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On Retention of Chromophore Configuration of Rhodopsin Isomers Derived from Three *dicis* Retinal Isomers

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By the chromophore extraction–HPLC method, it has been shown that an isomeric mixture of pigments (9,11-*dicis*, 9-*cis*, and 9,13-*dicis*) was formed from interaction of 9-*cis*,11-*cis*-retinal with bovine opsin. That from 7-*cis*,9-*cis*-retinal retained the original geometry and that from 7,13-*dicis* contained almost completely the isomerized 7-*cis* pigment.

Isomers of retinal (*1*) and its analogs (*2*) have been used extensively for examining stereoselectivity of the binding site of the visual protein, opsin. In the past, the formation of a spectrally (uv–vis) distinct new pigment was generally taken as a positive indication of compatibility of the new isomer with the binding site. But clearly the method would not be able to exclude the possibility of partial isomerization of chromophore giving a mixture of isomeric pigments. Recently, the establishment of an analytical method of HPLC for retinyl chromophores of visual pigment analogs, extracted under mild conditions in the form of retinal or, more efficiently, retinyl oximes (*3*), has provided a reliable means for determining the polyene configuration in a pigment analog and/or the extent of isomerization of the chromophore during binding interaction. Thus, it was shown that the original geometrical configuration was retained in all regenerated *monocis* pigments: 11-*cis*, 9-*cis* (*3b*), and 7-*cis* (*4*).

For the *dicis* pigment analogs, this method of chromophore extraction and characterization has so far been applied to the 9,13-*dicis* isomer only (*5, 6*). The situation is more complex than the *monocis* analogs. Formation of the *dicis* pigment was shown to be accompanied by a larger amount of 9-*cis*-rhodopsin (*6*). Apparently the slower rate of reaction of the *dicis* retinal with opsin allowed formation of 9-*cis*-rhodopsin from the more reactive 9-*cis*-retinal to which the *dicis* retinal isomerized under the reaction condition. This work raised the question of geometrical integrity of other reported *polycis* rhodopsin analogs (*1b*). In this paper we report results of chromophore extraction/HPLC studies of three *dicis* rhodopsin analogs (9,11-*dicis*, 7,9-*dicis*, and 7,13-*dicis*).

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EXPERIMENTAL

Materials and Methods

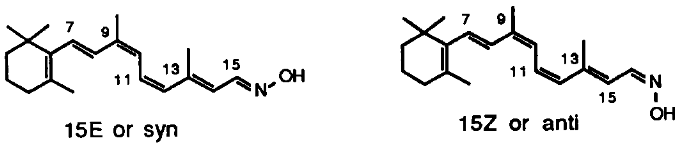
General information. The uv–vis absorption spectra were recorded on a Hitachi-330 spectrometer interfaced with a NEC PC-9801F computer or on a Perkin–Elmer Lambda-5 uv–vis spectrophotometer connected to a 7500 data station. For HPLC analysis, a Shimadzu-DuPont LC-1 system equipped with a Zorbax SIL column (4.0 × 150 mm) and a Shimadzu SPD-1 detector with the detecting beam set at 360 nm was used. The solvent used was a mixture of benzene, diethyl ether, and isopropyl alcohol in the ratio of 95 : 4.4 : 0.6. An Ultraturrax (Janke-Kunkel) homogenizer was used for chromophore extraction experiments. All samples were handled under dim red light and ice-chilled conditions.

Synthesis of retinal isomers. 7-*cis*,13-*cis*-, and 7-*cis*,9-*cis*-retinals were prepared according to the established $C_{15} + C_5$ procedure (*7*). 9-*cis*,11-*cis*-retinal was synthesized using the same modified Horner Emmons reagent used for the synthesis of all-*cis*-retinal (*8*). 7-*cis*- and 9-*cis*-retinal isomers were obtained as by-products of the syntheses mentioned above. Irradiation of all-*trans*-retinal, dissolved in acetonitrile, with light >380 nm provided the 11-*cis* and 13-*cis* isomers. All of the retinal isomers were purified by preparative HPLC (column, 10 × 250 mm 5-μm Lichrosorb; solvent, 5% diethyl ether in hexane).

Preparation of retinal oximes. Retinal oximes (standard for the HPLC analysis) were prepared as described by Shichida *et al.* (*6*). For the synthesis of retinal oximes on a preparative scale, a solution of 50 mg (0.18 mmol) of a retinal isomer was mixed with 2 ml of the hydroxylamine solution (1 M, pH 7.0). Formation of the retinal oxime was monitored by uv–vis spectroscopy or TLC analysis. The reaction was usually complete within 5 min. The reaction mixture was diluted with an equal amount of water and extracted with petroleum ether. After drying over Na_2SO_4 and evaporation of solvent, a mixture of *E*- and *Z*-oximes of the retinal isomer was obtained (46 mg, 88% yield). They were separated by preparative HPLC. (Structures of the two oximes from 9-*cis*,11-*cis*-retinal are shown in Scheme 1 to clarify possible confusion with the *E*,*Z* and *syn*,*anti* designations.)

The absorption characteristics of the isomeric oximes prepared from the *dicis* retinal isomers are listed in Table 1 along with other reported values. These values were used for calculation of composition of extracted oximes.

Preparation of cattle opsin. Membranes of cattle rod outer segments (ROS) were isolated from homogenized retina by a stepwise sucrose flotation method (*6*).



15E or syn

15Z or anti

SCHEME 1

TABLE 1

A List of Absorption Maxima and Molar Extinction Coefficients at Absorption Maxima and at 360 nm for *E*- and *Z*-Oximes of Retinal Isomers (in Benzene)

Retinal oxime[a]		λ_{max} (nm)	ε_{max} (M cm^{-1})	ε_{360} (M cm^{-1})
9,13-*dicis*	-*E*	353	40,200	38,300
9,13-*dicis*	-*Z*	356	31,400	30,900
9,11-*dicis*	-*E*	348	36,000	33,600
9,11-*dicis*	-*Z*	352	33,500	32,300
9-*cis*	-*E*	356	39,900	39,300
9-*cis*	-*Z*	361	30,600	30,600
7,13-*dicis*	-*E*	348	34,500	32,800
7,13-*dicis*	-*Z*	352	31,800	30,200
7-*cis*	-*E*	350	49,000	47,000
7-*cis*	-*Z*	355	47,000	46,200
all-*trans*	-*E*[b]	357	55,500	54,900
all-*trans*	-*Z*	361	51,700	51,600
11-*cis*	-*E*[b]	347	35,900	35,000
11-*cis*	-*Z*	351	30,000	29,600
13-*cis*	-*E*[c]	352	55,500	49,000
13-*cis*	-*Z*	356	53,500	52,100

[a] Represented by geometry of polyene (9,13-*dicis*, etc.) and oxime (*E*/*Z*).

[b] Ref. (*3a*), in hexane.

[c] Hamanaka, T., Hiraki, K., and Kito, Y. (1986) *Photochem. Photobiol.* **44,** 75–78.

They were then bleached with orange light (λ > 520 nm) in the presence of 100 mM hydroxylamine. The mixture of opsin and retinal oximes, so obtained, was washed 10 times with 10 mM Hepes buffer to remove hydroxylamine. After lyophilization, the ROS were washed with petroleum ether to remove retinal oximes and were used for the extraction of opsin by 2% digitonin solution (in Hepes buffer, pH 7.0).

Pigment formation. An ethanolic solution of the retinal isomer was incubated with digitonin extract containing opsin (in 2.5 M excess) at 20°C. The concentration of digitonin used for 9-*cis*,11-*cis*-retinal was 0.67% in the reaction mixture whereas in the cases of the 7,13-*dicis* and 7,9-*dicis* isomers due to slow rate of pigment formation it was reduced to 0.2%.

Extraction of chromophores from pigments. Procedures for denaturation of the pigment analogs and extraction of the chromophores in the form of retinal oximes were the same as those described in the literature (*6*).

Thermal denaturation of opsin. A solution of opsin in 2% digitonin was suspended in a hot water bath (60–70°C) for 50 s and immediately chilled in ice until use. Denaturation of the sample was verified by its lack of activity for binding of 11-*cis*-retinal (no indication of absorption at 500 nm).

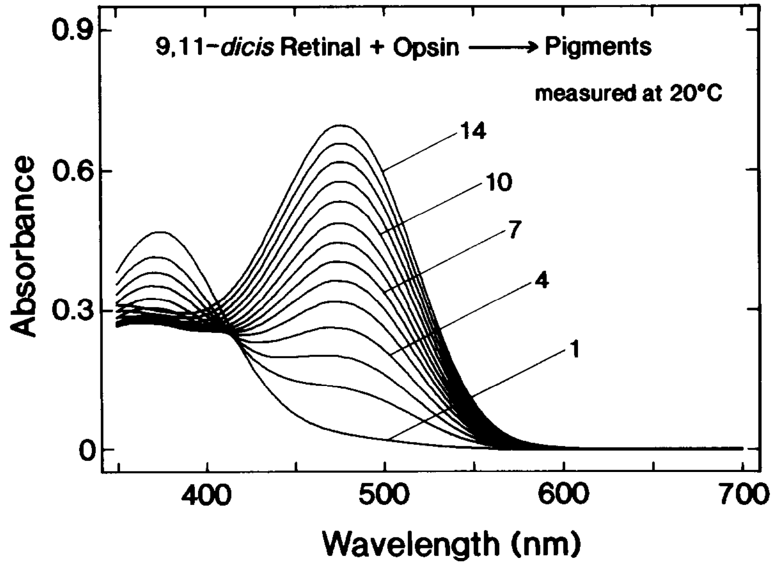


FIG. 1. Formation of rhodopsin isomers incubation of 9-*cis*,11-*cis*-retinal and cattle opsin at 20°C. The absorption spectra were recorded successively at intervals of 2 h (curves 1 to 14).

RESULTS

The 9,11-dicis Isomer

The course of pigment formation upon addition of 9-*cis*,11-*cis*-retinal to opsin is shown in Fig. 1. The different absorption maxima of the difference absorption spectra obtained during the progress of the reaction indicated the presence of more than one pigment.

A portion of the pigment formed was incubated with hydroxylamine by addition of 1 M hydroxylamine to a final concentration of 200 mM. The absorbance of the pigment, monitored at 480 nm, decreased reaching a constant value after about 10 h indicating that the remaining pigment was stable to hydroxylamine. The pigment mixture was irradiated at a wavelength greater than 520 nm. Chromophore extraction experiments were carried out with three different samples. The results are shown in Fig. 2.

The oxime mixture obtained from the original pigment consisted of three pairs of different isomers, 9,11-*dicis*, and the isomerized 9-*cis* and 9,13-*dicis* with the 9,11-*dicis* and 9-*cis* being the major pigments (in approximately 1 : 1 ratio) (Table 2). Small amounts of all-*trans* and 13-*cis*-retinal oximes (<5%) were also detected.

The HPLC chromatograms of the extracted chromophore from the sample irradiated after incubation in the presence of hydroxylamine (corresponding to curve 17 in Fig. 2) are shown in Fig. 3. The major peaks are those of the *E*- and *Z*-oximes of the all-*trans*-retinal and 9-*cis*,11-*cis*-retinal, formed respectively, from photobleaching of the 9-*cis*-rhodopsin and dark reaction of 9,11-*dicis*-rhodopsin

TABLE 2

Composition of Isomers of Retinal Oximes Extracted from the Pigment Produced by Incubation of 9-*cis*,11-*cis*-Retinal with Cattle Opsin[a]

	Molar % of isomers				
	9,11-*dicis*	9-*cis*	all-*trans*	9,13-*dicis*	13-*cis*
Original pigments	46.3	41.2	1.4	8.1	3.0
Irradiated 10 h after the addition of NH_2OH	45.0	3.0	40.3	8.5	3.2
Irradiated immediately after the addition of NH_2OH	12.7	1.0	73.6	3.8	8.8

[a] After combining *E*/*Z* isomers of each oxime.

with hydroxylamine. The next in size are those from 9-*cis*,13-*cis*-retinal. Those of 9-*cis* and 13-*cis* are also formed in small quantities.

In a separate run, the pigment was irradiated immediately after addition of hydroxylamine. The major products were the oximes of all-*trans*-retinal, formed from photoreactions of 9-*cis*- and 9,11-*dicis*-rhodopsin. Results of chromophore recovery under three different conditions are summarized in Table 2.

The difference absorption spectrum (λ_{max} = 485 nm) of the hydroxylamine

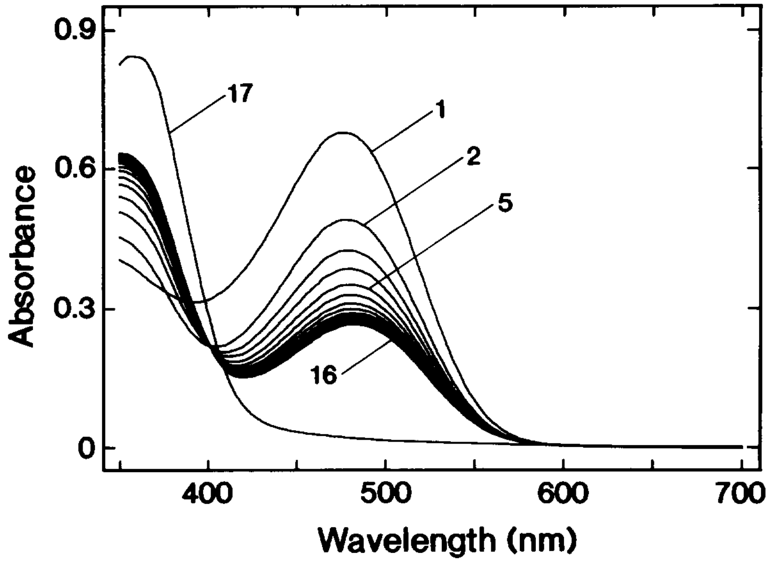


FIG. 2. Effect of hydroxylamine and subsequent irradiation with orange light on the pigments produced from 9-*cis*,11-*cis*-retinal and cattle opsin. Curve 1 shows the absorption spectrum of the pigments. Then, after addition of neutralized hydroxylamine (1 M) to a final concentration of 200 mM (curve 2), the spectrum of the sample was successively measured at intervals of 30 min (curves 3 to 16). The sample was then irradiated with light >520 nm (curve 17).

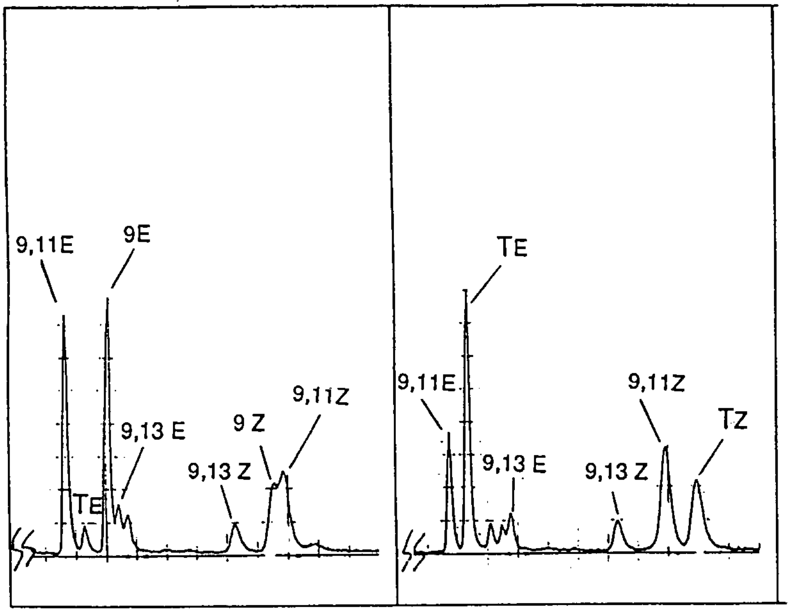


FIG. 3. HPLC patterns of the chromophore extracted from the pigments produced from 9-*cis*,11-*cis*-retinal and cattle opsin. Left, original pigment. Right, an identical sample of the pigment but allowed to stand for 10 h and followed by addition of hydroxylamine (to 200 mM) and irradiation with light >520 nm.

stable pigment obtained by subtraction of the spectra after prolonged standing with excess NH_2OH (curve 16 from Fig. 2) and after photobleaching (curve 17) was found to be identical to that of 9-*cis*-rhodopsin. This result is in agreement with that of the extraction experiment. That for the hydroxylamine unstable pigment (λ_{max} = 474 nm) was obtained by subtraction of the same curve 16 from that after immediate addition of NH_2OH (curve 2 in Fig. 2). The absorption must be due to the 9,11-*dicis* pigment analog and to a lesser degree the minor (Fig. 3) but also unstable 9,13-*dicis* pigment (*6*). The calculated difference absorption spectrum of the 9,11-*dicis*-rhodopsin (472 nm) shown in Fig. 4 was obtained by subtracting from the 474 curve the component (Table 2) contributed by the 9,13-*dicis*-rhodopsin. Also shown are those of 9-*cis*-rhodopsin and 9,13-*dicis*-rhodopsin.

The 7,13-dicis and 7,9-dicis Isomers

When 7-*cis*,13-*cis*-retinal was incubated with a 2% digitonin extract of opsin, formation of a pigment with absorption maximum around 450 nm was observed. The rate of pigment formation was found to be very slow, but accelerated upon decreasing digitonin concentration. After 48 h, the pigments were denatured and the extracted chromophores analyzed by HPLC (Fig. 5). The major peaks in the chromatogram are due to 7-*cis*-retinal oximes with the 7-*cis*,13-*cis*-retinal oximes

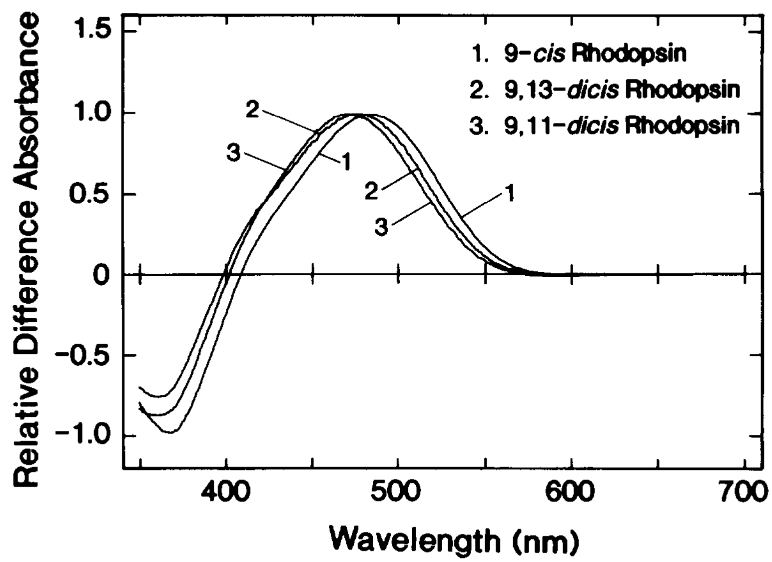


FIG. 4. Difference absorption spectra of 9,11-*dicis*-rhodopsin (λ_{max} = 472 nm) and 9-*cis*-rhodopsin (λ_{max} = 485 nm). The absorption spectrum of 9,13-*dicis*-rhodopsin (λ_{max} = 478 nm) (Shichida *et al.* (6)) has been overlayed for comparison. All absorption spectra have been normalized at absorption maxima.

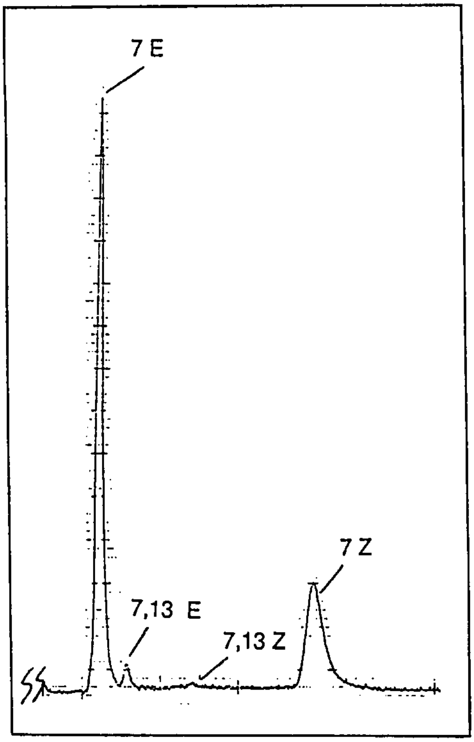


FIG. 5. An HPLC chromatogram of the chromophore extracted from the pigments produced from 7-*cis*,13-*cis*-retinal and cattle opsin.

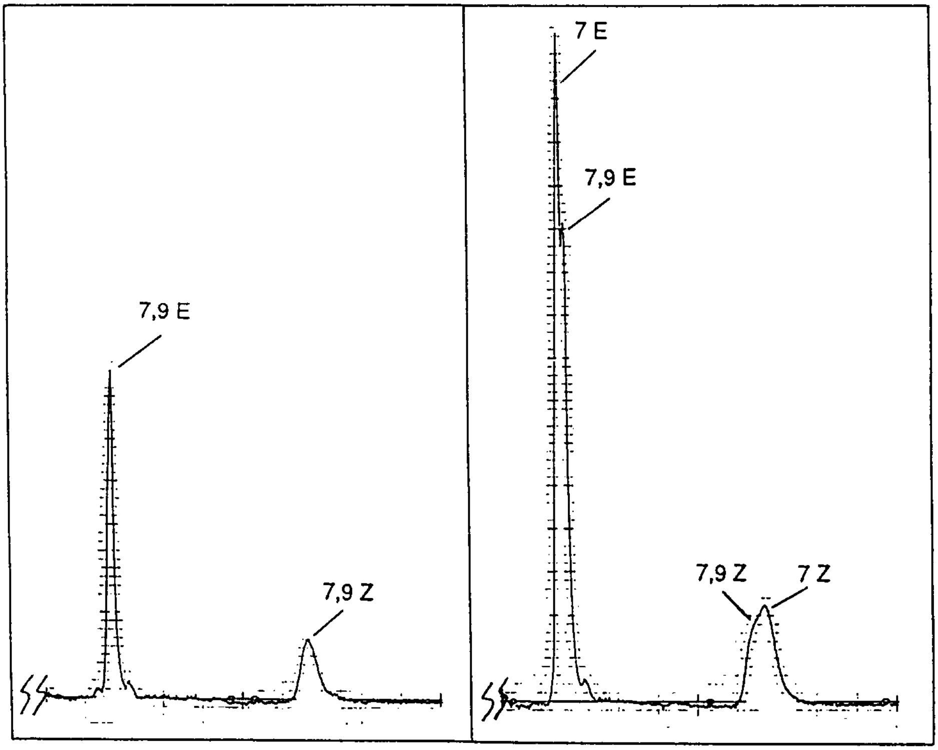


FIG. 6. HPLC chromatograms of the chromophore extracted from the pigments produced from 7-*cis*,9-*cis*-retinal and cattle opsin. Left, original pigment. Right, above, coinjected with 7-*cis*-retinal oximes.

present only as a minor fraction of the mixture (2.5%). Obviously extensive configurational changes occurred during the incubation of 7-*cis*,13-*cis*-retinal with opsin.

The reaction of 7-*cis*,9-*cis*-retinal with opsin was followed for 32 h (until absorption at 456 nm became constant). The pigments, so obtained, were denatured and analyzed by HPLC. The presence of only peaks due to 7-*cis*,9-*cis*-retinal oximes, confirmed by coinjecting the *E*- and *Z*-oximes of 7-*cis*-retinal (Fig. 6), indicates the exclusive formation of 7,9-*dicis*-rhodopsin.

To answer the question whether the opsin catalyzed isomerization of a *dicis* retinal took place prior to its reaction with opsin, 9-*cis*,11-*cis*-retinal or 7-*cis*,13-*cis*-retinal was incubated separately with thermally denatured opsin for 24 h. Extracted chromophores of aliquots taken were analyzed by HPLC. Results showed that extensive isomerization took place during a period comparable to that required for pigment formation. The case of 9-*cis*,11-*cis*-retinal is shown in Fig. 7 as a representative example.

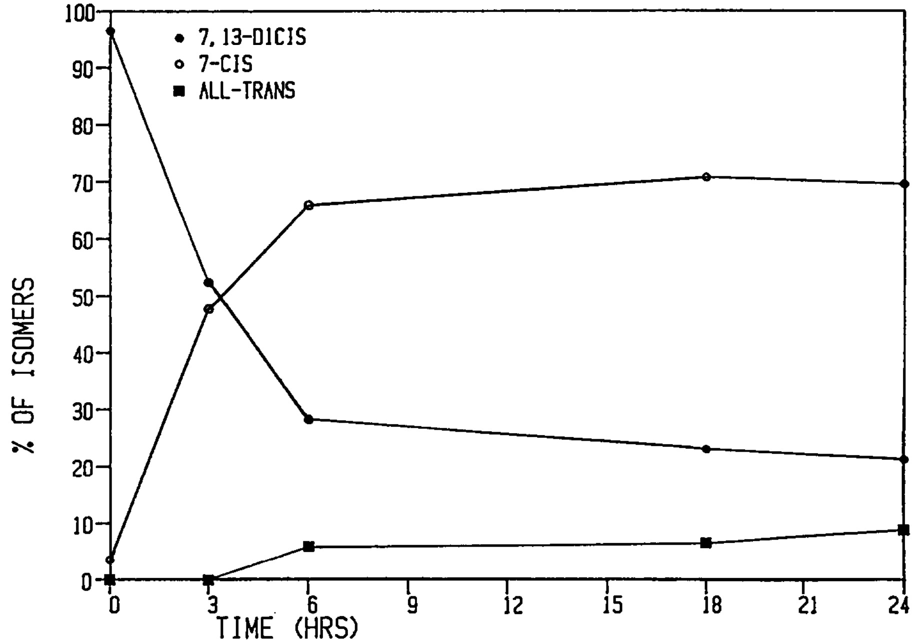
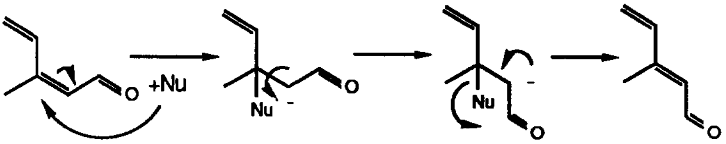


FIG. 7. Incubation of the 9-*cis*,11-*cis*-retinal with thermally denatured opsin at 20°C for 24 h. Small amounts of the sample were removed immediately after addition and also after 3, 6, 18, and 24 h of incubation, and the chromophore was extracted in the presence of hydroxylamine and then subjected to HPLC analyses. The molar percentages of various isomers have been plotted vs time.

DISCUSSION

The combined HPLC chromatographic data of the retinyl chromophores extracted from pigments derived from the three *dicis* retinal isomers (this paper) with bovine opsin and those reported on the 9,13-*dicis* system (*6*) show that the extent of retention of configuration during binding interaction with opsin varies drastically from one *dicis* isomer to another. Complete retention of configuration was observed in the case of 7,9-*dicis*-rhodopsin (Fig. 6) and nearly complete isomerization in 7,13-*dicis*-rhodopsin (Fig. 5). In between these two extremes are the 9,11-*dicis* (Table 2) and 9,13-*dicis* pigments (*6*). Their formations are accompanied by substantial amounts of the isomerized 9-*cis* product. The 9,11-*dicis* system yielded an amount of pigments retaining the original geometrical integrity (40% for the *dicis* pigment) higher than that of the 9,13-*dicis* (0–28%) (*6*), its mixture is also more complex, forming a not insignificant amount (~8%) of the seemingly two bond isomerized 9,13-*dicis* isomer (see below).

Experiments involving incubation of 9-*cis*,11-*cis*-retinal (Fig. 7) and 7-*cis*,13-*cis*-retinal with denatured opsin showed that isomerization of the chromophores, catalyzed by opsin, most likely took place concurrently with pigment formation. Apparently those double bonds nearer the carbonyl group are more sensitive to



SCHEME 2

the catalyzed isomerization. Since the *dicis* isomers are known to react more slowly with opsin (*1*) than the corresponding *monocis*, preferential formation of the isomerized 9-*cis* pigments from 9-*cis*,11-*cis*-retinal and 9-*cis*,13-*cis*-retinal and 7-*cis* pigment from 7-*cis*,13-*cis*-retinal is not surprising. Especially notable is the almost negligible amount (~3%) of the 7,13-*dicis* pigment detected in the current study.

On the basis of the work of Sack and Seltzer (*9*), the mechanism of catalyzed isomerization most likely involves the reversible Michael addition of a nucleophile from the protein to an electrophilic carbon of the polyene (see Scheme 2). Preferential isomerization at the 13,14 bond is then a reflection of the more electrophilic character of the odd numbered carbons near the carbonyl group. Hence only for the 7,9-*dicis* isomer where the *cis* double bonds are sufficiently removed from the carbonyl group, the *dicis* geometry is unaffected during binding interaction. Formation of the 9,13-*dicis* pigment from 9-*cis*,11-*cis*-retinal can also be rationalized by such a catalyzed process. Isomerization of the 11,12 bond (to the 9-*cis* isomer) should take place concurrently with isomerization of the 13,14 bond. The latter will lead to the unstable 9-*cis*,11-*cis*,13-*cis*-retinal, isomerizing to the 9,13-*dicis* isomer (*10*).

It is worth noting that the extrapolated uv–vis absorption maxima of the *dicis* isomeric pigment analogs retain the trend of those containing the 7-*cis* geometry absorbing in the blue shifted region of 440–455 nm, those with the 9-*cis* geometry in a more red shifted region of 472–485 nm and the 11-*cis* most red shifted at 498 nm. The opsin shifts (*11, 12*) for the 11-*cis*, 9-*cis*, and 7-*cis* pigments are, respectively, 2624, 2434 (*11, 12*), and 975 cm^{-1} (*13*). This trend is likely due to combined effects of a distorted chromophore from a twisted 6,7 bond (*14*) and increased longitudinal lengthes for, e.g., the 7-*cis* chromophore (*15*). Hence the blue shift of the 7-*cis* pigment could be either due to a weakened protonation as a result of a misplaced counterion (*16*) or due to a relocated second point charge (*11, 12*). The lower stability of 9,13-*dicis*-rhodopsin and 9,11-*dicis*-rhodopsin toward added hydroxylamine must also be a reflection of altered protein/substrate interaction.

Results of isomeric rhodopsin pigments have been used for mapping the shape of the binding site of opsin (*15, 17*). While the current and earlier (*6*) investigations showed extensive isomerization in some of the *dicis* pigments, the original geometry of the retinyl chromophore was retained in varying amounts in the final pigment mixture. Hence the assumption that the binding site of opsin is sufficiently flexible to accommodate these doubly bent *dicis* chromophores remains valid.

From the current study, one might infer that the two reported *tricis* pigment isomers, 7,9,11-*tricis* and 7,9,13-*tricis* (*1b*), are likely to contain mixtures of isomeric pigments. The loss of the 11-*cis* or the 13-*cis* geometry to give the stable 7,9-*dicis* pigment is a likely possibility.

ACKNOWLEDGMENTS

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REFERENCES

1. (a) Hubbard, R., and Wald, G. (1952) *J. Gen. Physiol.* **36,** 269–315. (b) Liu, R. S. H., Matsumoto, H., Kini, A., Asato, A. E., Denny, M., Kropf, A., and DeGrip, W. J. (1984) *Tetrahedron* **40,** 473–482.
2. Derguini, F., and Nakanishi, K. (1986) *Photobiochem. Photobiophys.* **13,** 259–283.
3. (a) Groenendijk, C. W. T., de Grip, W. J., and Daemen, F. J. M. (1979) *Anal. Biochem.* **99,** 304–310; (b) Groenendijk, C. W. T., de Grip, W. J., and Daemen, M. (1980) *Biochim. Biophys. Acta* **617,** 430–438.
4. (a) Maeda, A., Ogurusu, T., Shichida, Y., Tokunaga, F., and Yoshizawa, T. (1978) *FEBS Lett.***92,** 77–80; (b) Maeda, A., Shichida, Y., and Yoshizawa, T. (1979) *Biochemistry* **18,** 1449–1453.
5. Crouch, R., Purvin, V., Nakanishi, K., and Ebrey, T. G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1538–1542.
6. Shichida, Y., Nakamura, K., Yoshizawa, T., Trehan, A., Denny, M., and Liu, R. S. H. (1988) *Biochemistry* **27,** 6495–6499.
7. Liu, R. S. H., and Asato, A. E. (1982) *in* Methods in Enzymology (Packer, L., Ed.), Vol. 88, pp. 506–516, Academic Press, San Diego, CA.
8. Trehan, A., and Liu, R. S. H. (1988) *Tetrahedron Lett.* **29,** 419–422.
9. Sack, R. A., and Seltzer, S. (1978) *Vision Res.* **18,** 423–426.
10. Knudsen, C. G., Carey, S. C., and Okamura, W. H. (1980) *J. Amer. Chem. Soc.* **102,** 6355–6356.
11. Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gavinowicz, M. A., Arnaboldi, M., and Motto, M. G. (1979) *J. Amer. Chem. Soc.* **101,** 7084–7086.
12. Sheves, M., Nakanishi, K., and Honig, B. (1979) *J. Amer. Chem. Soc.* **101,** 7086–7088.
13. Unpublished results of Mead, D., and Liu, R. S. H.
14. Liu, R. S. H., Zingoni, J. P., Kini, A., Trammell, M., Chu, D., Asato, A. E., and Bopp, T. (1983) *J. Org. Chem.* **48,** 4817–4821.
15. Liu, R. S. H., and Mirzadegan, T. (1988) *J. Amer. Chem. Soc.* **110,** 8617–8123.
16. Blatz, P. E., Mohler, J. H., and Navagul, H. V. (1972) *Biochemistry* **11,** 848–855.
17. Liu, R. S. H., Asato, A. E., Denny, M., and Mead, D. (1984) *J. Amer. Chem. Soc.* **106,** 8289–8300.