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Properties of Several Sterically Modified Retinal Analogs and Their Photosensitive Pigments†

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ABSTRACT: Retinal analogs which have extra (14-methylretinal and methylretinone) or displaced (13-desmethyl-14-methylretinal) methyl groups were synthesized and their isomers isolated by high-pressure liquid chromatography. The 11-cis isomer of 14-methylretinal has its λ_{max} 30 nm to shorter wavelengths than 11-*cis*-retinal; we suggest that this is due to the fact that the 14-methyl analog is unable to assume the same twisted s-cis conformation available to 11-*cis*-retinal. Thus, the seemingly anomalous long-wavelength position of the absorption band of 11-*cis*-retinal is due to its being primarily in a twisted ($\phi_{12-13} \simeq 40°$) s-cis conformation. Three isomers of 14-methylretinal, 11-cis, 9-cis, and 9,13-di-cis, reacted with cattle opsin to form photosensitive pigments. The absorption spectra, circular dichroism spectra, extinction coefficients, and photosensitivities of the 11-cis pigments of retinal (rhodopsin) and 14-methylretinal were similar. This suggests that the conformation of these two chromophores, when attached to opsin, is similar and different from that of crystalline 11-*cis*-retinal. The 9-cis isomer of 13-desmethyl-14-methylretinal also combines with opsin. No reaction of opsin with any isomer of methylretinone (15-methylretinal) was detected.

The 11-cis isomer of retinal (or its 3,4-dehydro derivative) is the chromophore of all known visual pigments (Wald, 1968). A great deal is understood about the spectroscopic properties of retinals and it now appears that many of the important differences between their spectra and those of visual pigments can be explained in terms of the protonated Schiff-base linkage that is formed between the retinal and a lysine of the apoprotein opsin. However, the detailed spectroscopic and photochemical properties of visual pigments are not well understood. In addition, a number of important problems pertaining to retinal itself are unresolved (see Honig and Ebrey, 1974; Ebrey and Honig, 1975; Honig et al., 1975). In this paper we continue our studies of the spectroscopic and photochemical properties of sterically modified retinal analogs and the photosensitive pigments that result from their combination with opsin (Chan et al., 1974).

In the absence of steric hindrance, π-electron systems may be assumed to be planar. All isomers of retinal have a

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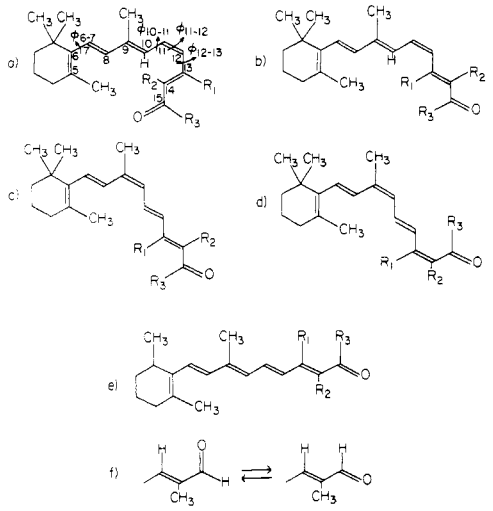

FIGURE 1: Conformations of retinals and retinal analogs. All are drawn 6-s-cis: retinal—$R_1 = CH_3$, $R_2 = H$, $R_3 = H$; 14-methylretinal—$R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$; 13-desmethyl-14-methylretinal—$R_1 = H$, $R_2 = CH_3$, $R_3 = H$; methylretinone (15-methylretinal)—$R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$; (a) 12-s-cis, 11-cis; (b) 12-s-trans, 11-cis; (c) 12-s-trans, 9-cis; (d) 12-s-trans, 9,13-di-cis; (e) all-trans; (f) 14-s-cis ↔ 14-s-trans.

steric hindrance, which precludes a planar ring–chain linkage at 6-C–7-C, and as a result are twisted about ϕ_{6-7}. While a twisted ring–chain conformation appears to be common to all retinal isomers, the existence of considerable steric hindrance in the side chain is unique to the 11-cis isomers, in which steric hindrance between 13-CH_3 and 10-H causes out-of-plane twisting in the 10-C to 13-C region. It is clear that the molecule must twist about either the 10-C–11-C or 12-C–13-C bond in order to alleviate this steric hindrance, and several studies have shown that the primary mode of twisting is about the 12-s bond (Patel, 1969; Honig and Karplus, 1971; Rowan et al., 1974). Both s-cis (Figure 1a) and s-trans (Figure 1b) conformations[1] are possible for this bond, which is predicted to be highly flexible (Honig and Karplus, 1971; Warshel and Karplus, 1974). In crystalline 11-*cis*-retinal the s-cis form seems to predominate (Gilardi et al., 1971; Hamanaka et al., 1972), but there is evidence that both conformations are present in solution (Rowan et al., 1974).

11-*cis*-Retinal absorbs slightly to the red of the planar side-chain cis isomers. This is surprising since, in general, out-of-plane twisting of the π-electron system of 11-*cis*-retinal would be expected to shift the spectrum to shorter wavelengths relative to planar isomers. This anomaly has been explained by assuming that 11-*cis*-retinal has a 12-s-cis conformation (Honig and Karplus, 1971) since s-cis structures in polyenes are known to absorb at longer wavelengths than the s-trans conformers (Simmons, 1970). Thus, Honig and Karplus suggested that the shift to shorter wavelengths, due to twisting, would cancel the shift to longer wavelengths due to the s-cis conformation, resulting in a spectral maximum in the vicinity of the other isomers. Evidence supporting this explanation has been presented by us in a preliminary study (Chan et al., 1974).

The conformation of the chromophore affects several of the most important properties of visual pigments. These include the specificity of opsin for certain isomers of retinal, the isomeric composition of the products of photoisomerization of the 11-cis chromophore in the pigment compared to 11-*cis*-retinal in solution, and the high quantum efficiency of bleaching. In addition, there is some evidence that in the first two intermediates of the bleaching process, prelumi- and lumirhodopsin, opsin has a similar conformation, which suggests that differences in the conformation of the chromophore are responsible for the spectral differences between these two intermediates (Ebrey and Honig, 1972). The specific geometry of the 11-cis chromophore when bound to the opsin is not known, but its conformation is likely to be different than that of free retinal in solution. Retinal itself is a highly flexible molecule and can assume a number of different possible conformations at room temperature which have the same or very similar energies. One purpose of this paper is to study what conformations are available to retinal when bound to the protein since these may be different than those that predominate in solution. First, the greater delocalization of the π electrons in the protonated Schiff base should make the double bonds more flexible and the single bonds less flexible. Second, the opsin itself might provide the energy necessary to force the isomers of the protonated Schiff base of retinal into geometries that differ from their equilibrium conformation in solution.

A number of studies indicate that the binding site of the chromophore is highly restrictive (see Ebrey and Honig, 1975). On the other hand, besides the 11-cis isomer, the 9- and 9,13-di-cis isomers also couple with opsin[2] so that an unanswered question is the degree of flexibility of the binding site.

In this paper we present additional experimental evidence in an attempt to understand the anomalous spectral properties of 11-*cis*-retinal and the influence of chromophore structure and conformation on pigment properties. Our approach has been to synthesize retinal-like molecules where the basic retinal structure has been modified in a specific and predictable way so that any changes in spectral properties should be explainable in terms of structural parameters.

Materials and Methods

All work was carried out under dim red light and the chromophores were stored under argon at Dry Ice temperature. A JASCO concave radiating monochromator, Model CRM-FA, equipped with a 3-kW xenon source was employed for irradiations. The absorption spectra were recorded on a Cary Model 14 spectrophotometer. The nuclear magnetic resonance (NMR) spectra were taken at three institutions; JOEL PS-100, Columbia University, New York, N.Y.; Varian HR-200, Rockefeller University, New York, N.Y.; and Bruker 270, Bell Laboratories, Murray Hill, N.J. All spectra were recorded in $CDCl_3$ and in the Fourier transform mode.

Preparation of Analogs. β-Ionylidenecrotonaldehyde (C_{17} aldehyde)–ethyl β-ionylidenecrotonate was obtained in low yields by Reformatsky reaction of β-ionone and ethyl α-bromocrotonate. After purification by thin-layer chromatography (TLC) the ester was converted by reduction with diisobutylaluminum hydride and immediate oxidation with

[1] In the following, s-cis is defined as 0° and s-trans as 180°; angles less than 90° are described as s-cis and angles larger than 90° as s-trans.

[2] We have recently learned that several 7-cis isomers of retinal also will form pigments (7-cis; 7,9-di-cis; 7,9,13-tri-cis) (R. Liu and W. DeGrip, private communication).

manganese dioxide to the methyl aldehyde with a 67% yield. The methyl aldehyde was a 50:50 mixture of 9-cis and all-trans isomers; these were separated by high-pressure liquid chromatography (HPLC) and identified by NMR.

14-Methylretinal. Reaction of the C_{18} ketone (mixture of trans and 9-cis) prepared from the C_{17} acid and methyllithium, with ethyl (diethylphosphono)-2-propionate gave the methyl ester in 95% yield. The aldehyde was obtained by reduction and oxidation of the methyl ester. The all-trans isomers were separated by HPLC and irradiated as above, and the resulting isomers (13-, 11-, and 9-mono-cis, and 9,13-di-cis) were separated by TLC and HPLC (Figure 2) and identified by NMR.

13-Desmethyl-14-methylretinal. Reaction of the C_{17} aldehyde (the 9-cis and trans isomers were reacted separately) and ethyl (diethylphosphono)-2-propionate as above gave the ethyl 13-desmethyl-14-methylretinate in 95% yield. Reduction and oxidation as above led to the respective aldehydes. Irradiation of the all-trans aldehyde (400 nm, 20 min, ethanol) followed by HPLC separation of the isomers afforded only the 9-cis and 13-cis in quantities sufficient to be characterized.

15-Methylretinal. Methyllithium (2 equiv) was added dropwise to all-trans vitamin A acid (1 equiv) in ether under nitrogen at 0°. After 1 hr the solution was poured onto ice and the ether layer was separated, dried, and concentrated. The yellow oil was purified by silica gel column chromatography, irradiated as above, and purified by HPLC. The cis isomers were separated from all-trans and combined for pigment formation. The isomeric mixture was identified by infrared (ir) (1670 cm^{-1}, C=O), mass (M^+ = 298), and ultraviolet (uv) [λ_{max} 367 nm (hexane)] spectroscopy.

Purification of Retinals. Waters PIC-100 and 202 liquid chromatography were used with uv 254-nm detectors. The columns were 3 × 3 ft × ⅜ in. Corasil II and the solvents were 1 to 6% ether in hexane. These conditions were sufficient to separate the retinal isomers on a single run, although some difficulty was encountered in the separation of pure 11-*cis*-14-methylretinal and a recycle technique had to be used. As expected from polarity considerations, the order of elution was the cis isomers first and trans isomers last. The relative order of cis isomers had to be checked carefully for, although the order was the same for the retinal and 14-methylretinals, it was different for the 13-desmethyl-14-methylretinals. Variations have also been noted in the order of elution of other retinal-like compounds which we have purified. The isomers were identified by NMR (see Tables I and II).

Preparation of the Pigments. The artificial photosensitive pigments studied below were prepared by a method similar to that of Zorn and Futterman (1971). The chromophores were allowed to react with bleached rod outer segment membranes, purified as previously described (Ebrey, 1971). The chromophores, dissolved in a small amount of Triton X-100 and ethanol, were added to the bleached rods and nitrogen placed over the mixture before incubation for 2–3 hr at 37°. The suspended rods containing the synthesized pigment were spun down, freeze-dried, and most of the unreacted chromophore removed by extracting overnight with petroleum ether. The rods were pelleted from the petroleum ether, again freeze-dried, and extracted with 2% Triton X-100 or 2% Ammonyx LO in 0.067 *M* phosphate buffer. It is especially important that the optical density at 380 nm, due to the free chromophore, not be greater than about 1 for samples used in the circular dichroism (CD) experiments or serious distortions of the CD spectra can occur. In a few cases, an attempt was made to purify the new pigment so that the entire absorption spectrum could be measured. To do this, a relatively large amount of the synthetic pigment in the rods was extracted with Ammonyx LO and then run on a $CaPO_4$ column as previously described (Shichi et al., 1969; Ebrey, 1971).

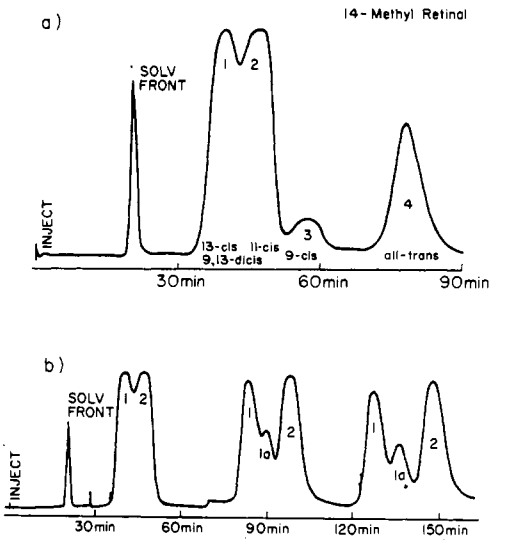

FIGURE 2: High-pressure liquid chromatography of (a) irradiated *all-trans*-14-methylretinal, Corasil II, 3 ft × 3 (prep); 3 ml/min; 1.5% ether in hexane; (b) recycling of bands 1 and 2: (1) 13-*cis*-; (1a) 9,13-di-*cis*-; (2) 11-*cis*-; (3) 9-*cis*-; and (4) *all-trans*-14-methylretinal.

Measurements of Pigment Properties. The absorption maxima of the purified pigments could be read directly from the spectra; the maxima of the unpurified pigments were determined by subtracting the spectrum before and after bleaching with orange light. It is common in such difference spectra measurements to add hydroxylamine to speed the disappearance of the intermediates of bleaching and prevent adventitious Schiff base formation. With the "strong" detergents used, either Triton X-100 or Ammonyx LO, the intermediates decayed rapidly without adding hydroxylamine. Moreover, in these detergents there is no adventitious Schiff base formation as demonstrated, not only by the lack of any pigment formation with the all-trans and 13-cis isomers, but also by the lack of any immediate change in the absorption spectrum upon addition of hydroxylamine.

The circular dichroism spectra of the pigments were measured on a JASCO J-40 recording spectropolarimeter. The spectra were scanned rapidly to minimize bleaching of the pigments by the measuring light. The rapid scanning speeds caused small shifts (3–5 nm) in the maxima of the circular dichroism bands that have been corrected in the spectra presented.

Photosensitivity and Extinction Coefficient. The photosensitivities of the pigments, relative to the photosensitivity of native rhodopsin, were directly determined by measuring the rate of bleaching of the pigment with monochromatic (λ 540 or 560 nm) light. Care was taken to see that the optical density at the irradiation wavelength was low, that the bleaching light uniformly illuminated the sample (Dartnall et al., 1936), and that the intensity of the bleaching light was constant during the entire time of measurement for both rhodopsin and the pigment whose photosensitivity was

Table I: Chemical Shifts of Retinals and Retinal Analogs in $CDCl_3$.

	6-CH_3	5-CH_2	9-CH_3	13-CH_3	13-H	14-CH_3	14-H	15-H	12-H	11-H	10-H	8-H	7-H
all-trans-Retinal[a]	1.04	1.72	2.03	2.33			5.98	10.12	6.37	7.15	6.20	6.18	6.36
14-Methyl	1.04	1.73	2.04	2.32		1.91		10.26	6.79	7.17	6.26	6.14	6.37
13-Desmethyl-14-methyl[b]	1.02	1.71	2.01		6.97	1.86		9.44	6.66	7.09	6.22	6.15	6.35
13-*cis*-Retinal[a]	1.04	1.72	2.02	2.14			5.85	10.20	7.28	7.05	6.23	6.18	6.35
14-Methyl	1.05	1.72	2.01	2.12		1.89		10.31	7.26	6.95	6.08	6.12	6.25
13-Desmethyl-14-methyl[b]	1.03	1.73	2.04			1.90		9.53					
9-*cis*-Retinal[a]	1.05	1.75	2.00	2.30			5.94	10.07	6.27	7.20	6.06	6.64	6.31
14-Methyl	1.06	1.77	2.03	2.31		1.92		10.25	6.73	7.31	6.16	6.71	6.31
13-Desmethyl-14-methylretinal[b]	1.05	1.75	2.03		6.95	1.87		9.43	6.59	7.14	6.12	6.69	6.32
11-*cis*-Retinal[a]	1.02	1.71	1.99	2.36			6.07	10.10	5.92	6.69	6.54	6.14	6.32
14-Methyl	0.98	1.69	1.97	2.32		1.72		10.22	5.92	6.59	6.07	6.08	6.30
9,13-Di-*cis*-retinal[a]	1.05	1.77	2.05	2.15			5.87	10.27	7.25	7.16	6.16	6.68	6.36
14-Methyl	1.05	1.75	2.01	2.11		1.88		10.34	7.23	7.00	6.10	6.67	6.28

[a] See Patel (1969). [b] Bruker 270-MHz NMR; Dr. Dinshaw Patel, Bell Laboratories. The others were with Varian HA-100 and HR-200 MHz instruments.

Table II: Coupling Constants (Hz) of 14-Methyl- and 13-Desmethyl-14-methylretinals.

Isomer	Compd	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$	$J_{12,13}$
All-trans	14-Methyl	16.1	11	15.1	
	13-Desmethyl-14-methyl	16.5	12	15	12
13-Cis	14-Methyl	16	11	15	
11-Cis	14-Methyl	16.1	12.3	12	
9-Cis	14-Methyl	17	11.8	15	
	13-Desmethyl-14-methyl	16	12	15	12
9,13-Di-cis	14-Methyl	16.2	10.3	15.1	

unknown. In control experiments with rhodopsin, the optical density fell exponentially with time, as expected when a single species is bleached uniformly. We assume that the quantum efficiency of bleaching is the same at λ_{max} and at λ_{bleach}; this has been shown to be true for rhodopsin (Dartnall, 1972) and in all likelihood is very nearly true for every pigment. If the photosensitivities and extinction coefficient of a pigment are known, then the quantum efficiency of bleaching relative to rhodopsin can be directly determined. Moreover, since the quantum efficiency for photobleaching rhodopsin has been found to be about 0.67 (see Dartnall, 1972) it is possible to calculate the quantum efficiency for the photobleaching of the artificial pigment.

The extinction coefficient of the 11-*cis*-14-methylretinal pigment was determined essentially by the method of Wald and Brown (1954). The pigment was bleached with long-wavelength light either in the presence or absence of hydroxylamine. From the known extinction coefficient of the presumed product of bleaching, *all-trans*-retinal or retinal oxime, the number of moles of pigment present in the sample can be determined and hence, from the optical density, the extinction coefficient. There are a number of uncertainties with this method and these will be discussed in the Results section.

Results

Free Retinals. 14-Methylretinal. Most isomers of 14-methylretinal have conformations and spectral properties similar to those of the corresponding retinals. NMR chemical shifts and coupling constants are given in Tables I and II. The values of the chemical shifts are similar to those of the analogous retinals, and suggest that the conformations of the isomer are similar (Patel, 1969). The data for the 11-cis isomers indicate, as pointed out by Patel, that the steric hindrance of these isomers is relieved by twisting about the 12-s bond. Although it is possible to tell from NMR the bond(s) at which twisting takes place, it is not possible to obtain information about the angle of twisting. The all-trans, 9-cis, 13-cis, and 9,13-di-cis isomers of 14-methylretinal, like the nonhindered isomers of retinal, appear to have planar chains.

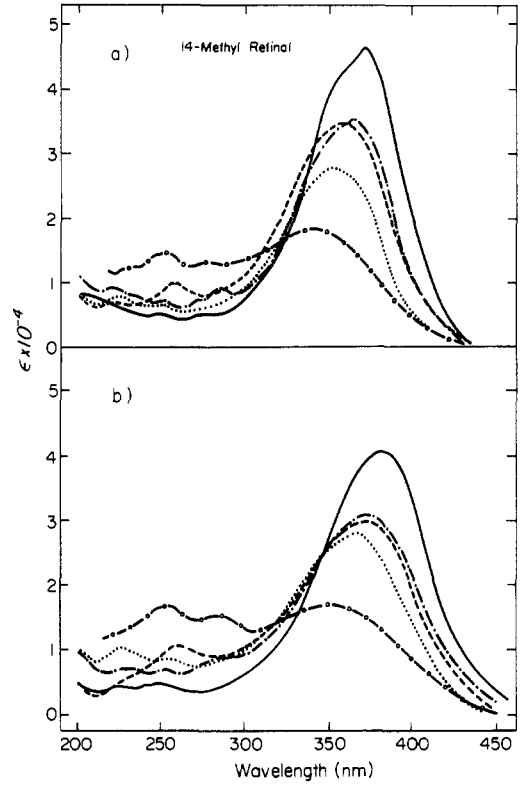

FIGURE 3: Absorption spectra of 14-methylretinal isomers at room temperature in (a) *n*-hexane; (b) ethanol; (—) all-trans; (- · -) 9-cis; (---) 13-cis; (· · ·) 9,13-di-cis; and (-O-) 11-cis.

The spectral data for the isomers are given in Figure 3 and summarized in Table III. The absorption spectra of most isomers of retinal and modified retinals are similar. However, there is a relatively large difference in the absorption spectrum of the 11-cis isomer in that the λ_{max} of 14-methylretinal is shifted toward shorter wavelengths by about 25 nm and the subsidiary bands are far more intense. To observe the temperature dependence of the absorption of

Table III: Absorption Maxima (nm) and Extinction Coefficients ($\epsilon \times 10^{-4}$) of Retinals and Modified Retinals at Room Temperature.

Isomer	Compound	*n*-Hexane	Ethanol
All-trans	Retinal[a]	368 (4.75)	381
	14-Methyl	373 (4.6)	382 (4.2)
	13-Desmethyl-14-methyl	368 (5.4)	377 (5.0)
13-Cis	Retinal[a]	366 (3.86)	372
	14-Methyl	358 (3.5)	372 (3.0)
	13-Desmethyl-14-methyl	366 (3.5)	378 (3.36)
11-Cis	Retinal[a]	363 (2.62)	376
	14-Methyl	338 (1.8)	350 (1.67)
9-Cis	Retinal[a]	363 (3.98)	
	14-Methyl	365 (3.5)	378 (3.1)
	13-Desmethyl-14-methyl	362 (3.5)	372 (3.6)
9,13-Di-cis	Retinal[a]	357 (3.58)	
	14-Methyl	353 (2.8)	367 (2.7)

[a] Hubbard, 1956.

Table IV: Circular Dichroism Data.[a]

Chromophore	Isomer	λ_{max} (nm) α	λ_{max} (nm) β	θ_{max}/A_{max}, α	R^c (dm) α	R^c (dm) β	R_α/R_β
Retinal	11-Cis	486	335	5.3	0.33	0.63	0.52
14-Methyl-retinal	11-Cis	497	338	6.9	0.43	0.60	0.79
13-Desmethyl-retinal	11-Cis	485	325	4.5			0.25
Retinal	9-Cis	480	432	6.2	0.31	0.22	1.4
Retinal	9,13-Di-cis	477	431	5.7	0.29	0.21	1.4
14-Methyl	9-Cis	485	334	9.2			2.1
14-Methyl	9-Cis	484	334	9.4			2.2
13-Desmethyl[d]	9-Cis	478	330	6.4			1.5
13-Desmethyl-14-methyl	9-Cis	487	330	6.8			2.4

[a] In 2% Triton X-100, unless indicated otherwise. [b] θ_{max} = ellipticity at λ_{max} in millidegrees; A_{max} = absorbance at λ_{max}. [c] The rotational strength (R) of a circular dichroic (CD) band is calculated from the formula $R = 1.23 \times 10^{-42} [\theta]_{max} \Delta/\lambda_{max}$, where $[\theta]_{max}$ is the molar ellipticity at λ_{max}, Δ is the wavelength interval in which $[\theta]$ falls from $[\theta]_{max}$ to $[\theta]_{max}/e$, and λ_{max} is the wavelength of maximum ellipticity for a particular CD band. The ϵ of the pigments is given in Table V. [d] In 2% digitonin solution; Kropf et al., 1973.

Table V: Photosensitivities of Natural and Artificial Pigments.

Chromophore	Isomer	Photosensitivity Rel. to Rhodopsin at λ_{max}	Extinction Coeff.[d] at λ_{max}	Quantum Efficiency for Bleaching
Retinal	11-Cis	1.00	42,000 ± 2000	0.67[a]
Retinal	9-Cis	0.45	44,500 ± 4000[b]	0.30
Retinal	9,13-Di-cis	0.45	42,000 ± 4000	0.30
14-Methylretinal	11-Cis	1.1	38,000 ± 5000	0.77
14-Methylretinal	9-Cis	0.37		
14-Methylretinal	9,13-Di-cis	0.37		
13-Desmethyl-14-methylretinal	9-Cis	0.67		
13-Desmethyl-retinal[c]	11-Cis	0.33		

[a] Dartnall, 1972. [b] Hubbard et al., 1972. [c] Kropf et al., 1973. [d] In Ammonyx LO.

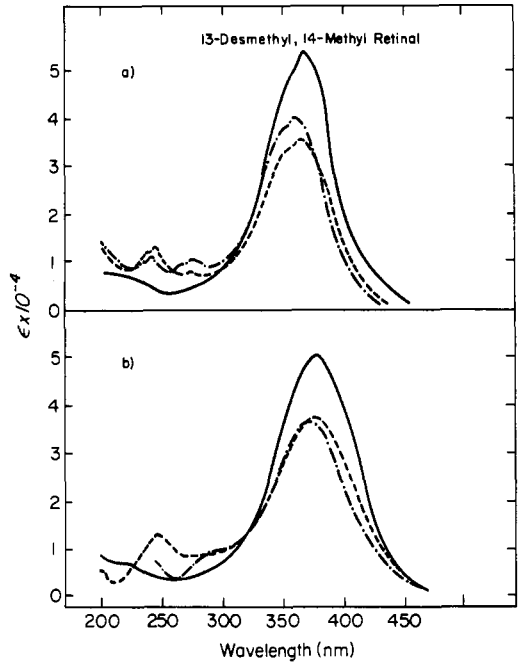

FIGURE 4: Absorption spectra of 13-desmethyl-14-methylretinal isomers at room temperature in (a) *n*-hexane; (b) ethanol; (—) all-trans; (---) 13-cis; and (– · –) 9-cis.

11-*cis*-retinal and the corresponding 14-methyl analog, absorption spectra were taken in EtOH at room temperature and at −105°. The main band of 11-*cis*-retinal had a red shift, from 376 to 387 nm with ϵ increasing to 1.31 of its room temperature value (see Jurkowitz et al., 1959). In contrast, 11-*cis*-14-methylretinal had a smaller shift, 350 to 353 nm, and ϵ increased to a value of only 1.13 of its room temperature value.

13-Desmethyl-14-methylretinal. The NMR spectra of the 13-desmethyl-14-methylretinals were more complicated due to the additional vinyl proton at 13-C (Tables I and II). The striking feature of the NMR spectra of this retinal analog is the large upfield shift for 15-H. The probable origin of this upfield shift is discussed below. Absorption data are given in Figure 4 and Table III.

Pigments. 14-Methylretinal Pigments. Five isomers of 14-methylretinal were tested for their ability to react with opsin to form photosensitive pigments. The all-trans and 13-cis isomers did not couple with opsin, but the 11-cis, 9-cis, and 9,13-di-cis isomers yielded pigments.

The 11-*cis*-14-methyl pigment was purified on a calcium phosphate column (Shichi et al., 1969; Ebrey, 1971). Using as a criterion for purity the ratio of the extinctions at 280 and 502 nm, it is possible to estimate that for the pure 11-*cis*-14-methyl pigment, the $\epsilon_{280}/\epsilon_{502} \simeq 1.7$. This is based on the following estimates and measurements: (i) the ϵ of the pigment at 502 nm is 38,000 ± 5000 (measured); (ii) the ϵ of the pigment at 280 nm is 65,000. Of this, that of opsin is 45,000 (Govindjee and Ebrey; unpublished), while the ϵ of the chromophore at 280 nm is on the order of 20,000 (estimated). The best sample we could obtain had 280 nm/502 nm ratios of 2.0, and this would indicate that the pigment is fairly pure.

The absorption spectrum of the 11-*cis*-14-methyl pigment is shown in Figure 5. λ_{max} of the α band is at 502 nm. A large and quite prominent β band has λ_{max} at 350 nm. The ratio of the $\epsilon_\beta/\epsilon_\alpha \simeq 0.33$ for the 11-*cis*-14-methyl pig-

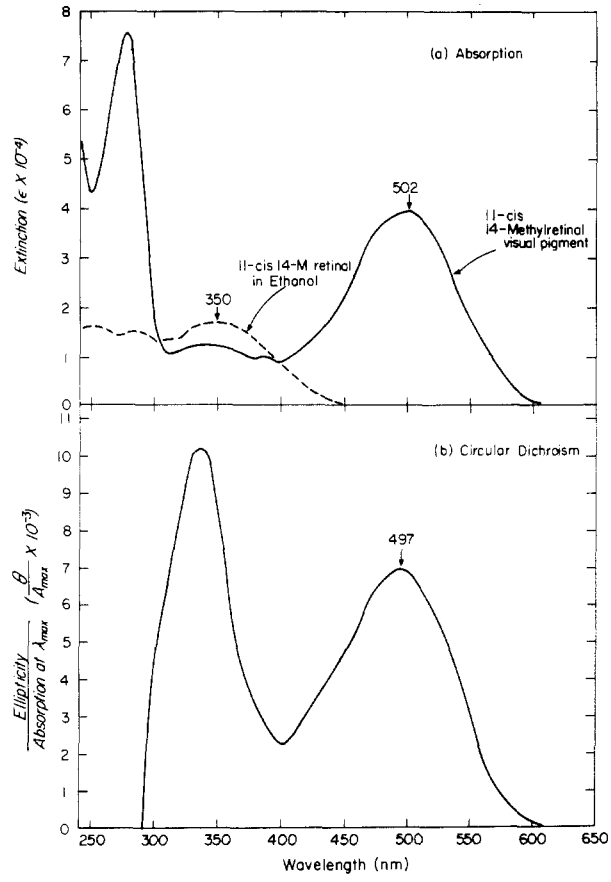

FIGURE 5: (a) Absorption spectra of 11-*cis*-14-methylretinal in ethanol, with ϵ 19,000 at 340 nm, and purified 11-*cis*-14-methylretinal pigment in 2% Ammonyx LO, with ϵ 40,000 at 502 nm. (b) circular dichroism spectrum of partially purified 11-*cis*-14-methylretinal pigment in 2% Ammonyx LO.

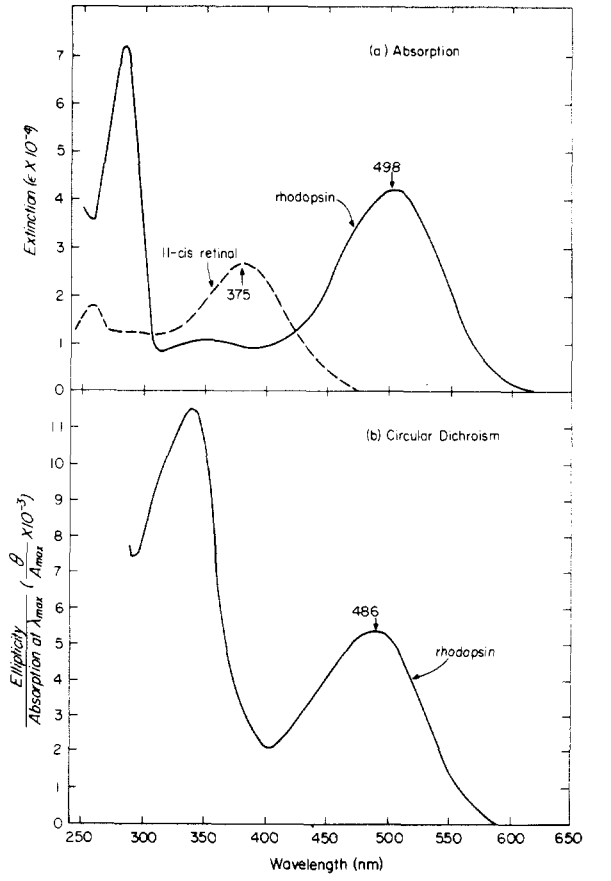

FIGURE 6: (a) Absorption spectra of 11-*cis*-retinal in ethanol with ϵ 26,000 at 375 nm, and purified 11-*cis*-retinal visual pigment (rhodopsin) in 2% Ammonyx LO normalized to an extinction of 42,000 at 498 nm; (b) circular dichroism spectrum of purified rhodopsin in 2% Ammonyx LO.

ment compared to 0.24 for the analogous ratio in rhodopsin (Figure 6). In Figures 7 and 8, the spectra of pure 9-*cis*- and 9,13-di-*cis*-retinal pigments are shown together with the difference spectra of the corresponding 14-methyl analogs. Because of the distortions inherent in such difference spectra, the true absorption maxima of the pigments are expected to be shifted a few nanometers to the blue of the difference maxima.

The extinction coefficient of the 11-*cis*-14-methyl pigment was measured by the method of Wald and Brown (1954). The method suffers from uncertainties about the impurities absorbing at 380 nm in the samples, and about the extinction coefficient (ϵ) of the product of bleaching, presumably *all-trans*-14-methylretinal. With these limitations, we estimated the extinction of the 11-*cis*-14-methyl pigment to be 38,000 ± 5000.

The circular dichroism (CD) spectra of all three 14-methyl pigments in Triton X-100 are shown in Figures 5, 7, and 8. Quantitative data are summarized in Table IV. The shapes of the circular dichroism spectra of rhodopsin and the 11-*cis*-14-methyl pigment are quite similar, as are the absolute magnitudes of the rotational strengths. Also, the shapes and magnitudes of the CD spectra of the 9-*cis*- and the 9,13-di-*cis*-14-methylretinal pigments are quite similar to each other and to the corresponding retinal based pigments. Because we have not been able to determine the extinction coefficient of the former pigments, it is not possible to compare their magnitude.

Figure 9 shows a typical experiment to determine the relative photosensitivities of the artificial pigments compared to that of rhodopsin. The photosensitivity, γ, is the product of the extinction coefficient, $\epsilon(\lambda)$, and the quantum efficiency for bleaching, ϕ, and thus depends on the wavelength of bleaching. Table V shows the relative photosensitivities of the pigments studied. For those pigments whose extinction coefficients are known, the calculated quantum efficiencies of bleaching are also given. Of special interest is the low quantum efficiency of the 9-cis and 9,13-di-cis pigments. In contrast, the photosensitivity of isomerization of 11-*cis*-14-methylretinal pigment is 1.1 times greater than that of rhodopsin, and the quantum efficiency is about 1.2 times greater.

13-Desmethyl-14-methylretinal Pigments. Only three 13-desmethyl-14-methylretinal isomers could be obtained in large enough quantities to test for pigment formation—all-trans, 13-cis, and 9-cis. Of these, only one, the 9-cis, reacted with opsin to form a pigment. This coupling between the 9-cis isomer and opsin was quite weak so that only small amounts of pigment were formed even with large excesses of chromophore. With some opsin preparations, almost no pigment was formed, again suggesting that pigment formation with 13-desmethyl-14-methylretinal is difficult.

The difference and circular dichroism spectra of this pigment are shown in Figure 10, and the photosensitivity is given in Table V.

Methylretinone (15-methylretinal) Pigments. A number of attempts were made to form a pigment from a mixture of isomers of methylretinone, but all of these failed.

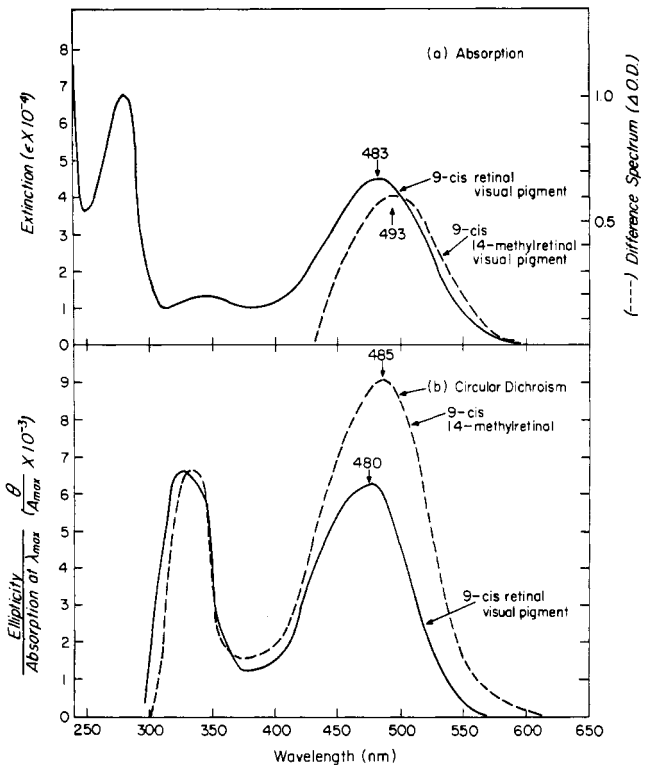

FIGURE 7: (a) Absorption spectrum of 9-*cis*-retinal pigment and difference spectrum of 9-*cis*-14-methylretinal pigment in 2% Ammonyx LO; (b) circular dichroism spectra of 9-*cis*-retinal pigment and 9-*cis*-14-methylretinal pigment with θ/A_{max} = 9.2 in 2% Ammonyx LO.

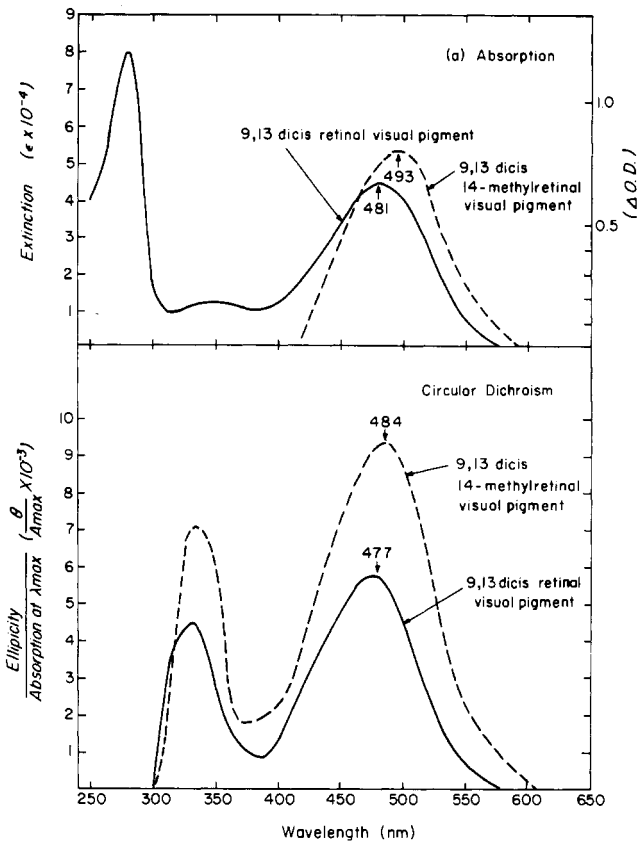

FIGURE 8: (a) Absorption spectrum of 9,13-di-*cis*-retinal pigment and difference spectrum of 9,13-di-*cis*-14-methylretinal pigment in 2% Ammonyx LO; (b) circular dichroism spectra of 9,13-di-*cis*-retinal pigment and 9,13-di-*cis*-14-methylretinal pigment in 2% Ammonyx LO.

Discussion

Free Retinals. It is expected that the absorption spectra of the twisted isomers of polyenes should have their λ_{max} at considerably shorter wavelengths than their planar conformers. However, what is in fact observed for 11-*cis*-retinal is a slight red shift. This anomaly has been explained (Honig and Karplus, 1971) by assuming that 11-*cis*-retinal has a 12-s-cis conformation; the s-cis conformations of polyenes are known to absorb at lower wavelengths than the s-trans conformation (Simmons, 1970).

To test this proposal we prepared the sterically hindered 11-cis isomer of 14-methylretinal, that could not assume the same conformation about the 12-C–13-C bond as that found crystalline 11-*cis*-retinal. This 11-cis isomer of 14-methylretinal has its λ_{max} 30 nm to shorter wavelengths than 11-*cis*-retinal; this wavelength shift appears to be due to changing the isomer conformation. As can be seen from Figure 1 the additional methyl group in 11-*cis*-14-methylretinal should have no steric effect on the s-trans conformer, but should be involved in strong nonbonded interactions with 10-H in the s-cis conformation. The effect can be seen in more quantitative fashion in Figure 11, which is a potential energy curve for twisting about the 12-C–13-C single bond. The potential curve for 14-methylretinal on the s-trans side is identical with that of retinal. However, on the s-cis side, the minimum is shifted to larger angles by about 20° with the minimum of 11-*cis*-retinal inaccessible to 11-*cis*-14-methylretinal.

Since other isomers are essentially unaffected by the 14-methyl group, and since the s-trans conformer of 11-*cis*-14-methylretinal should also be unaffected (see Figure 11), it must be concluded that the shift in λ_{max} to shorter wavelengths observed at both room and low temperature results from precluding s-cis conformations that are accessible to 11-*cis*-retinal but not to the more hindered 11-*cis*-14-methylretinal. Since both a more highly twisted s-cis conformation (e.g., $\phi_{12-13} \simeq 80°$) and the twisted s-trans conformer will absorb at shorter wavelengths than 11-*cis*-retinal in its equilibrium geometry ($\phi_{12-13} \simeq 40°$), either conformation or some combination of them could be responsible for the observed shift to shorter wavelengths. In either case our results indicate that the 12-s-trans conformer cannot be the predominant form in 11-*cis*-retinal at room or low temperature. If it were, the large wavelength shift that occurs upon methylation would not be observed.

This conclusion conflicts with the suggestion that the increased extinction observed for 11-*cis*-retinal at low temperature results from a predominantly 12-s-trans conformation (Honig and Karplus, 1971); an alternative explanation, that the increase in extinction of 11-*cis*-retinal upon lowering the temperature is due to a redistribution of intensities within the main band of the 12-s-cis conformer (Warshel and Karplus, 1974), seems a more likely possibility. (A theoretical calculation of the extinction coefficients of the main band is particularly difficult since it is probably a composite of two or three electronic states, each with its own conformation dependence.) Our conclusion also differs from a recent NMR study (Rowan et al., 1974) which reports that nuclear Overhauser effect (NOE) measurements indicate that 11-*cis*-retinal has a 12-s-trans form at low temperature in deuterioacetone. Similar studies on other modified retinals and retinals in other solvents may help resolve this problem.

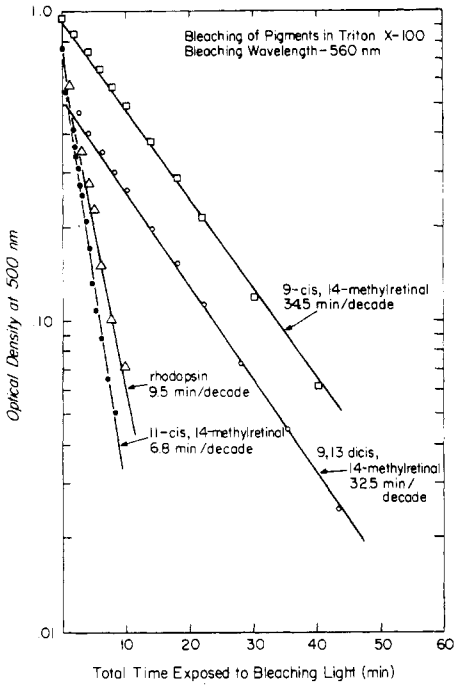

FIGURE 9: Relative photosensitivity of rhodopsin and 11-*cis*-14-methylretinal based pigments; bleaching wavelength = 560 nm. The sample was bleached for 15 sec to 5 min and the optical density at 500 nm has been plotted vs. the cumulative bleaching time. The intensity and wavelength of the bleaching light were constant throughout a set of bleaching experiments such as this one. Simple exponential reduction in the optical density was observed, indicating a single species was bleached. The slope of the line is proportional to the photosensitivity, the product of the quantum efficiency of bleaching, and the extinction coefficient of the pigment at the bleaching wavelength.

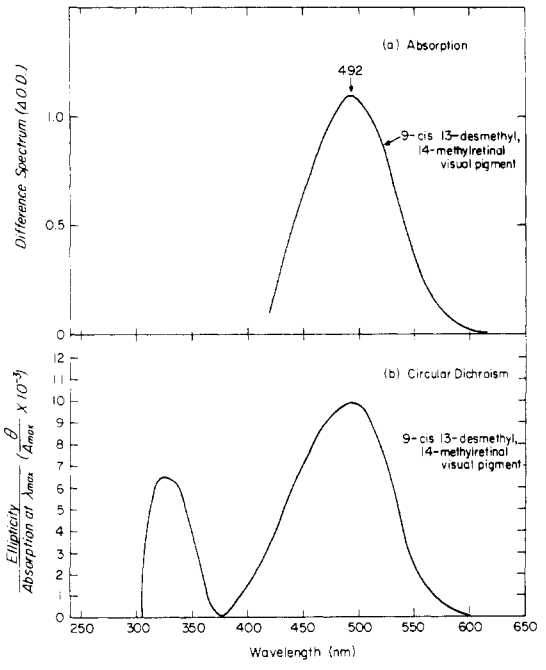

FIGURE 10: (a) Difference spectrum; (b) circular dichroism spectrum of 9-*cis*-13-desmethyl-14-methylretinal based pigments in 2% Triton X-100.

A rather unexpected finding from our studies concerns the conformation about the 14-C–15-C single bond. In the NMR spectra of 13-desmethyl-14-methylretinal, the position of the 15-H resonance was ca. 0.5 pp upfield of the retinals and 14-methylretinals. A downfield shift of the 15-H resonance would be expected in a 14-s-trans conformation, while in the s-cis conformation the proton would be pointed away from the polyene system and be shifted to higher field (Figure 1f). Thus, it is likely that the conformation of all the 13-desmethyl-14-methylretinals is 14-s-cis.

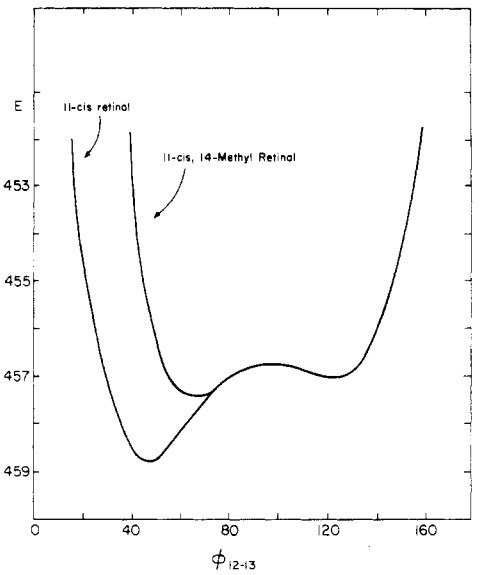

FIGURE 11: Potential energy function for 11-*cis*-14-methylretinal, rotating about the ϕ_{12-13} bond. $\phi_{12-13} = 0°$ corresponds to s-cis, $\phi_{12-13} = 180°$ corresponds to s-trans. At each point on the curve the energy $E = E^{n} + E^{nb}$, where E^{n} is the π-electron energy calculated in the Huckel approximation and E^{nb} is the pairwise sum over nonbonded interactions. The energy was minimized with respect to rotation about 10-C–11-C and 11-C–12-C at each point along the curve. All bond lengths and bond angles were fixed at standard values. ($\phi_{6-7} = 40°$ from Gilardi et al., 1971). Nonbonded parameters are from Williams (1966) but a point methyl group was used to simulate free rotation (see similar calculation in Nash, 1969; Honig and Karplus, 1971).

Pigments. Clearly one of the most interesting problems concerning visual excitation, but one of the most difficult to approach, is to determine the factors responsible for directing the pathways of isomerization with such a high specificity for the products of isomerization and with such a high quantum efficiency for the isomerization event. Since the photochemical properties of the bound chromophore differ from those of free 11-*cis*-retinal (or of the protonated Schiff base of 11-*cis*-retinal), and since the bleaching sequence must involve changes in the conformation of opsin, it is thought that retinal fits into its binding site and that its interactions with this binding site during photoisomerization control the pathways of isomerization and the subsequent changes in the opsin structure.

The 11-cis isomer of 14-methylretinal forms a pigment with opsin, whose spectroscopic and photochemical properties, i.e., the absorption spectrum, including band shape, λ_{max} of bands, and extinction coefficient (Figures 5 and 6, Table V), circular dichroism spectra, including shape and magnitude (Figures 5 and 6), and photosensitivity (Table V) are quite similar to the corresponding properties of rhodopsin. The similarity in these properties of the pigments is especially interesting in view of the considerable differences in the absorption spectra of the two 11-cis chromophores in solution (Figures 5 and 6). This latter difference has been attributed to a difference in conformation. The similarities observed in the pigment properties, on the other hand, suggest that the 11-cis isomers of both retinal and 14-methylretinal have similar conformations in their pigments. This,

in turn, would indicate that the angle about the 12-C–13-C bond of the chromophore in the pigment is different from that in crystalline 11-*cis*-retinal.

Another related and potentially important finding is the similarity in spectral and photochemical properties of the four pigments derived from the 9-cis and 9,13-di-cis isomers of retinal and 14-methylretinal (Figures 7 and 8; Table V). As in the case of the 11-cis pigments, the similarity in properties of these pigments suggests that the conformations of the four different chromophores may have many features in common.

An unexpected finding was that the 9,13-di-*cis*-14-methyl isomer forms a pigment. Since it was originally reported by Hubbard and Wald (1952) that of all the isomers of retinal they tested (all-trans, 11-cis, 9-cis, 13-cis, and 9,13-di-cis), only the 9-cis and 11-cis combined with opsin. Our result led us to a separate study which showed that the 9,13-di-cis isomer of retinal does, in fact, form a pigment (Crouch et al., 1975). This pigment, as is the case with the 14-methyl pigments, has spectral and photochemical properties similar to those of the 9-cis pigment.

The compound with an extra methyl at the 15 position, methylretinone, does not couple with opsin to form a pigment. The reason for this lack of reactivity is presumably steric.

Acknowledgment

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