# Divergent Pathways in Photobleaching of 7,9-*dicis*-Rhodopsin and 9,11-*dicis*-12-Fluororhodopsin: One-Photon–Two-Bond and One-Photon–One-Bond Isomerization†

Yun Zhu and Robert S. H. Liu*

*Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822*

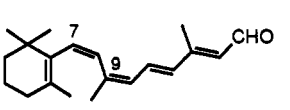
*Received April 2, 1993; Revised Manuscript Received July 13, 1993*®

ABSTRACT: Through low-temperature photochemistry, UV/vis spectroscopy, and chromophore extraction experiments, we have established that 7,9-*dicis*-rhodopsin undergoes one-photon–two-bond photoisomerization to a batho intermediate (its absorption maximum is slightly blue shifted from that of bathorhodopsin) containing the all-trans geometry, while 9,11-*dicis*-12-fluororhodopsin undergoes one-photon–one-bond isomerization to the corresponding 9-cis isomer and then the all-trans batho intermediate. The difference in the photochemical properties of the two dicis pigment analogs was rationalized by possible local protein perturbation, lability of the 11-cis geometry, and photochemical properties of the chromophores.

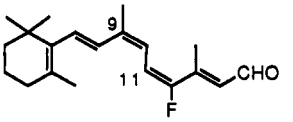
The primary photochemical process of the visual chromophore is the 11-cis to all-trans isomerization. Through low-temperature spectroscopy (Yoshizawa & Wald, 1964), the first stable, red-shifted intermediate was detected and named bathorhodopsin. The same intermediate was subsequently detected at physiological temperatures through time-resolved spectroscopy [see, for example, Shichida (1986)]. More recently, another earlier intermediate was detected (Shichida et al., 1988a; Schoenlein et al., 1991) that was named photorhodopsin by the Kyoto group. The all-trans geometry is believed to be present at the photorhodopsin stage as part of a highly twisted chromophore. Thus, subsequent decay to later intermediates in the dark merely allows gradual relaxation of the strained chromophore and the surrounding protein residues (Birge, 1990; Liu & Shichida, 1990).

Studies of bleaching sequences of other isomeric rhodopsins have been limited to 9-*cis*-rhodopsin [see, for example, Yoshizawa and Shichida (1982)] and 7-*cis*-rhodopsin (Kawamura et al., 1980; Shichida et al., 1991). In the former, its batho product was shown to be spectroscopically identical to that of bathorhodopsin (Kandori et al., 1988). However, for 7-*cis*-rhodopsin, a somewhat blue-shifted batho intermediate was detected instead. Only at later stages of the bleaching sequence (from lumirhodopsin) were spectra identical to those of native rhodopsin observed (Shichida et al., 1991). For dicis visual pigments, the only attempts were those on the 9,13-dicis isomer. The early claim of one-photon–two-bond isomerization (Crouch et al., 1975) was found to be inconsistent with more recent chromophore extraction experiments (Shichida et al., 1988b), where formation of the dicis pigment was complicated by extensive isomerization to the 9-cis pigment. The results from photoisomerization of a mixture of isomeric pigments seemed to indicate, instead, preferred isomerization of the 9,13-dicis pigment to the 13-cis isomer.

During the course of studying the configuration purity of a series of isomeric rhodopsin pigments (Trehan et al., 1990), it was shown that most of the dicis pigments failed to retain completely the original chromophore geometry. The exception was 7,9-*dicis*-rhodopsin, where retention of configuration was established by chromophore extraction experiments. Formation of the 9,11-dicis pigment, potentially the most interesting isomer because of the possibility of it yielding rhodopsin or 9-*cis*-rhodopsin through one-bond isomerization, was found to be accompanied by significant isomerization to 9-*cis*-rhodopsin. However, in a separate study (Colmenares & Liu, 1992), it was found that the presence of the 12-fluoro substituent greatly stabilized the 9,11-dicis geometry in that isomerically pure pigment could be formed. We have, therefore, undertaken a combined study of low-temperature photochemistry and chromophore extraction during the course of irradiation of these two stable dicis pigments: 7,9-*dicis*-rhodopsin and 9,11-*dicis*-12-fluororhodopsin.




7,9-dicis-retinal




9,11-dicis-12F-retinal

## EXPERIMENTAL PROCEDURES

*Materials.* 7,9-*dicis*-Rhodopsin was prepared from the reaction of bovine opsin with 7,9-*dicis*-retinal (Zhu et al., 1992) in the same manner as 7-*cis*-rhodopsin (Shichida et al., 1991). Briefly, bovine rod outer segments (ROS) were collected in 10 mM HEPES buffer (pH 7.0), 65 mM NaCl, and 2 mM $MgCl_2$ by the sucrose flotation method. Photobleaching of ROS was accomplished by irradiating the suspension with >540-nm light in the presence of 1 M $NH_2OH$. The bleached ROS (opsin) was resuspended in 10 mM HEPES buffer and divided into several fractions for analog preparation.

Rhodopsin analogs were prepared by incubation of the ROS suspension with excess concentrated ethanol solution of a retinal isomer at 5 °C for ~20 h, followed by purification by washing with HEPES and hexane to remove excess retinal. The pellet obtained was dried under a stream of nitrogen. The pigment was then extracted with 2% digitonin dissolved in 10 mM HEPES buffer (pH 7.0).

The 7,9-dicis pigment was found to be thermally less stable than rhodopsin, 9-*cis*-rhodopsin, and even the similarly hindered 7-cis. Hence, during hexane washing of the pigment

† This work was supported by grants from the U.S. Public Health Services (DK-17806) and HFSPO.

* Author to whom inquiries should be addressed.

® Abstract published in *Advance ACS Abstracts,* September 1, 1993.

0006-2960/93/0432-10233$04.00/0 

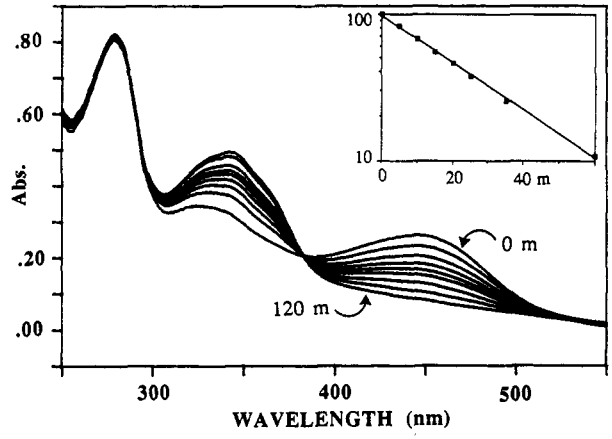



FIGURE 1: Low stability of 7,9-*dicis*-rhodopsin in 100 mM $NH_2OH$ at room temperature as indicated by spectra taken at 0, 5-, 10-, 15-, 20-, 25-, 35-, 60- and 120-m intervals after addition of all of the material. The insert is a log plot of the decrease in absorbance at 440 nm against time.

formed (eight times) for removal of excess retinal, pigment degradation unavoidably occurred. Yet, the possibility of a small amount of residual, unreacted 7,9-*dicis*-retinal remaining in the sample could not be rigorously ruled out. In the presence of a large excess (100 mM) of hydroxylamine, the pigment was also found not to be stable (Figure 1). Hence, for photochemical studies, $NH_2OH$ was not added to solutions of the 7,9-dicis pigment. Preparations of 9,11-*dicis*-12-fluororetinal and its rhodopsin analog have been reported (Colmenares & Liu, 1992). The pigment was found to be stable in $NH_2OH$, although prolonged standing at room temperature is known to result in isomerization to the 9-cis isomer.

*Low-Temperature Spectroscopy.* The setup was essentially that described in the literature (Yoshizawa & Shichida, 1982). In our case, an Oxford DN1704 optical cryostat was attached to a Perkin-Elmer λ5 spectrometer. The irradiation beam was filtered light from a 150-W Xe–Hg arc lamp, directed to the incident window through a light pipe. Opal glasses were inserted in the path of the sample and reference beams in order to minimize complications with scattered light. For clear glass formation, glycerol was added to a final concentration of 66%. The use of a precooled cell prior to the introduction of sample allowed rapid cooling of the sample, thus minimizing cracking of the glass.

*Chromophore Extraction.* The procedure for chromophore extraction was essentially that in the literature (Groenenduk et al., 1979). Because of improved separation of HPLC peaks corresponding to isomeric retinals (Zhu et al., 1992), in this work we decided to extract the chromophore without the addition of hydroxylamine, thus yielding retinal isomers instead of the oximes. Although this procedure caused partial isomerization to a 13-cis isomer (top of Figure 2), a complication known to occur for other isomeric pigments (Groenenduk et al., 1979), the small extent of isomerization did not affect the conclusions drawn in this work.

Thus, methylene chloride was used for protein denaturation followed by extraction of the chromophore with hexane. A concentrated solution was analyzed by HPLC (Dynamax 5-μm Microsorb silica gel column, 25 cm × 4.6 mm; solvent, 4–5% ether in hexane; detecting beam, 360 nm). Correction factors for the absorptivity difference of isomers at 360 nm as well as HPLC separation conditions are in the literature (Zhu et al., 1992).

For chemical studies at low temperatures, a sample (0.5 ml) of the pigment analog was introduced into a cylindrical

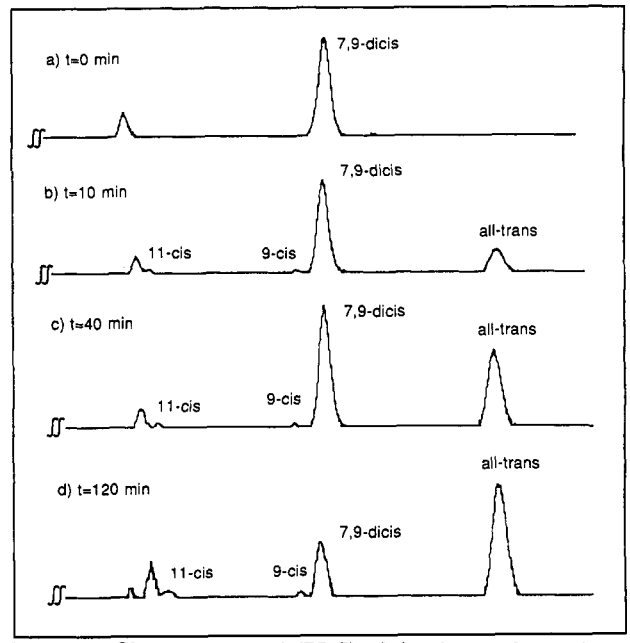



FIGURE 2: Chromatograms (HPLC) of the chromophore mixture extracted from 7,9-*dicis*-rhodopsin before (a) and after varying periods of irradiation with 437-nm light (b–d). All peaks were verified by retention time through coinjection with authentic samples. The minor peak at $t = 0$ corresponds to that of a 13-cis isomer. However, all isomers with the 13-cis geometry have nearly identical retention times (Zhu et al., 1992) making unambiguous identification difficult.

Pyrex cell of ~2 mm path length, which was then immersed in liquid nitrogen in a clear Dewar flask. A sample irradiated (437 nm) after a desired period was then denatured in the manner described above. The progress of the photochemical reaction was followed by HPLC analysis of the extracted chromophore mixtures. Those of 7,9-*dicis*-rhodopsin are shown in Figure 2, and those of 9,11-*dicis*-12-fluororhodopsin in Figure 3.

For comparison, a sample of rhodopsin was irradiated under the same conditions and the chromophore extracted for analysis by HPLC. The ratio of all-trans to 11-cis and 9-cis was found to be 60:20:10. Yields for retinal recovery were 50–60%.

## RESULTS AND DISCUSSION

*7,9-dicis-Rhodopsin.* The hindered 7-cis geometry is known to increase ring/chain distortion (Liu et al., 1983) and decrease pigment stability, e.g., in hydroxylamine (Kawamura et al., 1980; Shichida et al., 1991). The added 9-cis geometry in 7,9-*dicis*-rhodopsin appears to have further reduced pigment stability: at 23°C the half-life of the dicis pigment in 100 mM hydroxylamine was found to be ~20 min, while at 30°C the same half-life was reported for 7-*cis*-rhodopsin (Shichida et al., 1991).

A freshly prepared sample of 7,9-*dicis*-rhodopsin (444 nm)[1] was irradiated at liquid nitrogen temperature with 437-nm (interference filter) light. Progress of the photochemical reaction is shown by changes in the spectra in Figure 4. Formation of a batho product is revealed by the increased absorption in the 500–550-nm region. The direct conversion from the dicis pigment to the batho product is suggested by the presence of an isosbestic point and by the linear plot of change of absorbance at 440 versus irradiation time (insert in Figure 4).

[1] This value is lower than the 453-nm value reported earlier (Loppnow et al., 1990). This is probably due to the absence of hydroxylamine in the current study.

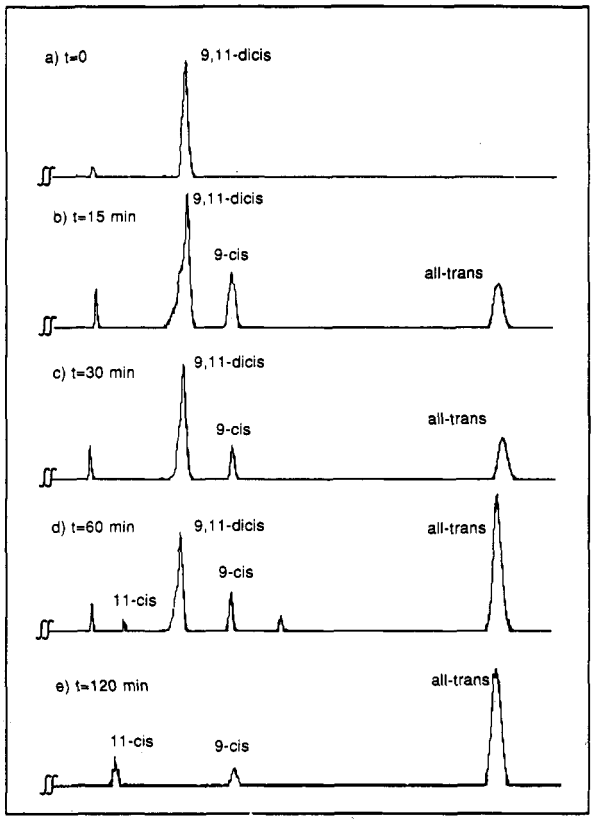



FIGURE 3: Chromatograms (HPLC) of the chromophore extracts obtained before (a) and during irradiation of a sample of 9,11-*dicis*-12-fluororhodopsin with 437-nm light (b–e). The nature of the major peaks was verified through coinjection with authentic samples.

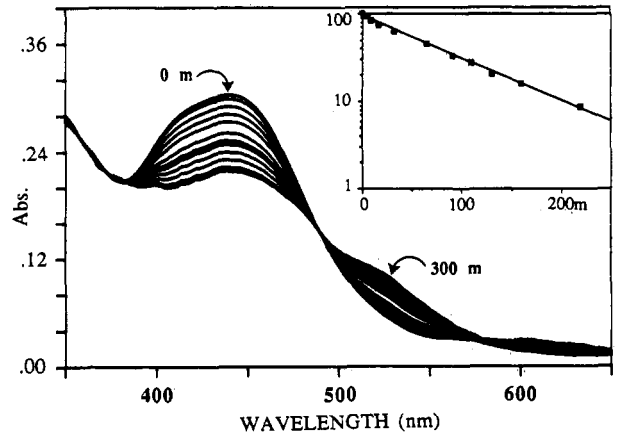



FIGURE 4: Low-temperature photochemistry of 7,9-*dicis*-rhodopsin. A sample of the pigment was irradiated (437 nm) at liquid nitrogen temperature with the spectra recorded after the following intervals of irradiation: 0, 4, 8, 16, 32, 64, 90, 110, 130, 160, 220, and 300 min. Insert: A log plot of decrease in 7,9-dicis pigment absorption at 440 nm upon 437-nm irradiation versus time.

The product mixture was subsequently irradiated with light >610 nm (Figure 5). The decrease in the batho product was accompanied by increased absorption in the 450–500 nm-region. This is similar to the well-known reversible reaction of bathorhodopsin to rhodopsin and 9-*cis*-rhodopsin (Yoshizawa & Shichida, 1982). It also shows that the photochemical reaction of the blue-shifted 7,9-*dicis*-rhodopsin is not reversible. Subsequent irradiation with 437-nm light regenerated the batho product.

The batho product absorbs slightly to the blue (8 nm) of that of bathorhodopsin (from rhodopsin), as shown in the difference spectra in Figure 5 (insert). The shift is reminiscent

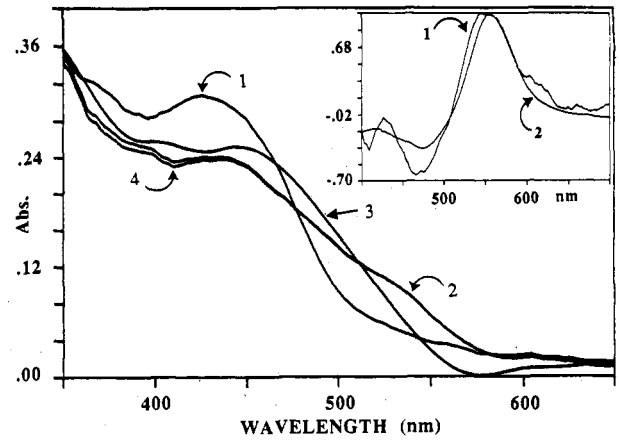



FIGURE 5: Photochemistry of isomeric rhodopsin pigments: the 7,9-*dicis*-rhodopsin (curve 1 and also the last curve in Figure 4) at liquid nitrogen temperature; after irradiation with 437-nm light (curve 2 and also the last curve in Figure 4), followed by prolonged irradiation with >610 nm (curve 3) and then with 437-nm light (curve 4). Insert: Difference spectra. Curve 1, the last curve in Figure 4 minus the first curve in the same figure; curve 2, corresponding spectrum of a rhodopsin sample.

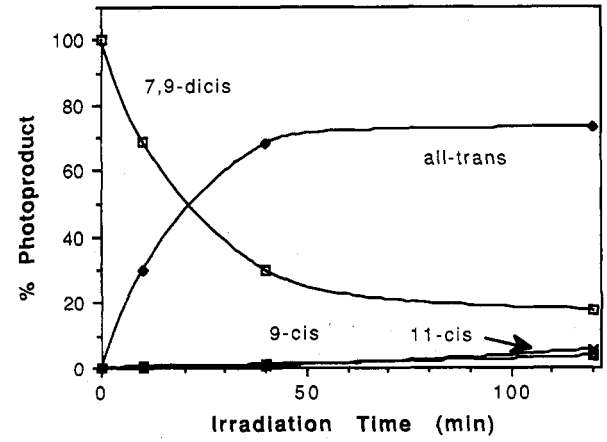



FIGURE 6: Time plot showing changes in isomeric composition of the chromophore during irradiation of 7,9-*dicis*-rhodopsin (data from Figure 2).

of that observed for the batho product from 7-*cis*-rhodopsin, where low-temperature spectroscopy as well as time-resolved spectroscopic studies revealed the convergence of photobleaching intermediates only after the lumi stage, suggesting that at the batho stage the chromophore (and/or the associated protein pocket) must have a shape different from that of bathorhodopsin (Shichida et al., 1991). The blue shift suggests that the batho products from the highly crowded 7-cis isomers are likely more twisted, possibly forced by a binding site retaining its shape of the highly hindered pigment.

Changes in the isomeric composition of the chromophore during the progress of the photoreaction were determined by HPLC analysis of chromophore extracts (Figure 2). The time plot is shown in Figure 6. It is clear from these data that formation of the all-trans chromophore is not preceded by either the 9-cis or the 7-cis isomer, the two one-bond-isomerized products. We believe that these data unambiguously establish the first case of one-photon–two-bond isomerization as the major photochemical pathway for a visual pigment analog.

Data in Figure 7 show that 7,9-*dicis*-rhodopsin is less photosensitive than 7-*cis*-rhodopsin. However, the difference is not sufficiently large to raise the possibility that the absence of one-bond-isomerized 7-cis product is due to the unusually high sensitivity of any one-bond-isomerized product.

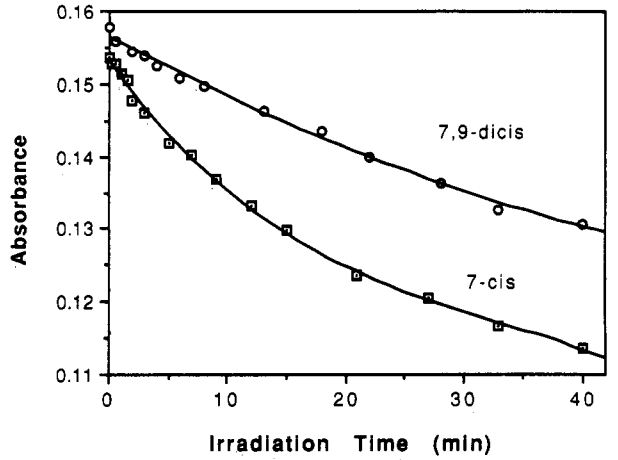



FIGURE 7: Relative photosensitivity of 7,9-*dicis*-rhodopsin and 7-*cis*-rhodopsin in 10 mM $NH_2OH$ during parallel irradiation with 437-nm light as indicated by changes in absorbance at 440 nm.

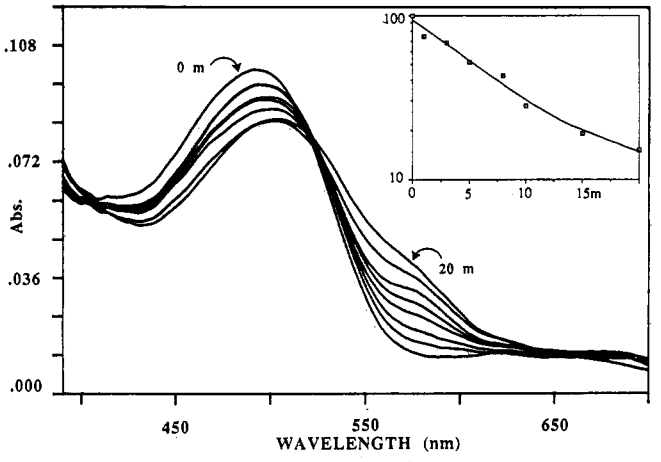



FIGURE 8: Low-temperature photochemistry of 9,11-*dicis*-12-fluororhodopsin. A sample of the pigment was irradiated (437 nm) at liquid nitrogen temperature with the spectra recorded after the following intervals of irradiation: 0, 1, 3, 5, 8, 10, 15, and 20 min. Insert: log plot of the disappearance of pigment absorption at 500 nm versus time.

*9,11-dicis-12-Fluororhodopsin.* The dicis pigment is known to undergo slow isomerization to the 9-cis pigment, as verified in the F NMR experiment (Colmenares & Liu, 1992). But its UV/vis absorption was found to be unaffected upon the addition of hydroxylamine.

A set of photochemical experiments was carried out for 9,11-*dicis*-12-fluororhodopsin ($\lambda_{max}$ = 484 nm) similar to that for 7,9-*dicis*-rhodopsin. The progress of the low-temperature photochemical reaction is shown by the spectra in Figure 8. Two features are evident: the formation of a batho product absorbing in the 500–550-nm region and the absence of a well-defined isosbestic point, as in Figure 4. The data points for the time plot for disappearance of the 9,11-dicis pigment (insert in Figure 8) are somewhat scattered. They do not fall on a straight line, as for the 7,9-dicis pigment.

Interconversions of photoproducts at liquid nitrogen temperature are shown in the spectra in Figure 9. The difference spectrum for the batho product from the 9,11-dicis pigment shows a maximum at 570 nm (insert, Figure 9), which is about the same value as that of the batho product of 12-fluororhodopsin (572 nm) obtained after irradiation of a sample of 9-*cis*-12-fluororhodopsin [see also Shichida et al. (1987)]. This is perhaps not surprising considering the fact that the 9-cis and the 11-cis pigments are known not to give

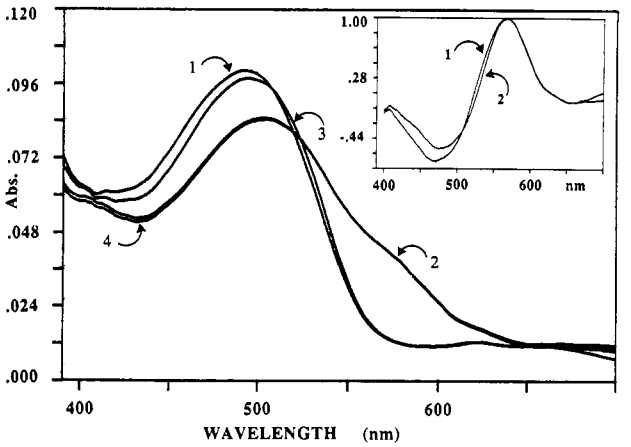



FIGURE 9: Photochemistry of isomeric 12-fluororhodopsin pigments: 9,11-*dicis*-12-fluororhodopsin (curve 1 and also the last curve in Figure 8) at liquid nitrogen temperature; after irradiation with 437-nm light (curve 2 and also the last curve in Figure 8), followed by prolonged irradiation with >610-nm light (curve 3) and then prolonged irradiation with 437-nm light (curve 4). Insert: Difference spectra. Curve 1, last curve in Figure 8 minus the first curve in the same figure; curve 2, same difference spectrum from irradiation of a sample of 9-*cis*-12-fluororhodopsin.

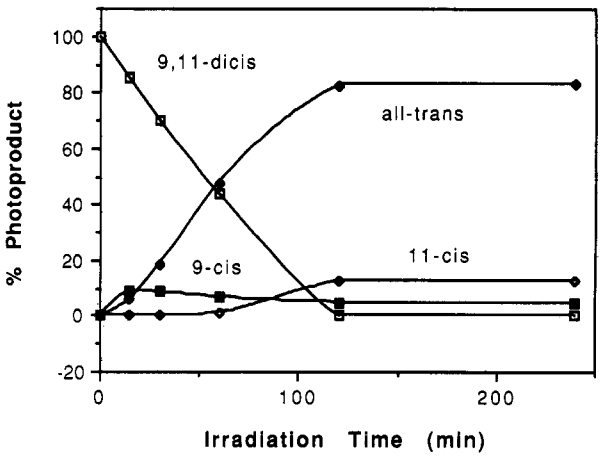



FIGURE 10: Time plot showing changes in isomeric composition of the chromophore during irradiation of 9,11-*dicis*-12-fluororhodopsin (data from Figure 3).

distinguishable absorption spectra for their batho intermediates (Kandori et al., 1988), i.e., they are likely to have minor structural differences.

HPLC analysis of the chromophore extracts obtained from irradiated mixtures during the course of the photochemical experiment is very revealing as to the possible cause for the absence of a well-defined isosbestic point. The data shown in the chromatograms in Figure 3 are replotted in the form of a time plot in Figure 10. It is clear that, while the original pigment is free from 9-cis and 11-cis pigments, the early irradiation mixtures contain a considerable amount of the 9-cis isomer. It disappears upon extended irradiation with a simultaneous increase in the all-trans isomer. Thus, the photoisomerization of the dicis pigment involves initial formation of the 9-cis pigment. Since the latter is more photosensitive than the original dicis pigment (Figure 11), the steady-state concentration of the 9-cis isomer remained low. Eventually, it decreased to a constant amount corresponding to that in the photostationary-state mixture of the isomeric 12-fluoro pigments (Shichida et al., 1987). The chemical results, when combined with those from low-temperature photochemical–spectroscopic studies (absence

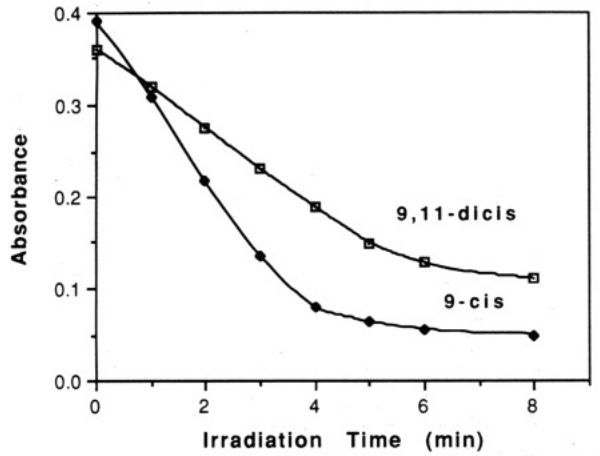



FIGURE 11: Relative photosensitivity of 9,11-*dicis*-12-fluororhodopsin and 9-*cis*-12-fluororhodopsin in 10 mM $NH_2OH$ during parallel irradiation with 437-nm light as indicated by changes in absorbance at 500 nm.

of an isosbestic point and a nonlinear rate of pigment disappearance), clearly show that the major pathway of photochemical reactions of 9,11-*dicis*-12-fluororhodopsin is one-photon–one-bond isomerization involving isomerization of the 11,12 bond to the 9-cis isomer first, followed by subsequent photoisomerization to the all-trans form.

*Possible Causes for the Different Reaction Pathways.* The finding that the two dicis pigment analogs exhibit different stereochemical consequences in the photoisomerization process was unexpected. However, upon reflection there are plausible causes for such divergent behavior.

For any chromophore with a relaxed geometry considerably different from that of 11-cis and the closely related 9-cis pigment, new steric interactions with the protein pocket are likely to be present, which could affect the isomerization process. Hence, it is not surprising that the photosensitivities of both pigment analogs have dropped. However, the 9,11-dicis chromophore exhibits a high preference for isomerization at the 11,12 bond, probably continuing to experience the specific protein perturbation that causes the exceptionally high quantum yield of isomerization of rhodopsin (0.67; Dartnell, 1968; Birge et al., 1988). No such accelerating perturbation is known to be near the 7,8 or 9,10 bond to affect isomerization of the 7,9-dicis pigment.

It is interesting to note that, in the recent study (Ganapathy & Liu, 1992) of the initial direction of photoisomerization of all 16 isomers of retinal, substantial amounts of two- or multiple-bond-isomerized products were detected for the hindered isomers in the excited singlet states as well as the triplet states. The exact nature of such a process was unclear but the possibility of a concerted process in the form of the "bicycle-pedal" model (Warshel, 1976) was mentioned. In the case of 7,9-*dicis*-rhodopsin, there now might be the interesting possibility that protein restrictions have made all one-bond isomerization processes competitively less favorable, giving way to an otherwise high-energy pathway, e.g., a two-bond "bicycle-pedal" isomerization process.

In Figure 12 are the overlaid structures of the tethered chromophores of the 11-cis, 9-cis, 7,9-dicis, and 9,11-dicis pigments anchored at the $\alpha$-carbons of the lysine residue and the cyclohexenyl rings. For the 7,9-dicis chromophore with the established 15-anti configuration (Loppnow et al., 1990), its ring/chain conformation and the positions of the C7–C10 atoms are seen to be substantially displaced from those of the 9-cis and 11-cis. Also, a larger dihedral angle is known for

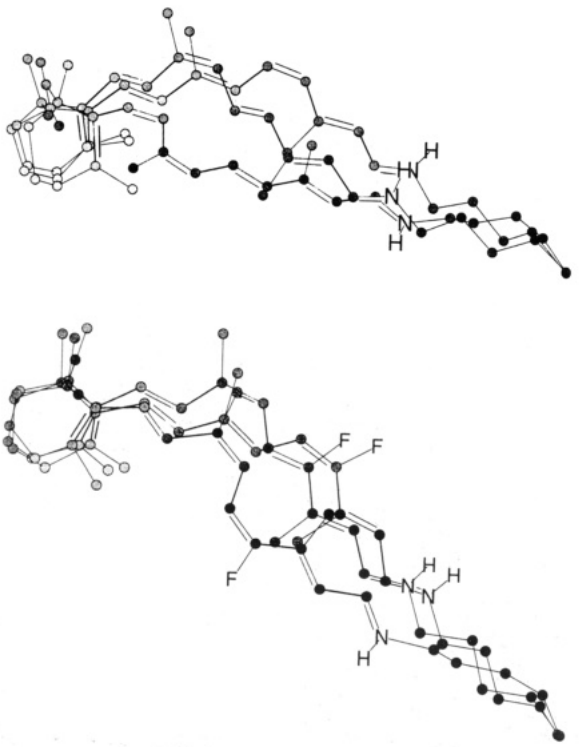



FIGURE 12: Computer-simulated chromophores of rhodopsin, 9-*cis*-rhodopsin, 7,9-*dicis*-rhodopsin, and 9,11-*dicis*-rhodopsin. The minimized isomeric retinal chromophores were replaced with iminium nitrogens and appended with the butyl tether and an extra carbon for the $\alpha$-carbon of the anchored lysine residue. They are essentially those reported by Loppnow et al. (1990) and Colmenares and Liu (1992), and details for their construction are in the literature

a 7-cis isomer (Liu et al., 1983). All of these could lead to a more crowded protein pocket that will hinder the volume-demanding one-bond isomerization processes. [For a related discussion on volume-conserving isomerization processes, see Liu and Browne (1986).] For the 9,11-dicis pigment, the 11,12 bond is not only less displaced from that of rhodopsin but is also shifted toward a relatively open portion of the binding pocket defined by the binding site map from rhodopsin isomers (Liu & Mirzadegan, 1989), leaving the one-bond isomerization processes relatively unperturbed.

It will be of interest to design model systems in order to mimic and test the concept of host restrictions impeding the normal one-bond isomerization process to the extent of favoring a less common two-bond process.

## ACKNOWLEDGMENT

The retinal isomers and analogs used in this work were supplied by Drs. A. E. Asato and L. U. Colmenares. We thank Dr. Y. Shichida for his valuable advice and Dr. E. Krogh for his assistance in setting up the low-temperature spectroscopic equipment at the University of Hawaii.

## REFERENCES

Birge, R. R. (1990) *Biochim. Biophys. Acta 1016*, 293–327.
Birge, R. R., Einterz, C. M., Knapp, H. M., & Murray, L. P. (1988) *Biophys. J. 53*, 367–385.
Colmenares, L. U., & Liu, R. S. H. (1992) *Photochem. Photobiol. 56*, 101–105.
Crouch, R., Purvin, V., Nakanishi, K., & Ebrey, T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1538–1542.
Dartnell, H. J. A. (1968) *Vision Res. 8*, 339–358.

Ganapathy, S., & Liu, R. S. H. (1992) *Photochem. Photobiol. 56*, 959–964.
Groenenduk, C. W. T., de Grip, W. J., & Daemen, M. (1979) *Anal. Biochem. 99*, 304–310. Groenenduk, C. W. T., de Grip, W. J., & Daemen, M. (1980) *Biochim. Biophys. Acta 617*, 430–438.
Kandori, H., Matuoka, S., Nagai, H., Shichida, Y., & Yoshizawa, T. (1988) *Photochem. Photobiol. 48*, 93–97.
Kawamura, S., Miyatani, S., Matsumoto, H., Yoshizawa, T., & Liu, R. S. H. (1980) *Biochemistry 19*, 1549–1553.
Liu, R. S. H., & Browne, D. T. (1986) *Acc. Chem. Res. 19*, 42–48.
Liu, R. S. H., & Mirzadegan, T. (1989) *J. Am. Chem. Soc. 110*, 8617–8623.
Liu, R. S. H., & Shichida, Y. (1991) in *Photochemistry in Organized and Constrained Media* (Ramamurthy, V., Ed.) pp 817–840, VCH publishers, Inc., New York.
Liu, R. S. H., Zingoni, J. P., Kini, A., Trammell, M., Chu, D., Asato, A. E., & Bopp, T. T. (1983) *J. Org. Chem. 48*, 4817–4821.
Loppnow, G. R., Miley, M. E., Mathies, R. A., Liu, R. S. H., Kandori, H., Shichida, Y., Fukada, Y., & Yoshizawa, T. (1990) *Biochemistry 29*, 8985–8991.
Schoenlein, R. W., Peteanu, L. A., Mathies, R. A., & Shank, C. V. (1991) *Science 254*, 412–415.
Shichida, Y. (1986) *Photobiochem. Photobiophys. 4*, 287–307.
Shichida, Y., Ono, T., Yoshizawa, T., Matsumoto, H., Asato, A. E., Zingoni, J. P., & Liu, R. S. H. (1987) *Biochemistry 26*, 4422–4428.
Shichida, Y., Imamoto, Y., Yoshizawa, T., Takahashi, T., Tomioka, H., Kamo, N., & Kabatake, Y. (1988a) *FEBS Lett. 236*, 333–336.
Shichida, Y., Nakamura, K., Yoshizawa, T., Trehan, A., Denny, M., & Liu, R. S. H. (1988b) *Biochemistry 27*, 6495–6499.
Shichida, Y., Kandori, H., Okada, T., Yoshizawa, T., Nakashima, N., & Yoshihara, K. (1991) *Biochemistry 30*, 5918–5926.
Trehan, A., Liu, R. S. H., Shichida, Y., Imamoto, Y., Nakamura, K., & Yoshizawa, T. (1990) *Bioorg. Chem. 18*, 30–40.
Warshel, A. (1976) *Nature (London) 260*, 679–683.
Warshel, A., & Barboy, N. (1982) *J. Am. Chem. Soc. 104*, 1469–1476.
Yoshizawa, T., & Wald, G. (1963) *Nature 197*, 1279–1286.
Yoshizawa, T., & Shichida, Y. (1982) *Methods Enzymol. 81*, 333–354.
Zhu, Y., Ganapathy, S., Trehan, A., Asato, A. E., & Liu, R. S. H. (1992) *Tetrahedron 48*, 10061–10074.