis* structure. Since fraction B is so closely associated with the all-trans fraction, it is tempting to assign it as the 6-*cis* conformation. However, the observed *cis*-peak intensity, vibronic structure, and steric hindrances would argue for a 12-*cis* assignment. At this point it seems reasonable to assign 8-*cis* to D and 12-*cis* to B, but this is only tentative.

At present, direct physical proof for the geometries of the isomers is not available. While a proton nuclear magnetic resonance (NMR) assignment has been published for anhydrovitamin A (16), the geometry was assumed rather than derived from the spectrum and no

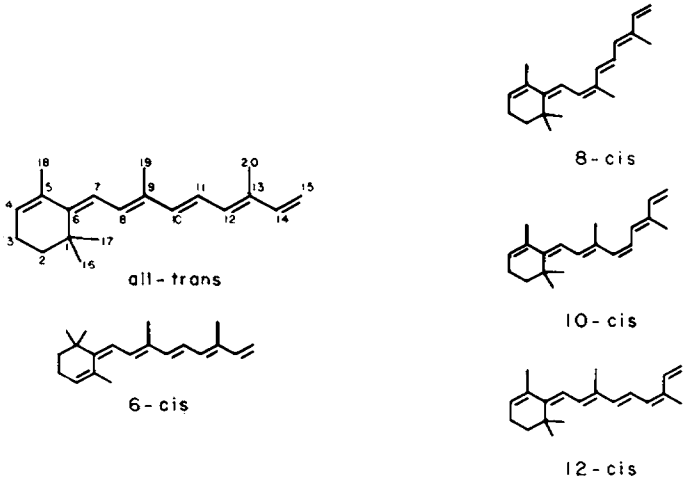

FIGURE 4 All-*trans* and mono-*cis* forms of anhydrovitamin A. The numbering system is from the International Union of Pure and Applied Chemistry (45).

mention was made of the other isomers we have isolated. The X-ray structure of all-*trans* retrovitamin A acid has been reported (17), but it is impossible to compare this with anhydrovitamin A, because the absorption spectra of other retrovitamin A acid isomers has not been published.

Table I gives the positions and relative intensities of the peaks in the absorption spectra of fractions A–D. The values for all-*trans* anhydrovitamin A are somewhat insensitive to small amounts of isomer B as an impurity. A good optical measure of the purity of an all-*trans* (isomer C) sample appears to be the ratio of the first peak (391 nm) to the first valley (381 nm). This ratio is 1.96 for an HPLC pure sample. A 5% impurity of isomer B in isomer C will only change the observed peak ratios of (0.94, 1.0, 0.64) to (0.90, 1.0, 0.67). In contrast, the first peak to valley ratio drops to 1.75.

The final assignment of geometries to isomers A–D will ultimately be based on either X-ray diffraction studies or a detailed analysis of high resolution NMR spectra. In the meantime we are relatively certain that isomer C corresponds to the all-*trans* form.

RAMAN STUDIES OF ALL-*trans* ANHYDROVITAMIN A AT 77°K

When laser excitation is used to excite the fluorescence of anhydrovitamin A, sharp lines are observed on top of the broad fluorescence background. Because the energy separation between these lines and the exciting source remains constant as the laser source is shifted, and because no detectable lifetime for the lines can be determined ($\tau < 10$ ps vs. 3 ns for the fluorescence), they are assigned to Raman scattering.

Raman spectra were obtained with good signal-to-noise when a continuous wave argon ion laser was used for excitation. Although the background fluorescence is greatly reduced in fluid solutions, all measurements were made at 77°K to avoid photochemical or thermal degradation of the sample during the experiment. After several months of use, the UV-VIS absorption, fluorescence, and Raman spectra of these samples did not show any changes.

TABLE I

SPECTRAL CHARACTERISTICS OF ANHYDROVITAMIN A ISOMERS IN HEXANE

Fraction	Peak λ	ϵ/ϵ_{max}
	Å	
A	3,870	0.77
	3,665	1.00
	3,492	0.55
B	3,862	0.83
	3,652	1.00
	3,480	0.71
C	3,912	0.94
	3,695	1.00
	3,512	0.64
D	3,848	0.83
	3,647	1.00
	3,482	0.70

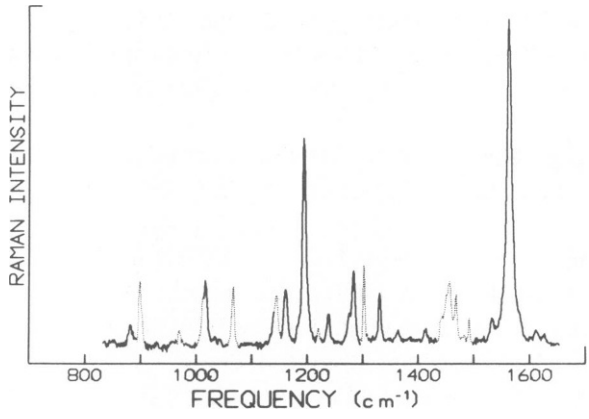

FIGURE 5 Overall view of all-*trans* anhydrovitamin A Raman spectrum in hexane at 77°K. Features attributable to the solvent are shown dotted. The excitation wavelength is 5,145 Å and the intensities have been corrected for instrumental response.

The Experiment

Excitation was by means of an argon ion laser (Spectra-Physics Inc., Laser Products Div., Mountain View, Calif., model 171-05) and the scattered light was analyzed with a one-meter monochromator. Degassed samples were prepared at concentrations of approximately 10^{-4} M in hexane and EPA. Polarized measurements were made on the EPA sample by using side excitation. The laser beam was focused to a long Gaussian waist and passed through the sample normal to the side of the sample cell and as close to the front face as possible. This greatly reduced the amount of reflected laser light and allowed the polarization of the transmitted beam to be visually checked as an indication of the integrity of the optics. The laser power was typically 100–200 mW. The instrumental resolution was better than 4 cm^{-1} in all cases.

Results

Raman spectra were recorded with the 4,545, 4,727, 4,765, and 5,145 Å lines of the laser. Figs. 5 and 6 show the observed spectrum in hexane by using the 5,145 Å laser line. Raman lines from the solvent could also be seen and these were identified by comparison with samples of pure solvent. Table II lists the observed lines and their relative intensities. Nine lines could be seen in all spectra and their shifts matched to within 2 cm^{-1}. The line widths were consistantly 9–11 cm^{-1}, so the height of the peak, corrected for instrumental response, was used as a measure of the intensity. The depolarization ratios[2] measured in EPA are listed in Table III.

DISCUSSION

General Features

The major source of resonance-enhanced Raman intensity in polyenes is a change of geometry in the ^1B_u excited state associated with changes in the π-electron delocalization. A decrease in bond alternation upon excitation to the ^1B_u state lengthens the double

[2]The ratio of $I_y(I_\perp)$ to $I_z(I_\parallel)$ for plane-polarized incident light as described by Woodward in reference 18.

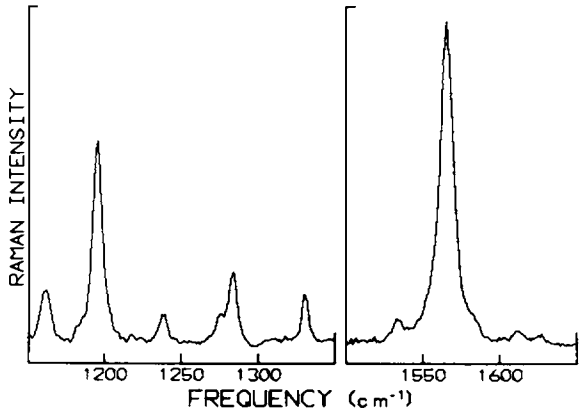

FIGURE 6 Expanded view of the double-bond and fingerprint regions of the all-*trans* anhydrovitamin A Raman spectrum.

bonds. As a result, vibrational progressions in single and double bond stretching modes dominate the absorption spectra, and the Raman intensities are enhanced through an Albrecht A-type mechanism (19). All of the all-*trans* polyenes and carotenoids studied so far show particular enhancement of vibrational modes around 1,500 and 1,200 cm^{-1} (20). According to several theoretical studies, these represent symmetric in-phase stretching modes of the double and single bonds, respectively (21–23). We can thus tentatively as-

TABLE II
RELATIVE INTENSITIES OF OBSERVED RAMAN LINES OF ALL-*trans* ANHYDROVITAMIN A IN HEXANE

Mode	Excitation wavelength			
	5,145 Å	4,765 Å	4,727 Å	4,545 Å
cm^{-1}				
880	0.13	*	*	*
969	0.13	*	*	*
1,016	0.63	0.68	*	*
1,138	0.40	0.36	0.40	0.80
1,160	0.72	0.92	1.00	2.00
1,194	2.80	3.72	4.05	8.00
1,238	0.37	0.60	0.65	1.20
1,275	0.40	0.64	0.69	0.80
1,283	1.07	1.44	1.55	2.20
1,330	0.72	1.24	1.35	2.40
1,413	0.19	<0.1	<0.1	<0.1
1,534	0.40	1.00	1.12	2.00
1,565	5.20	12.1	13.5	22.8
1,611	0.23	<0.1	<0.1	<0.1
1,625	0.13	<0.1	<0.1	<0.1

*Could not be seen because of the slit filter. The intensities are relative to the 1,453 cm^{-1} *n*-hexane Raman line. All values are ±10%. Frequencies are ±2 cm^{-1}.

TABLE III

OBSERVED RAMAN DEPOLARIZATION RATIOS FOR ALL-*trans* ANHYDROVITAMIN A IN EPA

Mode	$I_\perp/I_\parallel$		
	5,145 Å	4,765 Å	4,545 Å
cm^{-1}			
1,016	0.35	0.41	
1,138	0.30	0.32	
1,160	0.40	0.28	
1,194	0.29	0.31	0.33
1,238	0.63	0.30	0.30
1,283	0.37	0.34	0.34
1,330	0.53	0.38	0.32
1,565	0.38	0.32	0.33

All ratios are ±0.05.

sign the 1,565 cm^{-1} mode to a double bond stretch and the 1,194 cm^{-1} mode to a single bond stretch.

The double bond stretch is the strongest line observed in the spectra of polyenes. The number of double bond stretching modes is equal to the number of double bonds in the polyene chain and they are expected to fall within a 100 cm^{-1} range (23, 24). In Fig. 6, at least five Raman modes can be seen between 1,530 and 1,630 cm^{-1}. A blow-up of the single bond (fingerprint) region is also shown in Fig. 6. The single bond in-phase stretching seems to be more prominent in the spectra of all-*trans* carotenoids, as opposed to the *cis* isomers (24).

Several workers have commented on the correlation between the double-bond stretching frequency in Raman spectra and the wavelength of maximum extinction in absorption spectra for carotenoids (23, 25, 26). It is interesting to note here that if λ_{max} is taken to be in the second vibronic band, all-*trans* anhydrovitamin A falls nicely on the curve for hydrocarbon carotenoids (23) and doesn't fit at all on the curve for visual pigments, retinals, and retinal Schiff bases (26).

The features around 969 and 1,016 cm^{-1} are presumably due to methyl stretching modes (23). There has been a suggestion that the 965 cm^{-1} line observed in the protonated Schiff bases of retinal is due to an active out-of-plane C—N bend (27). The results on all-*trans* anhydrovitamin A are consistent with the theoretical interpretation that this mode has a significant admixture of C—CH_3 stretch (28).

Implications for Structural Studies of Visual Pigments

Comparisons of the Raman spectra of rhodopsin and the protonated Schiff base of 11-*cis* retinal, and of isorhodopsin and the protonated Schiff base of 9-*cis* retinal, have been presented in previous literature (29–31). On the basis of the similarities between the spectra of the pigments and the model compounds, the Raman spectra have been used as evidence for the nature of the chromophore in the pigments. While the *cis*-protonated Schiff-base spectra cannot be compared with those for the corresponding retro-derivatives, since the retro-derivatives would be single bond isomers, we can compare spectra for the all-*trans*

isomers. As shown in Fig. 7, there is a remarkable similarity between the positions and the intensities of lines in the Raman spectra of all-*trans* anhydrovitamin A and the protonated all-*trans* Schiff base of retinal.

The 1,658 cm^{-1} line in the protonated Schiff-base spectrum is due to the $C{=}\overset{\oplus}{N}H$ stretch and is not expected to appear in the anhydrovitamin A spectrum. The difference in the double-bond stretching frequency may not be significant, since the double bond frequency for other all-*trans* protonated Schiff bases varies from 1,547 to 1,584 cm^{-1} (25, 26, 29, 31, 32). Rimai has commented on the sensitivity of this frequency (relative to the fingerprint region) to environmental changes (25). Only the 1,330 cm^{-1} anhydrovitamin A line seems to be out of place. The line width difference is a result of the experimental conditions.

Several papers have commented on the multiplicity of ground state resonance structures for protonated Schiff bases and the visual pigments (33). Two of these structures are shown in Fig. 8. These two structures cannot contribute equally to the electronic ground state since the equalization of bond orders this implies is inconsistent with the observed Raman spectra that are qualitatively the same whether or not the molecule is protonated. The similarity shown in Fig. 7 suggests that the retro structure may be important in determining the Raman spectrum.

The clearest piece of structural information derived from Raman spectra appears to be the 1,660 cm^{-1} line, due to the protonated Schiff base. This line shifts to 1,630 cm^{-1} when a deuterated Schiff base is formed, as predicted by the change in reduced mass of the $C{=}\overset{\oplus}{N}H$ group, and is not present in nonprotonated Schiff bases. However, the disappearance of the 1,660 cm^{-1} line in one of the visual pigment intermediates does not necessarily prove that the Schiff base has been deprotonated at the lysyl nitrogen. Abstraction

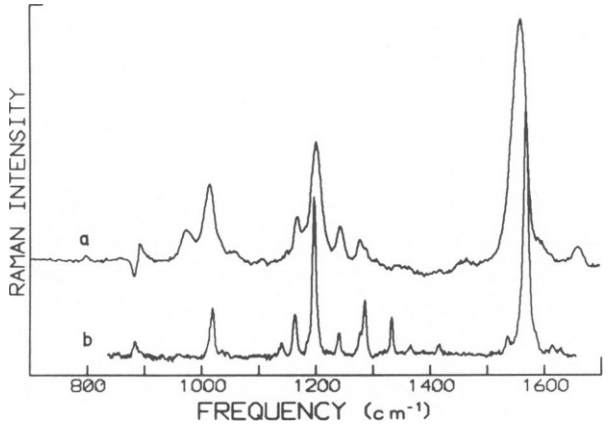

FIGURE 7

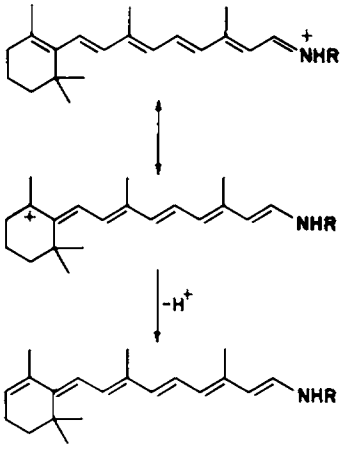

FIGURE 8

FIGURE 7 Comparison of the Raman spectra of (*a*) the chloride salt of *n*-butylamine protonated Schiff base of all-*trans* retinal in ethanol at room temperature and (*b*) all-*trans* anhydrovitamin A in hexane at 77°K. The Schiff base spectrum is reproduced from reference 36 with the permission of Drs. Stryer and Mathies.

FIGURE 8 Two resonance forms for the protonated Schiff base of retinal and its conversion to a retro compound by deprotonation.

of a ring proton, as shown in Fig. 8, would lead to an anhydrovitamin A analogue whose Raman spectrum in the 1,500–1,700 cm^{-1} region would look very similar to that of a non-protonated Schiff base.

Although a retro-structure has been proposed for bathorhodopsin (3, 4), we are not seriously advancing it here. Bathorhodopsin may or may not have a retro-configuration. What we wish to emphasize is the caution one must exercise in interpreting Raman spectra of visual pigments in terms of chromophoric structure.

Implications for Bacteriorhodopsin

Bacteriorhodopsin is found in purple patches of the cell membrane of halophilic bacteria (2). Although its physiological function differs from animal visual pigments, both contain the chromophore retinal bound as a Schiff base to a lysine residue of an apoprotein, and both undergo similar light-induced spectral changes. Upon absorption of light, the light-adapted form of bacteriorhodopsin (bR_{570}) decays through several steps to a blue intermediate (bM_{412}). This intermediate eventually cycles back to bR_{570}, forming a regenerative cycle. The net result of this cycle is to pump protons across the cell membrane, which is thought to drive the synthesis of ATP (2, 34, 35).

Unlike most pigment intermediates and retinal related compounds, bM_{412} exhibits a three-peaked absorption band (36). Intermediates of the reconstituted bacteriorhodopsin (37) and a reduced chromophore obtained by treatment of purple membranes with borohydride and light or acid (37–39) also show peaked absorption spectra. Such structure is consistent with a retro-configuration of the retinal chromophore and with chromophore-protein interactions leading to coplanarity of the hexene ring and side chain (37).

The product formed by photolysis or acid treatment of the borohydride-reduced purple membrane is most probably anhydrovitamin A. The structured absorption and excitation spectra are very similar to some of our anhydrovitamin A isomers. Treatment of the purple membrane with borohydride and light reduces the Schiff base to an amino compound. Protonation of the amine nitrogen by acid or by the protein under photolytic conditions could then lead to the formation of anhydrovitamin A by a mechanism directly analogous to that shown in Fig. 1. An observation that anhydrovitamin A is noncovalently bound by the bacterial opsin would help confirm this hypothesis.

Several Raman studies of bacteriorhodopsin and its intermediates have been done (26, 40–44). Their interpretation has led to the conclusion that bR_{570} is a protonated Schiff base, whereas bM_{412} is unprotonated. The correspondence between bacteriorhodopsin Raman spectra and model compounds is not as clear as that for the visual pigments, however. Similarities and differences between the Raman spectra of bM_{412}, all-*trans*-retinal *n*-butylamine, and 13-*cis*-retinal *n*-butylamine have been discussed by El-Sayed (42) and Ebrey (26), who conclude that the chromophoric structure found in bM_{412} is not identical with the structure found in solution.

We wish to reemphasize the caveat given in the previous section by comparing the spectral properties of bM_{412} with those of all-*trans* anhydrovitamin A. The prominent 1,567, 1,195, 973, 1,011, 1,124, 1,278, 1,338, and 1,623 cm^{-1} lines of bM_{412} are closely matched by the 1,565, 1,194, 969, 1,016, 1,138, 1,275, 1,330, and 1.625 cm^{-1} lines of anhydrovitamin A. The 1,194 cm^{-1} line is not as prominent in bM_{412}, however, and 1,283 cm^{-1} is absent. In addition, anhydrovitamin A shows no strong 1,183 cm^{-1} mode. The differences may be the

result of distortion of the polyene chain due to chromophore-protein interactions. If this distortion involved torsions of the polyene single bonds, it would also decrease the vibronic structure of the absorption spectrum, perhaps to that found for bM_{412}.

Quite clearly, considerable further work is necessary before Raman spectra can be confidently interpreted in terms of molecular structure for systems as complicated as rhodopsin. It is primarily to contribute to the pool of spectra that will be needed that we report these spectra for the retro-compounds. Of course, the striking similarity between the retro-spectra and those of the natural pigments could be interpreted as suggesting some new models for these light-driven processes, based on the idea that the retro-structure is dominant in the ground state. While this is a stimulating exercise, we also feel it is premature.

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