Tetrahedron Vol. 40, No. 3, pp. 473 to 482, 1984
Printed in Great Britain.

0040-4020/84 $3.00 + .00
Pergamon Press Ltd.

SEVEN NEW HINDERED ISOMERIC RHODOPSINS

A REEXAMINATION OF THE STEREOSPECIFICITY OF THE BINDING SITE OF BOVINE OPSIN

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(*Received in U.S.A. October* 1982)

Abstract—Results of interaction of seven new geometric isomers of retinal (7-*cis*; 7,9-*dicis*; 7,11-*dicis*; 7,13-*dicis*; 9,11-*dicis*; 7,9,11-*tricis*; 7,9,13-*tricis*) with bovine opsin are reported. All of them form pigments with absorption maxima varying between 450 and 480 nm. The rates of pigment formation were generally considerably lower than those of 11-*cis*-retinal and the yields were less than quantitative. Implications of these results for the stereospecificity of the binding site of opsin are discussed.

In a series of classical experiments, Wald first demonstrated that the chromophore of the visual pigment is a derivative of vitamin A. Its identity was later unambiguously identified by the regeneration of rhodopsin through the interaction of the apo-protein, opsin, with 11-*cis*-retinal.[1] At that time the stereospecificity of the binding site of opsin was tested with six geometric isomers of retinal (all-*trans*; 9-*cis*; 11-*cis*; 13-*cis*; 9,13-*dicis* and 11,13-*dicis*).[2] In addition to the 11-*cis* isomer, only the structurally similar 9-*cis* isomer was found to yield a pigment analogue. (The low yield of pigments formed with the two di*cis* isomers was believed to have resulted from the active mono-*cis* isomers which were already present or were formed in the di*cis* samples.) These observations led to the commonly accepted notion of a high stereospecificity of the binding site.

The limits of specificity of opsin's binding site have not been independently tested, even though much effort has gone into the study of the structure of opsin.[3] Even after the recent studies of Hargrave *et al.*,[4] its primary sequence is less than 50% determined.† Though two-dimensional frog rhodopsin crystals have recently been prepared,[5] a study of rhodopsin's tertiary structure has not yet been reported.

On the other hand, recent analogue studies following two independent lines have shed new light on the question of binding site specificity. First, the availability of new methods such as CD and high pressure liquid chromatography (hplc) allowed Nakanishi *et al.* to establish beyond doubt the retention of the di*cis* geometry of the chromophore in the pigment derived from 9-*cis*,13-*cis*-retinal which was clearly different from 9-*cis*-rhodopsin.‡[6] Secondly, the selective triplet sensitized photoisomerization method[7] allowed the introduction of the previously inaccessible hindered 7-*cis* geometry into the polyene chain of retinal. This then led to the preparation of seven new geometric isomers at the laboratory in Hawaii. They are 7-*cis*-, 7-*cis*,9-*cis*-, 7-*cis*,13-*cis*- and 7-*cis*,9-*cis*,13-*cis*-retinal,[8] the doubly hindered 7-*cis*,11-*cis*-retinal,[9] 7-*cis*,9-*cis*,11-*cis*-retinal[10] and the related 9-*cis*,11-*cis*-retinal[11] (Fig. 1). These isomers allow the testing of opsin binding site specificity on a broader basis.

Some of the preliminary results on interaction of these new retinal isomers with bovine opsin are already in the literature.[9-12] In this paper, we report in detail some properties of the pigment analogues derived from these isomers and discuss the implications for the binding site specificity of opsin.

†Since submission of this paper, two reports have appeared dealing with the complete sequence of bovine rhodopsin: [a]Y. A. Ovchinnikov, N. G. Abdulaev, M. Y. Feigina, I. D. Artamonov, A. S. Zolotarev, M. B. Kostina, A. S. Bogachuk, A. I. Miroshnikov, V. I. Martinov and A. B. Kudelin, *Bioorg. Khim.* **8**, 1011 (1982); [b]P. A. Hargrave, J. H. McDowell, D. R. Curtis, J. Wang, E. Juszczak, S.-L. Fong, J. K. M. Rao and P. Argos, paper presented at the *5th Int. Cong. Eye Research*, 3–8 October 1982, *Biophysics of Structure and Mechanism*, **9**, 235 (1983).

‡Also known as isorhodopsin. Now that many isomeric rhodopsins have been synthesized we have adopted a pigment designation which explicitly specifies the chromophore geometry.

EXPERIMENTAL

General information. All UV–VIS spectra were recorded on a Cary 14, 15 or a P.E. 124 spectrometer. The CD spectra were recorded on a JASCO J-40 spectrometer equipped with a Morvue Photoelastic Modulator. All HPLC separations were of the normal phase variety using silica gel based columns (Waters μ-Porasil or Altex Lic-

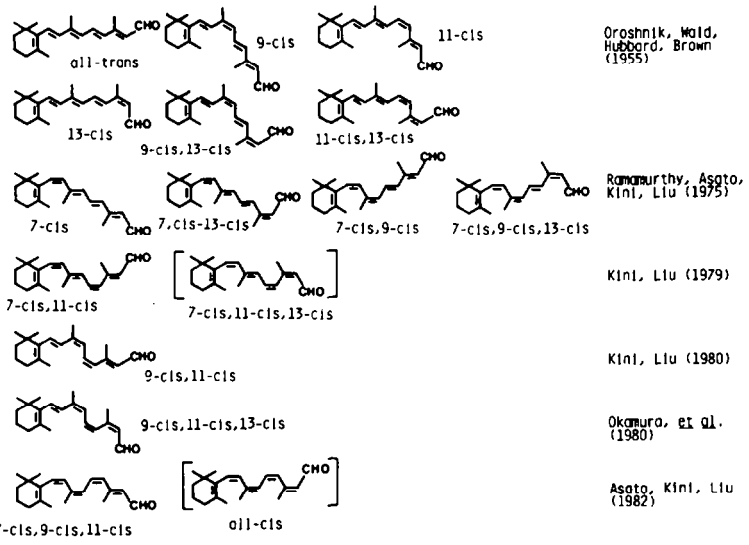

Fig. 1. The sixteen possible geometric isomers of retinal in chronological order of their preparation. The aldehyde form of those isomers in brackets have not been isolated although their ester or the alcohol form is known (Refs. 9 and 10).

hrosorb Si-60) with UV detection and hexane with varying amount of ether as eluent. The digitonin used in this study was purchased from Sigma or from Merck.

Retinal isomers. The principal source of four of the seven new geometric isomers of retinal was the mixture of 7-*cis*, 7,9-*dicis*, 7,13-*dicis* and 7,9,13-*tricis* isomers from a non-stereoselective synthesis of these isomers.[8a] Preparative HPLC readily provided the first two isomers in high purity (Fig. 2). The two 13-*cis* isomers (and most of the 13-*cis* retinals)[13] have nearly identical retention times and thus remained unseparable by this method. The 7,9,13-*tricis* isomer became available along with the readily separable 7,9-*dicis* isomer following a stereoselective synthesis of the key intermediate, 7-*cis*,9-*cis*-C_{15}-aldehyde.[8b] The 7,13-*dicis* isomer, however, was obtained only in small quantities in an accidental way. During an unsuccessful synthesis of 7-*cis*,11-*cis*,13-*cis*-retinal, a few milligrams of the isomer was isolated.[14] Therefore only a limited amount of work was carried out with this isomer. 7-*cis*,11-*cis*-Retinal, 9-*cis*,11-*cis*-retinal and 7-*cis*,9-*cis*,11-*cis*-retinal were prepared following a successful synthesis of the corresponding key intermediates, 7-*cis*,11-*cis*-C_{18}-ketone,[9] 9-*cis*,11-*cis*-C_{18}-ketone[11] and all-*cis*-C_{18}-ketone.[10] 7-*cis*-Retinal and 9-*cis*,11-*cis*-retinal were also obtained in small quantities after photoisomerization of all-*trans*-retinal followed by preparative HPLC separations.[13a,15]

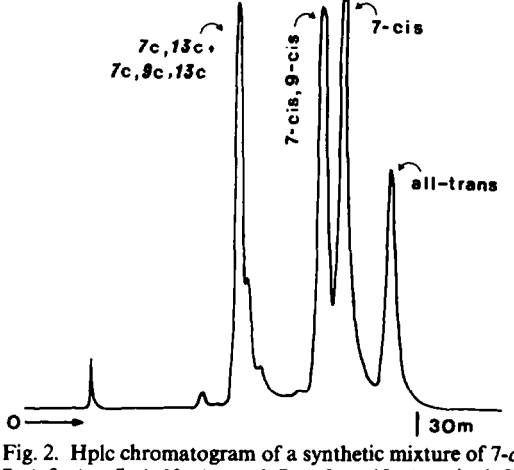

Fig. 2. Hplc chromatogram of a synthetic mixture of 7-*cis*-, 7-*cis*,9-*cis*-, 7-*cis*,13-*cis*- and 7-*cis*,9-*cis*,13-*cis*-retinal (Waters μ-Porasil column, 2% ether in hexane as solvent, 360 nm detection).

7-*cis*-Retinal was a yellow solid while all six other isomers remained as viscous oils even after purification by preparative HPLC. The UV–VIS absorption spectra of all seven isomers are shown in Figs. 3(a) and 3(b) along with those of several related, previously characterized isomers. The absorption maxima and extinction coefficients are listed in Table 1. Also included are absorption maxima of the corresponding oximes. The ^{1}H NMR spectra most useful for configurational assignments have already been presented in detail.[16] For all pigment analogue studies, the retinal isomers were purified by preparative HPLC and stored in deoxygenated hexane solutions at $< -10°$.

Preparation of isomeric rhodopsins. Most preparative procedures have been reported in detail in the lit.[17,18] and only certain key points will be described below.

Cattle rod outer segment (ROS) membranes were isolated from homogenized retinas either by continuous or stepwise sucrose density gradient centrifugation. They were then bleached during work-up with white light and retinal was converted to the oxime by washing with solutions of hydroxylamine (or into retinol by treatment with NADPH).[17] Opsin containing ROS were stored in pellets or in buffered solutions (pH 7) at $-10°$.

For pigment formation, an aliquot of a concentrated ethanol or acetone solution of the retinal isomer was added to the ROS membranes suspended in aqueous buffer. The opsin concentration was adjusted to give 0.5–1.0 OD unit of rhodopsin and the retinal isomer was added in greater than a five fold excess. Pigment formation was also performed with opsin solubilized in buffered 1–2% digitonin. Rates of pigment formation were generally slower when a detergent was used.[18] However, the difficulties with light scattering and solubility of retinal were eliminated.

For determination of pigment yield, the concentration of opsin (the limiting quantity) was first determined by the amount of rhodopsin it would form with an excess of 11-*cis*-retinal. The amount of isomeric rhodopsin formed was calculated on the basis of the absorbance of the pigment in the difference spectrum. A common extinction coefficient for rhodopsin of 42,000 was assumed.[18]

Determination of the rates of pigment formation. The case of 7-*cis*,11-*cis*-rhodopsin is described as a representative example. Upon mixing a 1% digitonin solution of opsin (final conc., 47.6 μM) and an EtOH soln of 7-*cis*,11-*cis*-retinal (117 or 59 μM) the progress of the reaction was followed by intermittent withdrawal of aliquots of soln for determination, by measurement of the difference absorption spectrum, of the isomeric pigment formed. UV–VIS absorption spectra were recorded before and after addition of a large excess of NH_2OH (final conc

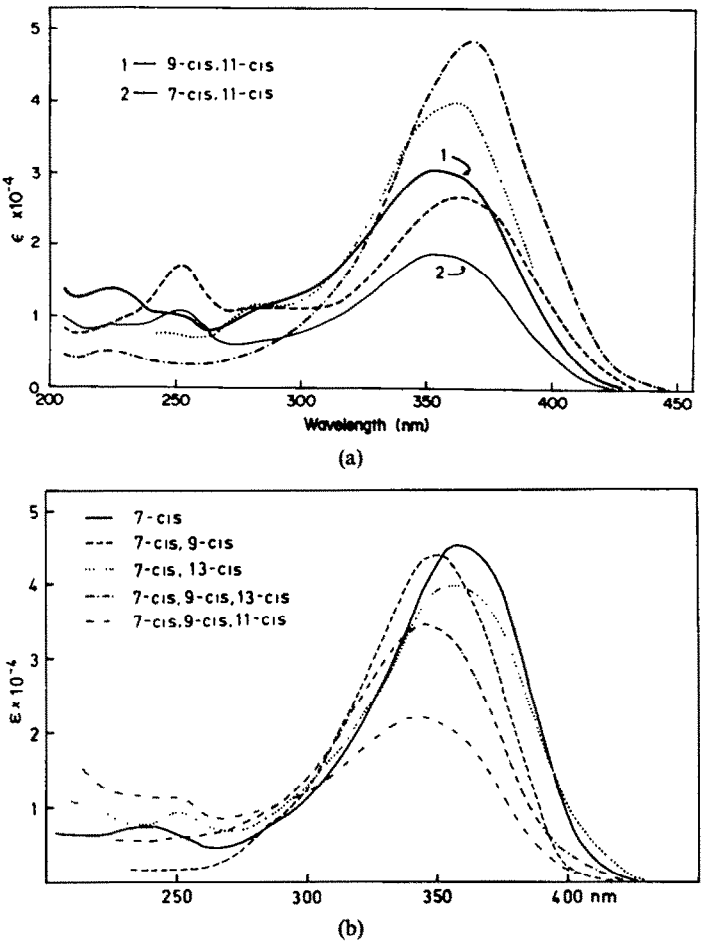

Fig. 3. (a) UV-vis absorption spectra of 9-*cis*,11-*cis*-retinal (line 1) and 7-*cis*,11-*cis*-retinal (line 2) in hexane. Spectra for three other known isomers are also shown for comparison: — · — ·, all-*trans*; ····, 9-*cis*; ----, 11-*cis* (data of Hubbard and Wald, Ref. 2). (b) UV-vis absorption spectra of five 7-*cis* isomers of retinal in hexane.

Table 1. Absorption maxima of new isomers of retinal and the corresponding oxime

Isomer	Retinal λ_{max} (ε), nm		Oxime,[b]
	hexane	ethanol	λ_{max}, nm
7-cis	359 (44,100)	377 (38,000)	353
7-cis,9-cis	351 (42,500)		345
7-cis,11-cis	355 (18,800)	374 (16,000)	345
7-cis,13-cis	357 (a)		344
9-cis,11-cis	352 (30,600)	368 (27,000)	c
7-cis,9-cis,11-cis	346 (22,000)		337
7-cis,9-cis,13-cis	346 (36,600)		343

a. Insufficient amount for accurate determination. An arbitrary value of 40,000 was used for plotting the curve in Figure 3.
b. In ethanol. c. Not determined.

50 mM) followed by orange light bleaching ($\lambda \geq 440$ nm). The absorbance change at 455 nm was plotted against time (Fig. 4). From the tangents of the initial portion of the two curves, the rates and the rate constants of reaction were determined.

When retinal was used in large excess, a pseudo first order rate constant was first calculated. The bimolecular constant was then obtained utilizing the appropriate conc of retinal. For 7-*cis*-rhodopsin the rates were measured at three different temps. Both the pseudo first order and second order rate constants were calculated. The values are listed in Table 2.

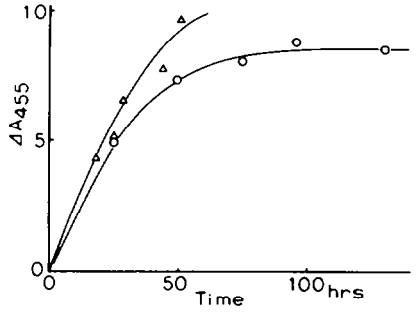

Fig. 4. Determination of rates of pigment formation of 7-*cis*,11-*cis*-rhodopsin at two different concentrations of retinal (△, 110 μM, ○, 59 μM) with 48 μM opsin solubilized in 1% digitonin. The reaction was monitored at 455 nm.

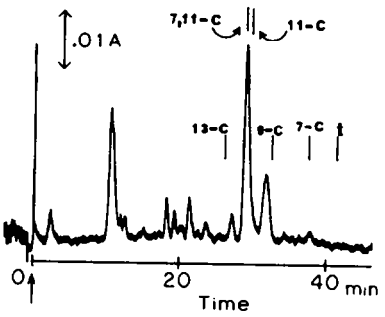

Fig. 5. Hplc chromatogram of the extract of denatured 7-*cis*,11-*cis*-retinal. The bars indicate positions of authentic samples of retinal isomers (see Ref. 13*a* for a chromatogram of retinal isomers).

Extraction of the chromophore of 7-cis,11-cis-*rhodopsin.*[19] To 5 ml of a 1% digitonin solution of 7-*cis*,11-*cis*-rhodopsin was added at 0° 5 ml of CH_2Cl_2. The mixture was thoroughly mixed by pumping through a syringe. An equal volume of hexane was then added. After mixing, the mixture was centrifuged at 2500 rpm for 20 m. The hexane fraction which was decanted off showed UV-absorption corresponding to a retinal isomer. After concentration, the mixture was analyzed on a μ-Porasil HPLC column. The chromatogram is shown in Fig. 5. Retention times of authentic retinal isomers were determined under identical conditions, and are marked in the Figure.

Measurement of CD spectra of pigment analogues. Pigment analogues were constituted from the appropriate aldehyde and a bleached ROS suspension. The pigment which formed was then extracted into a pH 6.5 phosphonate buffered 2% digitonin soln. Pigment concentration was measured with a Cary 15 spectrophotometer.

CD spectra were measured with a JASCO J-40A spectropolarimeter. Typically about 0.9 ml of the generated pigment extract filled a cylindrical cell with a 1.00 cm pathlength. Spectra were generally measured at around 23° from 600 to 300 nm in 5–10 mins, which resulted in no more than about a 5% pigment bleach during each CD scan. After a scan of the generated pigment, hydroxylamine was added to a concentration of about 0.01 M, another CD spectrum was taken, and then the soln was irradiated with white light until pigment bleaching was complete. The CD spectrum of the bleached pigment soln served as a CD baseline.

RESULTS

All seven newly synthesized geometrical isomers of retinal when incubated with opsin, whether in ROS suspension or in digitonin micelles, formed pigment analogues. The reactions were readily monitored by the appearance of long wavelength bands in the UV–VIS absorption spectra (Figs. 6, 7). The rates, however, were generally much slower than the case of 11-*cis*-retinal where rhodopsin regeneration was complete within a few mins.[18] Under similar conditions each of these new isomers usually took several hours to reach a steady level of absorption in the long wavelength region when ROS suspensions were used and longer when digitonin solutions were used.

The new pigments appeared to be quite stable at room temperature. However, upon addition of a molar excess of hydroxylamine for removal of unreacted retinal and scavenging random Schiff bases, the pigment absorption decreased. When monitored near its λ_{max} (see insert in Fig. 7), an initial rapid drop of pigment absorption was detected followed by a slower one. The initial intensity change is believed to be due to removal of random Schiff bases, the slower process due to direct attack of the pigment analogues by hydroxylamine. The overall rate of degradation was nevertheless sufficiently slow that addition of the

Table 2. Rates of pigment formation of opsin with new geometric isomers of retinal

Isomer	[opsin], μM[a]	[Retinal], μM	T °C	k_1,[b] m^{-1}	k_2,[c] $M^{-1}sec^{-1}$ S	D
7-cis	14	70	38	$(2.11\pm.02)10^{-2}$,[d]	5	--
	14	70	28	$(4.20\pm.03)10^{-3}$,[e]	1	--
	14	70	10	3.2×10^{-4},[f]	.08	--
7-cis,9-cis	9.5	74	23	--	1.3	--
7-cis,11-cis	47	59	25	--	--	0.04
7-cis,13-cis	36	40	25	--	--	0.007
9-cis,11-cis	5.1	15	25	--	--	0.34
7-cis,9-cis,11-cis	34	75	25	--	--	0.02
7-cis,9-cis,13-cis	9.5	70	23	--	.3[g]	--

a. Determined by amount of rhodopsin formed. b. Pseudo first order rate constant. c. Second order rate constant with ROS in suspension (S) opsin solubilized in digitonin (D). d. Five data points. e. Four data points. f. Two data points. g. Mixture of 7,9,13-tricis and 7,13-dicis (3:1).

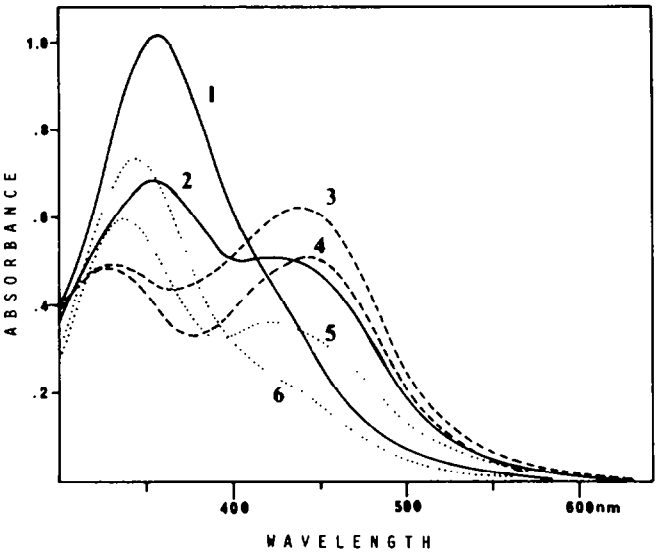

Fig. 6. Progress of formation of 7-*cis*,9-*cis*-rhodopsin at 23°. Curve 1: absorption spectrum of a mixture of 74 μM 7-*cis*,9-*cis*-retinal and 13 μM opsin in the form of ROS membranes in suspension (pH = 7.0); curve 2: 1.2 h after addition; curve 3: 4.7 h after addition; curve 4: immediately after addition of NH_2OH solution (final conc. 0.1 M); curve 5: 30 m after addition of NH_2OH; curve 6: after irradiation with yellow light ($\geq$ 450 nm) for 9 m.

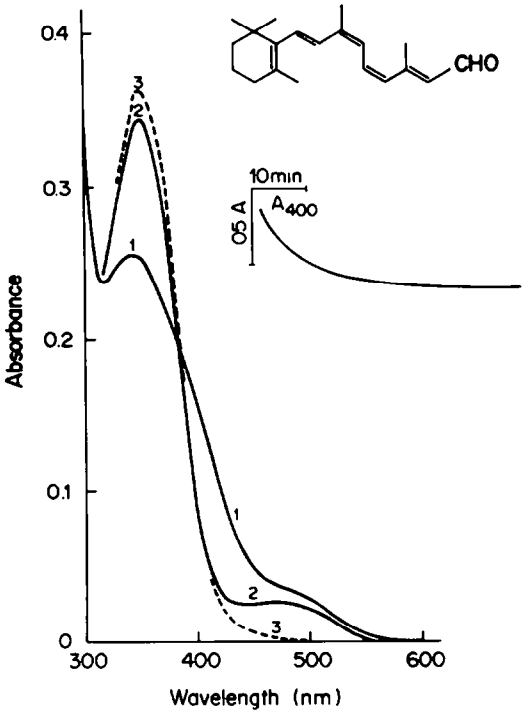

Fig. 7. Progress of formation of 9-*cis*,11-*cis*-rhodopsin. Curve 1: Absorption spectrum of a mixture (1 ml) of 15 μM 9-*cis*,11-*cis*-retinal and 5.2 μM opsin in 1% digitonin buffer (pH = 7.0) after incubation at 25°C for 12 h and a twofold dilution. Upon the addition of 50 mM NH_2OH (final conc.), A_{400} was monitored (the insert). Curve 2: Spectrum recorded 50 m after addition. Curve 3: After irradiation with > 520 nm light (40-W tungsten lamp with cutoff filter) for 5 m.

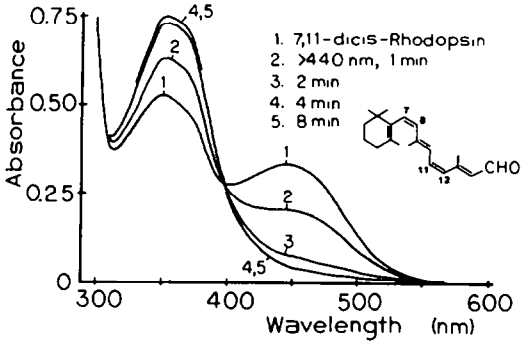

Fig. 8. Photobleaching of 7-*cis*,11-*cis*-rhodopsin. Curve 1: absorption spectrum of the dicis pigment in 1% digitonin. Curves 2–5: successive photobleaching with > 440 nm light.

reagent helped to obtain more meaningful absorption spectra of the pigments. After addition of hydroxylamine the pigment was photobleached with yellow light (Fig. 8). The difference absorption spectrum of a pigment obtained by subtracting the spectrum before from the spectrum after light bleaching was thus obtained (Figs. 9a, b). The absorption maxima for all new pigment analogues (450–480 nm) were found to be blue shifted from those of the earlier known isomeric rhodopsins (Discussion).

Pigment stability was also found to be detergent dependent. In digitonin all pigments appeared to be stable when not delipidated. When solubilized in Triton X-100 or CTAB, however, the pigments were found to be unstable. Their half life in Triton X-100 averaged 60 m while that in CTAB was only 8 m.

Attempts to purify the isomeric pigments were only partially successful. 7-*cis*- and 7-*cis*,9-*cis*,13-*cis*-rhodopsins were found to be unstable during attempts to purify samples by affinity chromatography on Con A-glass in 2% digitonin.[17] On the other hand, the 7,9-*dicis* pigment was partially purified under the same conditions. Its UV–VIS absorption spectrum (Fig. 10) showed peaks at 460 and 340 nm and a high 280/460 ratio (~4) indicating the possible presence of opsin.

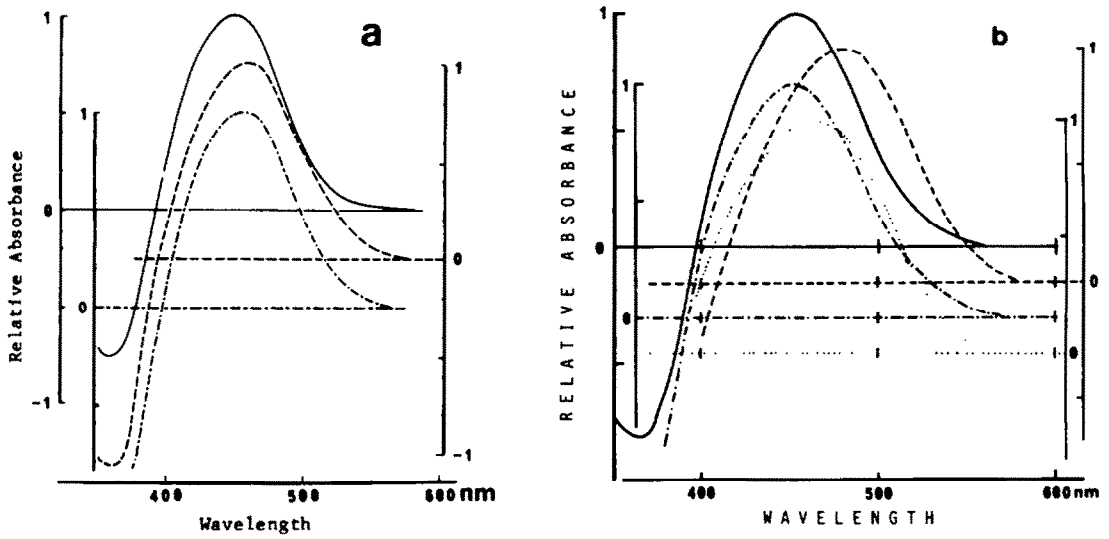

Fig. 9. (a) Difference absorption spectra of 7-*cis*- (—); 7-*cis*,9-*cis*- (---); 7-*cis*,9-*cis*,13-*cis*- (— · — ·) rhodopsin obtained by, e.g., subtracting curve 5 by curve 6 in Fig. 6. (b) Difference absorption spectra of 7-*cis*,11-*cis*-(—), 7-*cis*,13-*cis*- (— · — ·) 9-*cis*,11-*cis*- (----) and 7-*cis*,9-*cis*,11-*cis*-rhodopsin by, e.g. subtracting curve 3 from curve 2 in Fig. 7 or curve 5 from curve 2 in Fig. 8.

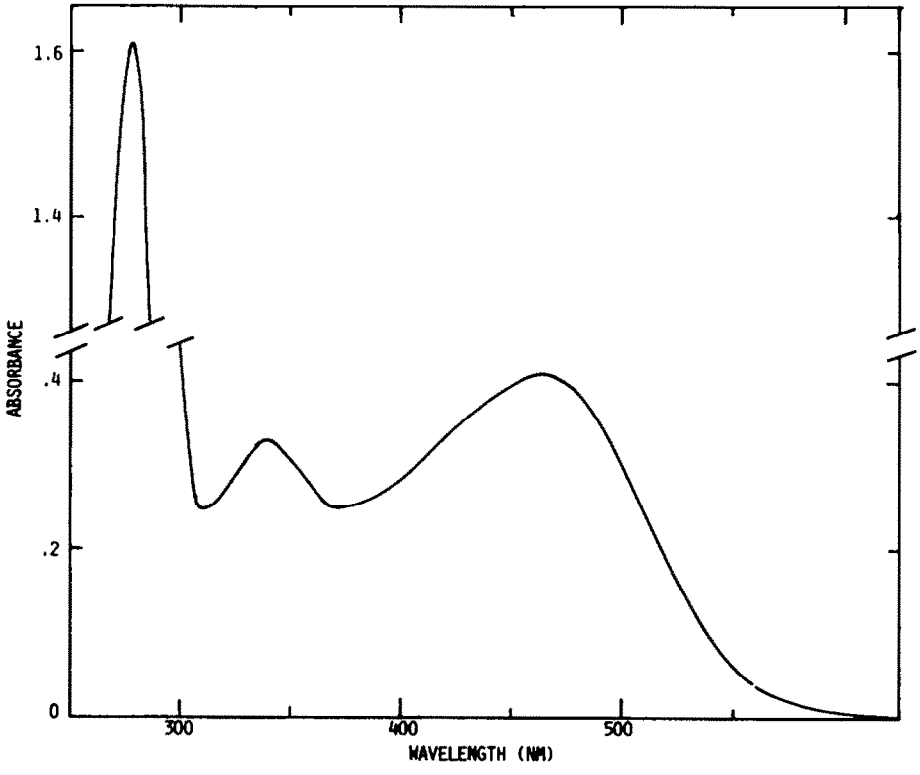

Fig. 10. Absorption spectrum of 7-*cis*,9-*cis*-rhodopsin, after purified by affinity chromatography on Con A-glass.

In one case (7-*cis*,11-*cis*-rhodopsin) the chromophore of the isomeric pigment was recovered following the procedure of Nakanishi *et al.*[19] While the result was complicated by the obvious presence of other inpurities, mostly with retention times much shorter than those of the retinal isomers (Fig. 5), there was obviously one major peak in the lc chromatogram. Its retention time was identical to that of 7-*cis*,11-*cis*-retinal. No other peaks of significant size had a retention time identical to other known retinal isomers. These observations argue strongly for the retention of the doubly hindered geometry in the pigment analogue.

The rates of pigment formation (Table 2) were estimated at room temperature with the accuracy limited by the lack of knowledge of accurate extinction coefficients of the isomeric pigment analogues. In the case of 7-*cis*-rhodopsin, the rates of pigment formation measured at three different temperatures (Table 2) were fit to an Arrhenius plot

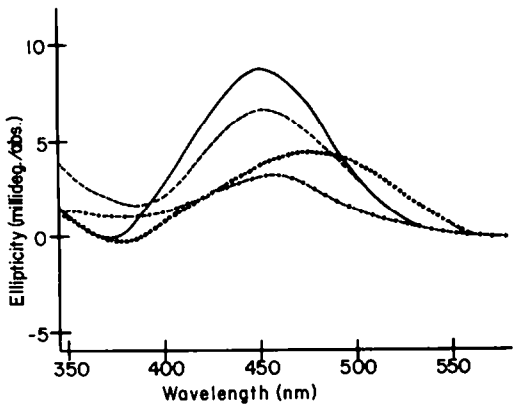

Fig. 11. Circular dichroism of 7-*cis*-rhodopsin (—), 7-*cis*,9-*cis*-rhodopsin (— · — ·) 7-*cis*,11-*cis*-rhodopsin (----) and 9-*cis*,11-*cis*-rhodopsin (· · · ·). Ellipticity values in millidegrees divided by the absorbance at λ_{max} for each pigment are plotted as the ordinate.

which yielded an activation energy of 26 ± 2 kcal/mol for the reaction.

The CD spectra of five of the seven isomeric rhodopsins discussed in the paper were measured. Four are shown in Fig. 11 with additional data listed in Table 4.

DISCUSSION

New hindered isomeric rhodopsins. Because of the fact that the seven new isomeric pigments are formed only after prolonged periods of incubation (thus distinctly different from 9-*cis*-rhodopsin, the earliest known isomeric rhodopsin) one might reasonably infer the possibility of pigment formation as a result of the slow isomerization of the hindered retinal isomers to other active isomers. This suggestion is, however, not compatible with the observation that the absorption maxima of the pigments derived from retinal isomers containing the 7-*cis* geometry are considerably blue shifted from the two earlier known isomeric analogues (9-*cis* and 9,13-*dicis*). Furthermore, the retention of the original chromophore geometry has been substantiated in the case of 7-*cis*,11-*cis*-rhodopsin, where HPLC analysis of the extract of denatured pigment showed the absence in any significant amounts of isomers other than 7-*cis*,11-*cis*-retinal. The slow rates of pigment formation are, therefore, most likely due to the decreased activation entropy associated with the conformational adjustment by the protein in order to accommodate those isomers with shapes considerably different from 11-*cis*-retinal. It follows that the activation energy required for formation of 7-*cis*-rhodopsin is 3–4 kcal/mole higher than that of rhodopsin.[18]

The lower stability of these new isomeric pigments in hydroxylamine suggests that the conformational readjustment does not result in a sufficiently enclosed binding site to shield the imine linkage between the chromophore and the protein from attack by external reagents, as is the case with 11-*cis*- and 9-*cis*-rhodopsins. This difference in shape and conformational properties of the binding sites of the isomeric pigments from those of rhodopsin is also consistent with the interpretation of the blue-shifted absorption properties of the 7-*cis* pigments by the external point charge model.[20]

We might add that the lower stability of these new isomeric pigments compared to rhodopsin is not due to the involvement of different portions of the protein chain in the respective binding sites. This was established earlier in the case of 7-*cis*-rhodopsin in a study of the photo-bleaching sequence of the pigment.[21] It was found that the primary photo-product was identical to that of rhodopsin (namely, bathorhodopsin). The subsequent stepwise thermal transformations were also identical to those in rhodopsin. Therefore, it appears safe to assume that identical binding sites are involved in all isomeric rhodopsins.

The cause for the low yield of the new isomeric pigments is not entirely clear. An unfavorable equilibrium, however, can be safely ruled out by the observation that the addition of a molar excess of 11-*cis*-retinal to a mixture of, e.g. ROS suspension and 7-*cis*-retinal after first allowing the mixture to complete formation of 7-*cis*-rhodopsin, did not lead to immediate formation of rhodopsin. Apparently some of the 7-*cis*-retinal molecules, even though not successful in forming stable pigment analogues, were able to inhibit the immediate reaction of the "unreacted" opsins. This could take place either in the form of 7-*cis*-retinal induced denaturing of opsin or formation of irreversible complexes in the form similar to those from β-ionone and homologues.†[22]

Photobleaching properties of seven isomeric pigments appear to be quite similar to those of rhodopsin. In the presence of hydroxylamine, irradiation leads to the appearance of an oxime band around 359 nm, identical to that reported for all-*trans*-retinal oxime.[2c] In the case of 7-*cis*-rhodopsin, this conclusion was independently confirmed by experiments with low temperature spectroscopy.[21]

CD spectroscopy played an important role in distinguishing 9-*cis*,13-*cis*-rhodopsin from 9-*cis*-rhodopsin.[6] The CD spectra of five of these new isomeric rhodopsins also show features which differentiate among the new pigments and which distinguish them from the previously known isomers. Some of the key data for rhodopsin,[23] 9-*cis*-rhodopsin[23] and 9-*cis*,13-*cis*-rhodopsin[6] are tabulated in Table 4 together with those of the new isomers. Most strikingly different are the ratio of the intensities of the two main bands. Considering the similarity of the UV-visible absorption spectra of these isomeric rhodopsins containing the 7-*cis* geometry, we suggest that the CD spectra may better serve to characterize these pigments.

The α- or long-wavelength CD band and the β- or short-wavelength CD band show λ_{max} values close to those of the corresponding absorption spectral maxima, but their intensity ratios differ considerably.[23] Though the α/β ratios are somewhat dependent upon the solubilizing detergent[24] and the method of pigment preparation,[23] Table 4 makes clear that the ratio for each rhodopsin isomer is unique. Pigments con-

†We have not rigorously ruled out another explanation of unusually low extinction of these hindered analogues for the apparent low yield of pigments. However, the similar extinction coefficients for the free retinals do not appear to support this explanation.

Table 3. Properties of pigment analogues derived from bovine opsin and the fourteen known geometric isomers of retinal

Isomer	Pigment Analogue	Rhodopsin λ_{max}, nm	k_2, $M^{-1}sec^{-1}$ S	D[a]	Pigment Yield	Stability in NH_2OH[b]
All-trans[c]	-	None	--	--	0	--
7-cis	+	450	1	--	51%	unstable
9-cis[c]	+	483	80[g]	--	>90%	stable
11-cis[c]	+	498	5600[h]	50[h]	100%	stable
13-cis[c]	-	None	--	--	0	--
7-cis,9-cis	+	460	1.3	--	41%	unstable
7-cis,11-cis	+	455	--	.04	45%	unstable
7-cis,13-cis	+	450	--	.007	>5%	--
9-cis,11-cis	+	480	--	.34	21%	unstable
9-cis,13-cis[d]	+	481	10[g]	--	~90%[d]	--
11-cis,13-cis[e]	+	498(?)	--	--	20%	--
7-c,9-c,11-c	+	462	--	.02	35%	--
7-c,9-c,13-c	+	455	.3	--	40%	unstable
9-c,11-c,13-c[f]	?					

a. S ≡ ROS suspension; D ≡ in 1-2% digitonin. b. In excess; final conc. 50-100 μM. c. Ref. 2c. d. Ref. 6. e. Ref. 2a, b. f. C. G. Knudson, S. C. Carey and W. H. Okamura, J. Am. Chem. Soc., 102, 6355-6356 (1980). Test result not available. g. Calculated based on the graph in ref. 6. h. Ref. 18.

Table 4. Circular dichroism spectral data for cattle rhodopsin pigments

Isomeric Pigments	CD λ_{max} α	β	θ_{max}/A_{max}[a] α	β	Reference
11-*cis*-rhodopsin	486	330	7.9	14.0	23
9-cis	474	330	8.3	7.5	23
9,13-dicis	476	330	5.7	4.5	6
7-cis	450	330	8.7	2.5	this work
7,9-dicis	458	--	3.2	--	this work
7,11-dicis	455	--	7.0	--	this work
9,11-dicis	475	338	4.5	1.0	this work
[7,9,13-tricis][b]	450	--	5.0	--	this work

a. A_{max} is the absorption at λ_{max} of the pigment solution whose value of maximum ellipticity, θ_{max}, is tabulated.
b. Retinal sample contained a small amount of 7-*cis*,13-*cis*-retinal.

taining the 7-*cis* geometry show a weak β-band of the CD in the near UV. Such variations in β-band intensity might well reflect a lessened interaction of the ionone ring portion of the chromophore with opsin, since studies of a number of visual pigment analogues show that the β-band intensity in CD is especially variable where the analogue chromophore has a structural alteration in that part of the molecule farthest from the covalent, Schiff-base binding site.[23,25,26]

On the stereospecificity of the binding site of opsin. Properties of the new isomeric rhodopsins are listed in Tables 3 and 4 along with those of the known isomers. Column 2 of Table 3 indicates the presence (+) or absence (−) of a pigment analogue with a given geometric isomer of retinal. The data reveal that out of the 13 geometric isomers tested, only two do not form pigment analogues. This trend is diametrically opposed to the early observation of the formation of pigments from only two out of six geometric isomers. Since these new geometric isomers are quite different in shape from one another, ranging from the singly bent *mono-cis* isomers to the triply bent *tricis* isomers, the conclusion that there is an almost complete lack of absolute stereospecificity at the binding site of opsin appears inevitable.

However, before abandoning the notion of high stereospecificity of the binding site of opsin, we should examine the rates of pigment formation for different geometric isomers of retinal. Since the values for k_2 were obtained at different times, in different laboratories and sometimes under slightly different conditions, they are probably only qualitatively meaningful. Nevertheless, some obvious conclusions can be safely drawn. The numbers in the fourth and fifth columns of Table 3 indicate an overwhelming preference of opsin for the 11-*cis* isomer whether when combining with opsin in ROS suspension or opsin solubilized in digitonin. Opsin reacted with other isomers at rates up to three orders of magnitude slower. The data also indicate a slight preference for 9-*cis*-retinal, which reacted less than seventy times slower than 11-*cis*. This preference for *mono-cis* isomers bent at the center of the polyene chain is also reflected in the pigment yield data. 11-*cis*- and 9-*cis*-retinal regenerate pigments in much higher yields (column 6 in Table 3). Therefore, we believe that the binding site of opsin does exhibit a degree of stereospecificity but, the criteria for stereospecificity should be restated in terms such as rates of recombination and pigment yields rather than in terms of the existence of a pigment analogue. The limited data on the stability of pigment analogues in an excess of hydroxylamine (column 5) also can be taken to support the notion of binding site specificity in that 11-*cis* and 9-*cis* pigments are more stable than other chromophoric configurations toward this reagent.

It is worth noting that the failure of the *trans* and the 13-*cis* isomers to form pigments with opsin can be explained by the presence of longitudinal restrictions of the binding site. This concept was first proposed by Mastumoto and Yoshizawa,[27] and was subsequently refined with more exact molecular dimensions.[28] The critical distances of the stable or low energy twisted conformers of all active isomers fall within the range of 10.1–10.9 Å from C_{15} to the center of the cyclohexenyl ring. On the other hand, the lengths of the all-*trans* and the 13-*cis* isomers exceed the above limit.

Acknowledgements—The work done at Hawaii and Amherst were supported respectively by grants from the U.S. Public Health Service (AM-17806 and EY-00102), the work at Nijmegen was supported by the Netherlands Organization for the Advancement of Basic Research (ZWO). Some of the early work was carried out at the Biological Laboratories of Harvard University. RSHL (a Guggenheim fellow) and WJD (a ZWO fellow) wish to thank Prof. George Wald and Paul Brown for their hospitality and the use of their equipment, and the respective foundations for the fellowships.

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