introduced, the total volume, and the equilibrium constant for dimer ⇄ 2 trityl,[16] by assuming 100% yield for the reduction of trityl chloride.

Kinetics of Reaction of 5 with 12 and 13. Radicals **12** and **13** were used as received from the Aldrich Chemical Co. The appropriate solutions of **5** and **12** (or **13**) in benzene containing dimethyl carbonate as internal standard were degassed and sealed into NMR tubes as already described above for analogous experiments.

Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the Americal Chemical Society, and to the Natural Sciences and Engineering Research Council of Canada for support of this work.

Synthesis and Properties of 12-Fluororetinal and 12-Fluororhodopsin. A Model System for ^{19}F NMR Studies of Visual Pigments

Robert S. H. Liu,*,† Hiroyuki Matsumoto, Alfred E. Asato, Marlene Denny, Yoshinori Shichida, Toru Yoshizawa, and F. W. Dahlquist

Contribution from the Department of Chemistry, University of Hawaii, Honolulu, Hawaii 96822, Department of Biophysics, Faculty of Science, Kyoto University, Kyoto, Japan, and Department of Chemistry, University of Oregon, Eugene, Oregon 94703. *Received April 28, 1981. Revised Manuscript Received July 28, 1981*

Abstract: The synthesis and spectroscopic properties of 12-fluororetinal and 12-fluororhodopsin are described. A comparison of these properties and the photobleaching sequence with the parent retinal and rhodopsin is made. A ^{19}F NMR spectrum of the pigment is reported.

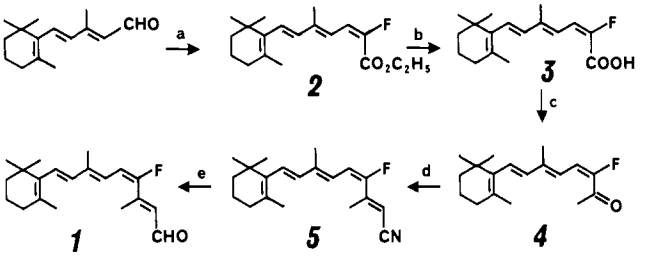
The use of fluorine labels to study structural properties of enzymes is a well-established technique. The label offers the obvious advantage of being able to employ the nondestructive nuclear magnetic resonance method without any interfering signals from the protein or the detergent. On the other hand the highly electronegative substituent could introduce other complications. In applying the method one must therefore examine with care that a fluorinated analogue still retains all characteristic properties of the molecule of concern.[1] Even with this possible complication, the method has been successfully applied to several systems, including the following: alkaline phosphatase,[2] α-chymotrypsin,[3] and aspartate transaminase.[4]

Recently we reported the preparation and absorption properties of 10-fluoro- and 14-fluororhodopsin.[5] Subsequently the preparation of a fluorinated aromatic rhodopsin analogue was also described.[6] We now wish to report the successful preparation of 12-fluororetinal and 12-fluororhodopsin. The location of the fluorine label on the configurationally important 11,12-double bond makes the pigment analogue an interesting one. More importantly the results will show that the analogue has properties more akin to the visual pigment rhodopsin, thus making it an ideal substitute. Some preliminary ^{19}F NMR data will also be presented.

Results

11-*cis*-12-Fluororetinal [(*all*-*E*)-12-Fluororetinal] (1). The synthetic route we used was essentially that described by Machleidt and co-workers in their syntheses of fluorinated vitamin A analogues.[7] The fluorine atom at C_{12} was introduced by the Horner reaction of β-ionylideneacetaldehyde with lithium triethylphosphonofluoroacetate to give (11*E*)- and (11*Z*)-tetraene ester **2** (11*E*/11*Z* ≈ 3) in 84% yield. Separation of the isomers was readily effected by flash column chromatography. Subsequent elaboration of the carbon skeleton (**2** → **1**) proceeded with complete retention of the 11*E* geometry, thereby obviating the necessity of photochemically introducing this critical cis linkage. All spectroscopic properties of the intermediates agreed with their assignments and are listed in the Experimental Section. The configuration of the 12-fluororetinal isomers was clearly established by the magnitude of the three bond H,F coupling constants ($J_{H,F}^3$(cis) ≈ 20–25 Hz and $J_{H,F}^3$(trans) ≈ 30–40 Hz)[8] and the proton chemical shifts. The NMR data of **1** along with that for the corresponding all-trans (11*Z*), 9-cis (9*Z*,11*Z*) and 13-cis (11*Z*,13*Z*) isomers which were obtained in a separate synthetic sequence[9] are listed in Table I.

Scheme I



a, $(EtO)_2P(=O)CHFCO_2Et$, LDA; b, KOH, MeOH; c, excess MeLi, d, $(EtO)_2P(=O)CH_2CH$, LDA; e, $(i\text{-}Bu)_2AlH$

* Address correspondence in the following manner: R.S.H.L., H.M., A. E.A., and M.D., University of Hawaii. Y.S. and T.Y., Kyoto University. F.W.D., University of Oregon.

† R.S.H.L. wishes to dedicate this paper to George S. Hammond on the occasion of his 60th birthday.

(1) For a leading reference on ^{19}F NMR on proteins see: Sykes, B. D.; Hull, W. E. *Methods Enzymol.* **1978**, *49*, 270–95.
(2) Hull, W. E.; Sykes, B. D. *Biochemistry* **1976**, *15*, 1535–46.
(3) Gerig, J. T.; Halley, B. A.; Ortiz, C. E. *J. Am. Chem. Soc.* **1977**, *99*, 6219–25.
(4) Critz, W. J.; Martinez-Carrion, M. *Biochemistry* **1977**, *16*, 1554–8, 1559–64.
(5) Asato, A. E.; Matsumoto, H.; Denny, M.; Liu, R. S. H. *J. Am. Chem. Soc.* **1978**, *100*, 5957–60.
(6) Matsumoto, H.; Asato, A. E.; Denny, M.; Baretz, B.; Yen, Y.-P.; Tong, D.; Liu, R. S. H. *Biochemistry* **1980**, *19*, 4589–94.
(7) Machleidt, H.; Wessendorf, R. *Justus Liebigs Ann. Chem.* **1964**, *674*, 1–10; *679*, 20–25; **1965**, *681*, 21–29.
(8) Emsley, J. W.; Phillips, L.; Wray, V. *Prog. Nucl. Magn. Reson. Spectrosc.* **1977**, *10*, 85–756.

0002-7863/81/1503-7195$01.25/0

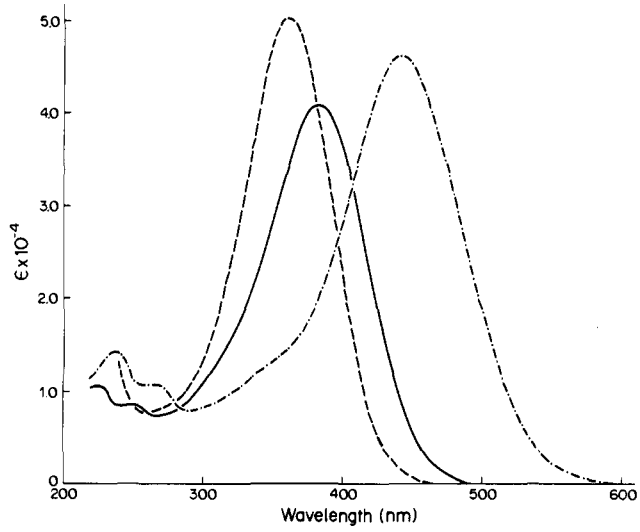


Figure 1. Absorption spectra of *all-trans*-12-fluororetinal and its Schiff base in ethanol: (—) free aldehyde ($\epsilon_{max} = 4.11 \times 10^4$); (- - - and -·-) *all-trans*-12-fluororetinylidenemethylamine ($\epsilon_{max} = 5.05 \times 10^4$) and its protonated form ($\epsilon_{max} = 4.64 \times 10^4$), respectively.

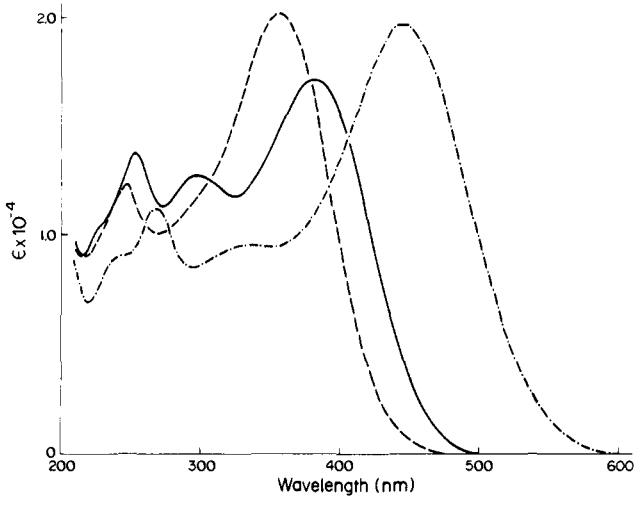


Figure 2. Absorption spectra of 11-*cis*-12-fluororetinal and its Schiff base in ethanol: (—) free aldehyde ($\epsilon_{max} = 1.71 \times 10^4$); (- - - and -·-) 11-*cis*-12-fluororetinylidenemethylamine ($\epsilon_{max} = 2.02 \times 10^4$) and its protonated form ($\epsilon_{max} = 1.98 \times 10^4$), respectively.

The UV–vis absorption spectra of the all-trans and the 11-cis isomers of 12-fluororetinal, the methylamine Schiff bases, and the protonated Schiff bases are shown in Figures 1 and 2. They generally exhibit features identical with those of the parent retinal system.[10]

12-Fluororhodopsin. The procedure to prepare the pigment analogues was the same as that described earlier.[10,11] Immediately after mixing a 1% digitonin solution of cattle opsin extract with a slight excess of 11-*cis*-12-fluororetinal, absorption in the long wavelength region (>450 nm) became detectable. The reaction was a rapid one, almost complete within 10 min (Figure 3). When conducted in digitonin, the regeneration rate ($k_2 = 3 \times 10^2$ M^{-1} s^{-1}) is approximately the same as the parent retinal.[11]

Just as rhodopsin,[12] the pigment was found to be photobleachable giving *all-trans*-12-fluororetinal as the end product,

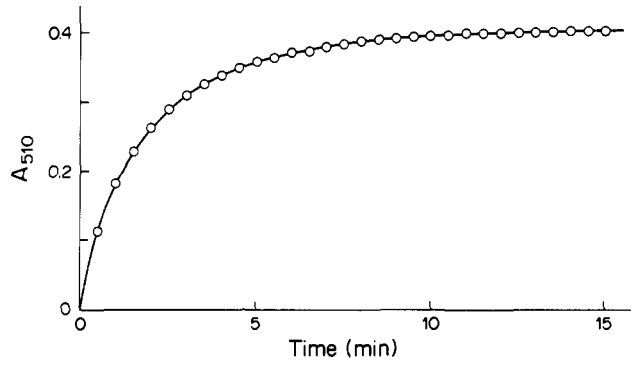


Figure 3. Formation of 12-fluororhodopsin from 11-*cis*-12-fluororetinal and cattle opsin in 1% digitonin, pH 7.0 at 25 °C, as indicated by increase of absorbance at 510 nm.

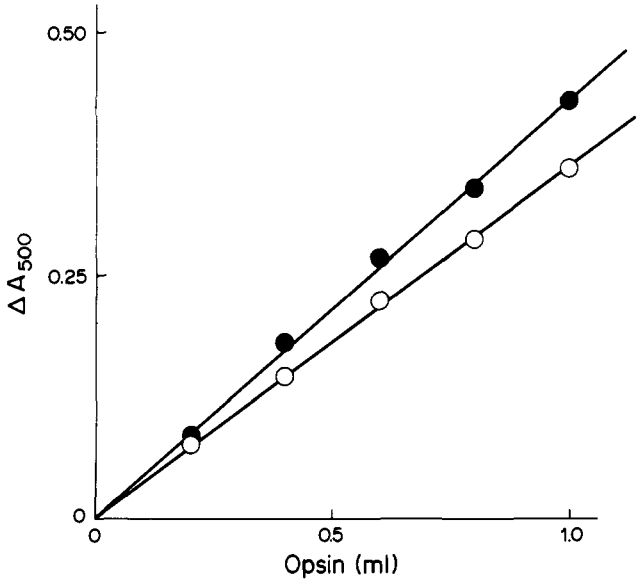


Figure 4. Determination of the molar extinction coefficient of 12-fluororhodopsin. Rhodopsin (●) and 12-fluororhodopsin (○) were formed from limited amounts of cattle opsin (abscissa) and the corresponding retinal. See Experimental Section for detail.

identified by UV–vis absorption properties of the oxime. When formed in digitonin extract, the pigment was stable both in the absence and presence of hydroxylamine. However, when the pigment was extracted with 1% Ammonyx LO from the reconstituted rod outer segment (ROS) membranes, it was apparently less stable than rhodopsin. For example upon standing in a high concentration of hydroxylamine, the pigment gradually deteriorated (see below). This behavior appears to be characteristic of many of the rhodopsin analogues.[5,6,13]

Under the same conditions the all-trans and 13-cis isomers of 12-fluororetinal were found not to give pigment analogues while the 9-cis isomer gave a pigment with the absorption peak centered at 493 nm.[14]

The extinction coefficient of 12-fluororhodopsin has been measured relative to that of rhodopsin (42 000).[15] Equimolar concentrations of 11-*cis*-retinal and 11-*cis*-12-fluororetinal in two separate cuvettes were titrated against a solution of opsin extract. The ratio of rhodopsin and 12-fluororhodopsin absorption was found to be constant (1:1.186) throughout the fivefold variation of opsin concentration (Figure 4). Since the opsin–11-*cis*-retinal complex formation is believed to be complete,[16] it appears safe

(9) Asato, A. E.; Liu, R. S. H., unpublished results.
(10) Hubbard, R.; Brown, P. K.; Bownds, D. *Methods Enzymol.* **1971**, *18*, 615–53.
(11) Matsumoto, H.; Horiuchi, K.; Yoshizawa, T. *Biochem. Biophys. Acta* **1978**, *501*, 257–68.
(12) (a) Yoshizawa, T.; Wald, G. *Nature (London)* **1963**, *197*. 1279–86. (b) Kawamura, S.; Miyatani, S.; Matsumoto, H.; Yoshizawa, T.; Liu, R. S. H. *Biochemistry* **1980**, *19*, 1549–53.
(13) Matsumoto, H.; Asato, A. E.; Liu, R. S. H. *Photochem. Photobiol.* **1979**, *29*, 695–8.
(14) Matsumoto, H.; Liu, R. S. H., unpublished results.
(15) Shichi, H.; Lewis, M. S.; Irreverre, F.; Stone, A. L. *J. Biol. Chem.* **1969**, *244*, 529–36.

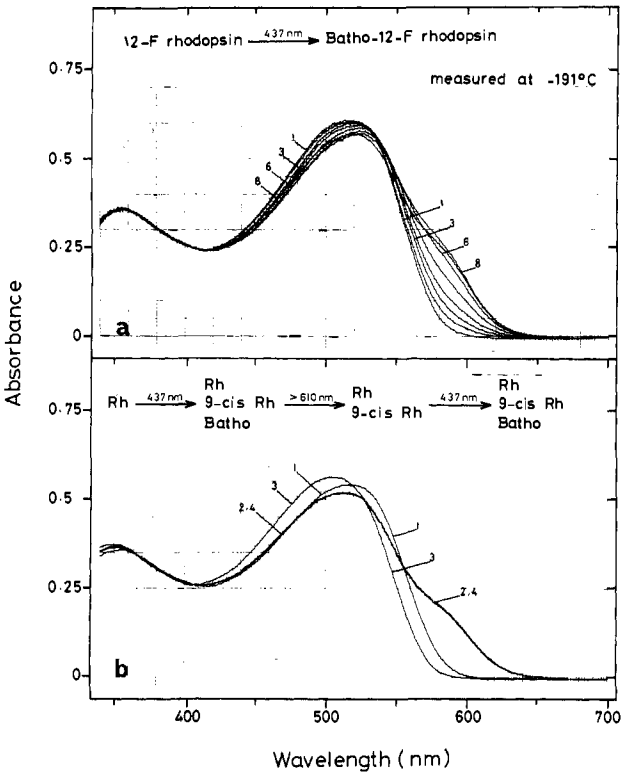


Figure 5. (a) Spectral changes in the course of conversion of 12-fluororhodopsin to batho-12-fluororhodopsin. A 12-fluororhodopsin–glycerol mixture (pH 7.0, curve 1) was successively irradiated with 437-nm light at −191 °C for 5, 10, 20, 40, 80, 160, and 320 s (curves 2–8). (b) Photoreversibility among 12-fluororhodopsin, 9-*cis*-12-fluororhodopsin, and batho-12-fluororhodopsin at −191 °C. A 12-fluororhodopsin–glycerol mixture (pH 7.0, curve 1) was irradiated with 437-nm light at −191 °C for 42 min and 40 s (curve 2). The sample was then irradiated with light longer than 610 nm for 21 min and 20 s. After measurement of the spectrum (curve 3), the sample was irradiated with 437-nm light again for 42 min and 40 s. Another spectrum was recorded (curve 4).

to conclude that the same situation exists for the fluorinated analogue. Therefore, using the determined ratio, we obtained the value 35 500 for the extinction coefficient of 12-fluororhodopsin (at 507 nm, λ_{max}).

Intermediates in Photobleaching by Low-Temperature Spectroscopy. The photobleaching process of 12-fluororhodopsin has been examined at liquid nitrogen temperature (−191 °C). The results clearly showed the similarity between this system and the parent rhodopsin.[12] The pigment in glycerol at −191 °C with an absorption maximum now at 514 nm exhibited a red shift immediately after irradiation with light of 437 nm (Figure 5a) indicating the formation of a batho product. After ~40 min, a photostationary state mixture was produced with the absorption maximum shifted to 526 nm and a discernible shoulder beyond 550 nm.

Similar to bathorhodopsin, batho-12-fluororhodopsin was found to be unstable toward light and heat. When the mixture was irradiated with 610-nm light, batho-12-fluororhodopsin was selectively bleached giving now a mixture of 12-fluororhodopsin and 9-*cis*-12-fluororhodopsin. At this temperature the interconversion of the three isomeric pigments is apparently completely reversible (curves 2–4, Figure 5b).

Upon warming, batho-12-fluororhodopsin underwent stepwise transitions eventually giving the *all-trans*-12-fluororetinal. This process was followed carefully between −191 and 0 °C. The above photostationary mixture of isomeric 12-fluororhodopsin was allowed to warm up briefly to a given temperature and the absorption spectrum rerecorded at −191 °C or −85 °C. In Figure 6 is shown the change of the absorption maximum with temperature. It is clear that the bathointermediate is stable up to −150 °C. At higher temperatures there were two additional bleaching intermediates. Similar to the parent rhodopsin these are labeled as lumi-12-fluororhodopsin (stable up to −50 °C) and meta-I-12-fluororhodopsin (>−20 °C). At the end of the experiment the sample was warmed to room temperature. The absorption spectrum corresponded to those of 12-fluororhodopsin (15%) and 9-*cis*-12-fluororhodopsin (50%). Knowledge of concentrations of those species allowed the calculation of the amount of bathorhodopsin in the original mixture (35%). The absorption spectrum of batho-12-fluororhodopsin was then obtained (Figure 7) which shows an absorption maximum at 572 nm, considerably longer than that of rhodopsin.

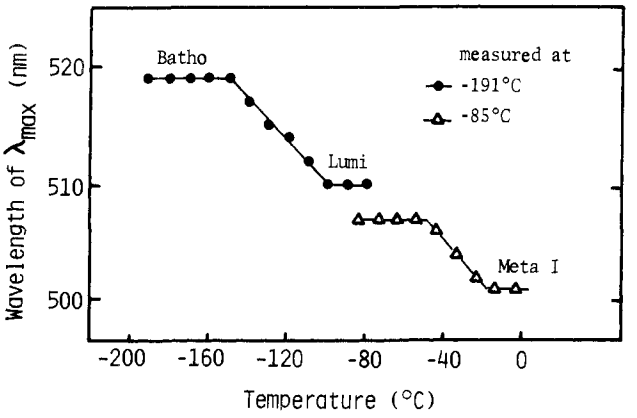


Figure 6. Changes in λ_{max} of an irradiated mixture of 12-fluororhodopsin by stepwise warming. A 12-fluororhodopsin–glycerol mixture (pH 7.0) was irradiated with 437-nm light for 320 s at −180 °C, resulting in a mixture composed of 12-fluororhodopsin, 9-*cis*-12-fluororhodopsin, and batho-12-fluororhodopsin. After irradiation the mixture was warmed to various temperatures (abscissa) and then re-cooled to −191 °C (in those cases where warming was below −90 °C) and −85 °C (in those cases where warming was above −85 °C) for measurement of the spectra.

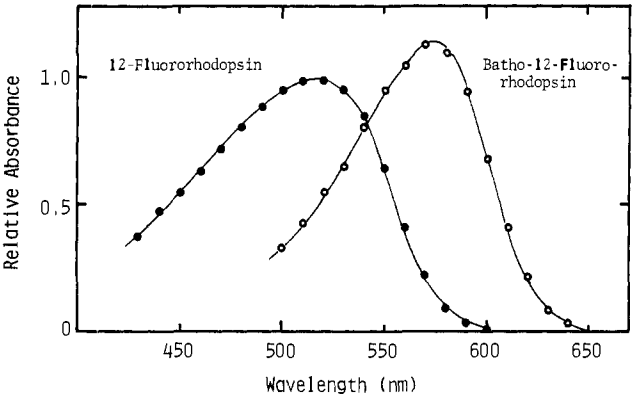


Figure 7. Absorption spectra of 12-fluororhodopsin (λ_{max} = 514 nm) and batho-12-fluororhodopsin (λ_{max} = 572 nm) at −191 °C.

^{19}F NMR Studies. The procedure for preparation of samples for NMR studies will be described in detail. It represents the typical method for our NMR studies.

A suspension of ROS from 200 retinae was reacted with a slight excess of 11-*cis*-12-fluororetinal. The regenerated pigment was extracted with a solution of 1% Ammonyx LO buffered in 10 mM Hepes. The pigment was then purified by preparative column chromatography on a hydroxylapatite column at 4 °C in the dark. The column containing 12 g of Bio-Gel HTP was suspended in the above detergent and buffer solution. The 12-fluororhodopsin solution after concentrated to 7 mL (10.5 A units) was applied, followed successively by these eluants: 200 mL of 1% Ammonyx LO and 10 mM Hepes (pH 7.0), 100 mL of 0.25 M NaCl in Ammonyx LO-Hepes, and 100 mL of 1% Ammonyx LO-Hepes and 0.15 M sodium phosphate (pH 6.5). Sixty fractions (8 mL each) were collected. Each fraction was analyzed at 280 and 500

(16) Kropf, A.; Whittenberger, B. P.; Goff, S. P.; Waggoner, A. S. *Exp. Eye Res.* **1973**, *17*, 591–606.

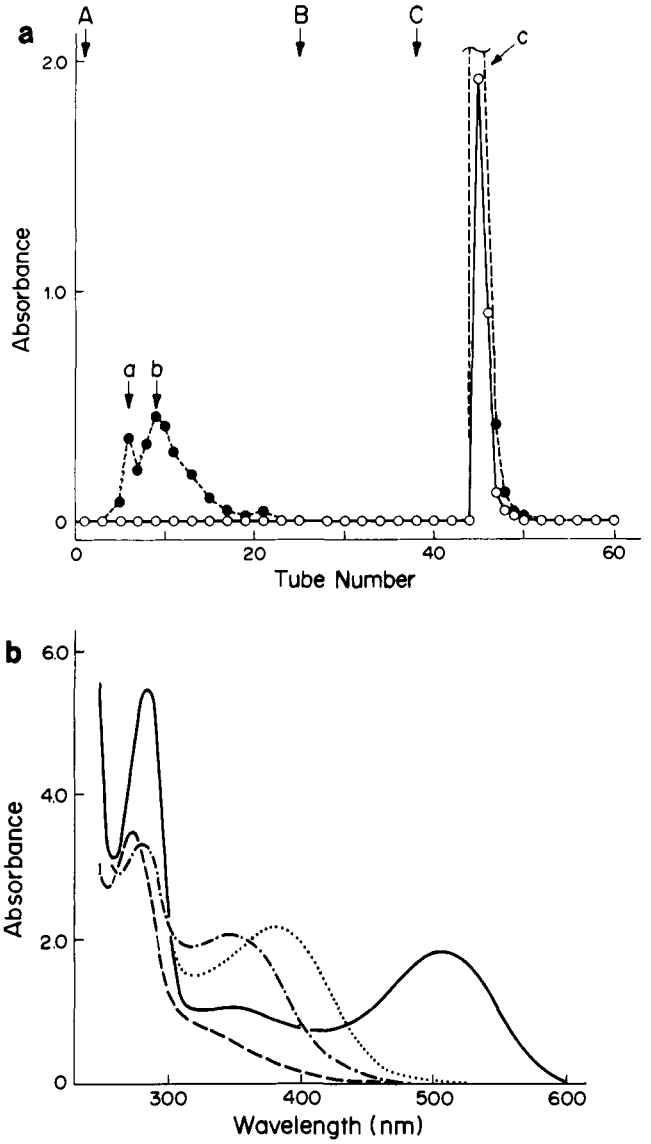


Figure 8. Chromatographic purification of 12-fluororhodopsin. (a) Elution profile of 12-fluororhodopsin on Bio-Gel HTP column. See Experimental Section for eluants A, B, and C. (○) A_{500}; (●) A_{280}. (b) Absorption spectra of peak a (- - -), peak b (-·-), and peak c (—). (···) Bleached peak c.

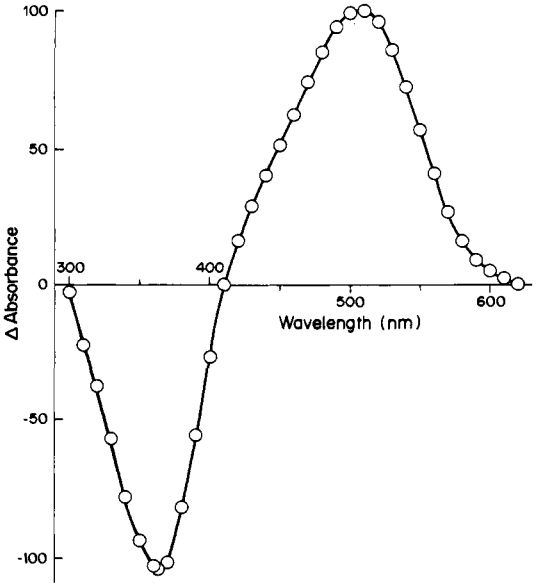


Figure 9. Room temperature bleaching difference spectrum of 12-fluororhodopsin in 1% Ammonyx LO in the presence of 10 mM hydroxylamine. Purified 12-fluororhodopsin was bleached with orange light.

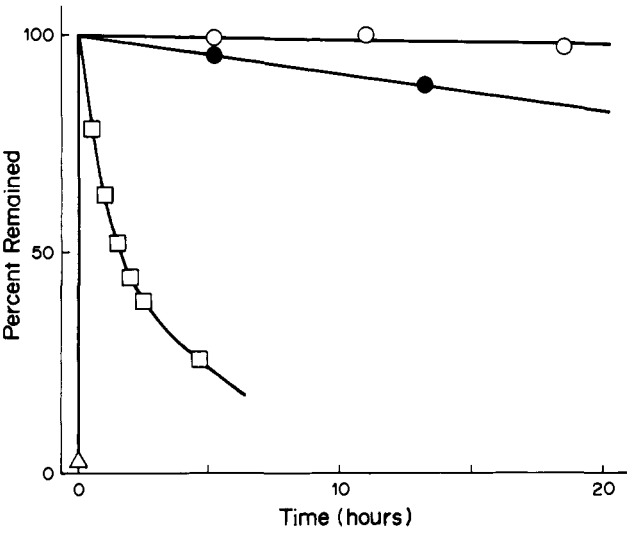


Figure 10. Heat stability of chromatographically purified 12-fluororhodopsin in 1% Ammonyx LO: (○) 0 °C; (●) 9 °C; (□) 26 °C; (△) 45 °C.

nm. The complete analyses are summarized in Figure 8.

The early fractions exhibiting no absorption at 500 nm were those of unreacted retinal or degradation products. Fraction 45 was found to contain the pigment analogue. The difference absorption spectrum of this fraction obtained from the spectra before and after photobleaching in the presence of hydroxylamine is shown in Figure 9. Its absorption is slightly different from that obtained in low-temperature glass (Figure 7).

At room temperature in Ammonyx LO, the pigment degraded slowly with the absorption at 500 nm decreasing by half after 20 min. At lower temperatures the degradative process was considerably slower (Figure 10). Therefore for storage purpose, the purified pigments (fractions 45–48) were kept at a temperature below −50 °C to prevent degradation. After concentration and addition of D_2O to a final concentration of 5%, the sample was used for NMR studies.

The ^{19}F NMR spectrum of 12-fluororhodopsin was recorded on a Nicolet NT-150 spectrometer (141 MHz). A 12-mm sample tube was used. The sample temperature was kept at 4 °C. Deuterium oxide was used for the lock and also for internal standard. A spectrum was first recorded (Figure 11a,c) in the absence of any internal fluorine standard. A small amount of CF_3CO_2H was then added to the buffered solution. The spectrum was again recorded (Figure 11b). The fluorine signal was unchanged. The sample was subsequently bleached with ≥440 nm light at 0 °C. The spectrum was again recorded (Figure 11d,e).

Discussion

The close similarity between rhodopsin and 12-fluororhodopsin is clearly shown in Table II. The absorption properties of the retinals are almost identical and they exhibit the same selectivity and reactivity in their reaction with cattle opsin. Therefore, in this case the fluorine atom must have little effect on the conformation of the isomers with the result that the relative distances between the center of the ring and the carbonyl group remain the same as in the parent retinal isomers. Their reactivities are therefore again governed by the longitudinal restrictions of the binding site of opsin.[17] The same number of bleaching intermediates were observed with transition temperatures very close to each other. These are important observations because in preliminary experiments with the pigment derived from 10-

(17) Matsumoto, H.; Liu, R. S. H.; Simmons, C.; Seff, K. *J. Am. Chem. Soc.* **1980**, *102*, 4259–62.

Table I. ^1H and ^{19}F NMR Signals of Isomers of 12-Fluororetinal[a]

isomer	chemical shift, δ									coupling constants, Hz			
	CH_3-1	CH_3-5	CH_3-9	CH_3-13	H_7	H_8	H_{10}	H_{11}	F_{12}[c]	H_{14}	H_{15}	$J_{7,8}$	$J_{10,11}$	$J_{11,12}$	$J_{14,15}$
all-trans-III (7*E*,9*E*,11*Z*,13*E*)[b]	1.04	1.73	2.01	2.31	6.37	6.24	6.31	6.51	−44.2	6.44	10.14	16.0	11.8	35	7.8
9-*cis*-III[c] (7*E*,9*Z*,11*Z*,13*E*)	1.04	1.74	2.04	2.30	6.28	6.48	6.30	6.44	−45.4[d]	6.25	10.02	16	11.2	32.5	7.2
11-*cis*-III[c] (*all-E*)	1.01	1.70	1.96	2.40	6.32	6.10	6.15	6.46	−30.8	6.2	10.10	16	12	21	7.2
13-*cis*-III[b] (7*E*,9*E*,11*Z*,13*Z*)	1.04	1.75	2.07	2.10	6.47	6.55	?[e]	?[e]		5.90	10.19	?	?	?	8.0

[a] Varian XL-100 for ^1H and IBM NR-80 for ^{19}F. For ^{19}F signals, chemical shift from CF_3CO_2H. [b] In $CDCl_3$. [c] In CD_3COCD_3. [d] XL-100. [e] Chemical shifts too close to allow accurate measurements.

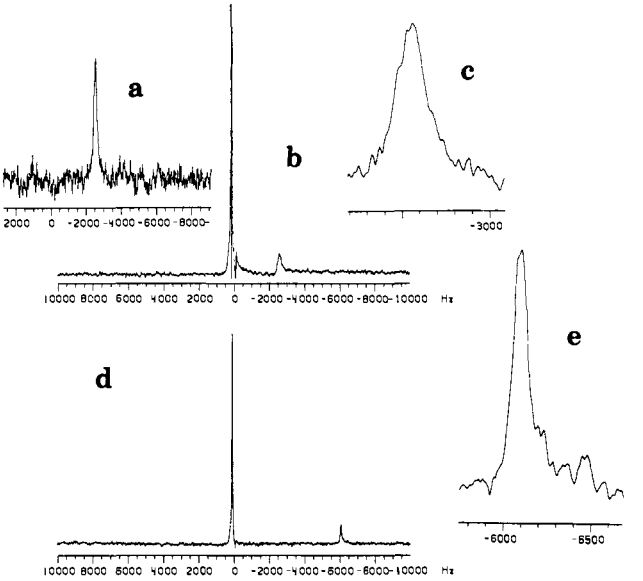


Figure 11. ^{19}F NMR spectra of 12-fluororhodopsin (~0.4 mM) solubilized in 10% Ammonyx LO with phosphate buffer (pH 7.0) recorded on a Nicolet NT-150 spectrometer at 141.182 MHz with a 12-mm sample tube. Deuterium oxide (5%) was added for locking. The probe temperature was maintained at 4 °C. (a) 12-Fluororhodopsin in the dark, number of scans 2000; acquisition time 204.8 ms. (b) Same sample after addition of small amounts of CF_3CO_2H. (c) Part a, expanded. Peak centered at −2570 Hz. (d) Sample b after photobleaching with >440 nm light at 0 °C. (e) Partial expansion of d.

Table II. A Comparison of Properties of Rhodopsin and 12-Fluororhodopsin

	parent systems	12-fluoro
absorption maximum of Isomers of retinal,[a] nm		
all-trans	368[b]	368
11-cis	365	362
9-cis	363	363
13-cis	363	
rates of pigment formation k_2, M^{-1} s^{-1}	3×10^2 [c]	3×10^2
absorption maximum of pigments, nm		
rhodopsin	499[d]	507
9-*cis*-rhodopsin	487	493
13-*cis*-rhodopsin	not formed	not formed
bathorhodopsin	540	572
lumirhodopsin	497	514
meta-I-rhodopsin	478	495
stability of pigments in Ammonyx LO		
rhodopsin	stable	stable <0 °C
batho	≤−150 °C	≤−150 °C
lumi	≤−50 °C	≤−45 °C
meta-I	≤0 °C	≤0 °C

[a] In ethanol. [b] Reference 10. [c] Reference 11.

fluororetinal[5] no long wavelength absorption attributable to the bathointermediate was detected.[18] In the latter case the location of the fluorine atom has apparently changed the course of the photobleaching process to one different from that of rhodopsin.

The 12-fluoro substituent, however, did introduce other minor but noticeable side effect. For example, the pigment is now less stable than rhodopsin. The reason could be due to a decrease of the stability of the protonated Schiff base by the presence of the highly electronegative substituent. The other possibility is a less tightly bound pigment because of the presence of the fluorine atom thus allowing diffusion of hydroxylamine into the binding site. However, the cause will have to be electronic rather than steric because the van der Waals radius of a covalently bonded fluorine

(18) Shichida, Y.; Yoshizawa, T., unpublished results.

atom is rather small.[1] Another noticeable difference is the absorption maximum of the two bathointermediates. It is tempting to suggest that secondary electrostatic interactions with the fluorine atom or carbon atoms close to the fluorine atom caused the red shift[19] in 12-fluororhodopsin. This point will be scrutinized in future studies.

At this stage the ^{19}F NMR data for 12-fluororhodopsin can only be described as preliminary. The successful recording of a signal within a reasonably short data acquisition period clearly established feasibility of the method as a general probe for structural information of visual pigments. The signal recorded at 4 °C is quite broad with the half-height width approximately equal to 120 Hz. At this stage we cannot ascertain to what extent the relaxation rate was controlled by chemical shift anisotropy or by dipolar contributions. The peak width however is too large to allow direct measurement of the three bond H,F coupling which is so useful for characterizing the geometry of an olefin. The system will be reinvestigated on a lower field spectrometer which may partially alleviate the peak width problem if chemical shift anisotropy is an important contribution to the relaxation rate.

The fluorine signal of the sample after photobleaching shifted considerably upfield into a region (43.2 ppm) found for 11-*trans*-retinal isomers (Table I). The shift is therefore consistent with the net chemical result of the release of retinal from the pigment upon irradiation. The width of the peak however indicates that the retinal is not completely free. Other detailed questions such as whether 12-fluororhodopsin is protonated[20] and the presence of other perturbing point charges along the polyene chain[19] will have to await detailed studies of model systems and other pigments analogues.

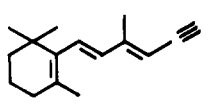
Experimental Section

All reactions were conducted using standard syringe transfer techniques under an atmosphere of argon. Unless otherwise stated normal workup consisted of drying of organic extracts over $MgSO_4$, filtering, and removing solvent on a rotary evaporator. Ethyl bromofluoroacetate was purchased from PCR Research Chemicals, Inc. Anhydrous THF and ether were prepared by distillation from calcium hydride followed by redistillation from Red-al (Aldrich Chemical Co.) and stored over activated 5A molecular sieves. HMPA was dried by storage over activated 13× molecular sieves. Unless otherwise specified, UV spectra were obtained on a Perkin–Elmer double-beam spectrophotometer, and NMR spectra recorded on a Varian EM-360 or XL-100 spectrometer. Chemical shifts are reported in parts per million relative to Me_4Si as internal standard. The ^{19}F NMR studies of the visual pigment were conducted on a Nicolet NT-150 spectrometer.

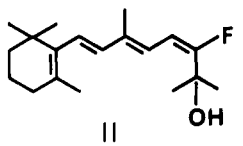
Ethyl (11*E*)-12-Fluoro Ester 2. A solution of β-ionylideneacetaldehyde (3.47 g, 15.9 mmol) in THF (14 mL) was added to a magnetically stirred solution of lithium triethylphosphonofluoroacetate (prepared from 4.84 g (20.0 mmol) of ethyl diethylphosphonofluoroacetate[7] and 1 equiv of lithium diisopropylamide in THF–hexane) at −78 °C. After 1.5 h at 0 °C the reaction mixture was triturated with water (75 mL), acidified with cold dilute 1 N HCl, and extracted with 50% ether–hexanes to give 4.97 g of crude product as an orange oil. Flash column chromatographic separation[21] on silica gel (110 g, 40–63 μm) with use of 3.7% ether–hexanes afforded the following fractions (0.1 L each): (a) Fractions 2 and 3 contained 2.42 g (49.7%) of the desired ester **2** as a yellow oil: UV (hexanes) λ_{max} 332 nm; ^1H NMR ($CDCl_3$) δ 1.03 (s, 6 H), 1.38 (t, J = 7 Hz), 1.73 (s, C_5-CH_3), 1.98 (s, C_9-CH_3), 4.31 (q, J = 7 Hz), 6.17 (d, C_8-H, J = 16.5 Hz), 6.38 (br d, C_7-H, J = 16 Hz), and 6.68–7.06 (5-line m, ABX, C_{10}-H and C_{11}-H). (b) Fraction 4 gave a mixture of (11*E*)- and (11*Z*)-ethyl esters (0.74 g, 15.2%). (c) Fractions 5–8 afforded 0.94 g (19.3%) of 11*Z* isomer of **2** as a yellow oil: UV (hexanes) λ_{max} 326 nm; ^1H NMR ($CDCl_3$) δ 1.04 (s, 6 H) 1.36 (t, J = 7 Hz), 1.73 (s, C_5-CH_3), 2.01 (s, C_9-CH_3), 4.29 (q, J = 7 Hz), 6.17 (d, C_8-H, J = 16 Hz), 6.32 (d, C_{10}-H, J = 12 Hz), 6.41 (br d, C_7-H, J = 16 Hz), and 6.99 (d of d, C_{11}-H, J = 12 and 30.0 Hz).

Fluoro Acid 3. A mixture of ester **2** (2.42 g, 7.9 mmol), potassium hydroxide (6.0 g, 0.11 mmol), methanol (60 mL), and water (4 mL) was stirred at room temperature for 3 h. At this time the homogeneous orange solution was cooled to 0 °C, acidified with cold 0.4 N HCl (0.3 L), and extracted with ether to give 2.05 g (93.5%) of crude acid **3** as an orange solid. Recrystallization of a small sample from acetonitrile afforded pure **3** as a yellow solid: mp 115–6 °C (uncorrected): UV (hexanes) λ_{max} 366 nm; ^1H NMR ($CDCl_3$) δ 1.06 (s, 6 H), 1.76 (s, C_5-CH_3), 2.02 (s, C_9-CH_3), 6.23 (d, C_8-H, J = 16.5 Hz), 6.46 (br d, C_7-H, J = 16 Hz), and 6.87–7.23 (5-line m, ABX, C_{10}-H and C_{11}-H).

Fluoro Ketone 4. To a magnetically stirred solution of crude fluoro acid **3** (1.00 g, 3.60 mmol) in dry ether (30 mL) cooled to −20 °C was added a solution of ethereal methyllithium (1.3 M, 8.8 mL, 10.4 mmol) over 5 min. After being stirred until it reached room temperature over 4 h the reaction mixture was cooled to 0 °C and decanted into a rapidly stirred mixture of ice–water (0.1 L) acidified with concentrated HCl (2 mL). Ether extraction followed by normal workup afforded 0.92 g of crude product which was flash column chromatographed on silica gel (125 g, 40–63 μm) with use of, consecutively, (a) 5.0% ether–hexanes (0.8 L), (b) 20% ether–hexanes (0.5 L), and (c) 25% ethyl acetate–hexanes (0.5 L). Forty fractions (25 mL each) were collected and analyzed by TLC (silica gel, 50% $CHCl_3$–heptane). [Two byproducts were also isolated: Fractions 3–8 contained I (0.29 g, R_f 0.76) and fractions 35–40 contained fluorocarbinol II (0.09 g, R_f 0.13).] Fractions 9–22



I



II

contained 0.46 g (44%) of the desired ketone **4** as a bright yellow oil: UV (hexanes) λ_{max} 352, 275 nm; ^1H NMR ($CDCl_3$) δ 1.03 (s, 6 H), 1.72 (br s, C_5-CH_3), 1.98 (s, C_9-CH_3), 2.31 (d, $COCH_3$, J = 5 Hz), 6.20 (d, C_8-H, J = 16 Hz), 6.41 (br d, C_7-H, J = 16 Hz), 6.64 (d of d, C_{11}-H, J = 12.9 and 20.6 Hz), and 7.14 (br d, C_{10}-H, J = 12 Hz).

(11*E*)-12-Fluororetinonitrile (5). A solution of ketone **4** (0.45 g, 1.54 mmol) in THF (2 mL) was added to a cold (−78 °C), magnetically stirred solution of lithiodiethylphosphonoacetonitrile (prepared from 0.70 g (4.0 mmol) of diethylphosphonoacetonitrile in THF (10 mL) and HMPA (3 mL) and 1 equiv of LDA). After being stirred at room temperature for 1.5 h the reaction mixture was diluted with water (75 mL), acidified with cold 1 N HCl (4 mL), and extracted with 25% ether–hexanes. After normal workup the resultant orange oil (0.54 g) was flash column chromatographed on silica gel (135 g, 40–63 μm) with use of 6% ether–hexanes to give 0.35 g (75%) of **5** as a yellow oil: TLC (50% $CHCl_3$–heptane) R_f 0.70; UV (hexanes) λ_{max} 356, 239 nm; ^1H NMR ($CDCl_3$) δ 1.05 (s, 6 H), 1.74 (s, C_5-CH_3), 1.98 (s, C_9-CH_3), 2.36 (s, C_{13}-CH_3), 5.61 (br s, C_{14}-H), 6.10 (d, C_8-H, J = 16.2 Hz), 6.26 (br d, C_{10}-H, J = 12 Hz), 6.35 (br d, C_7-H, J = 16 Hz), and 6.56 (d of d, C_{11}-H, J = 12.4 and 22.5 Hz).

(11*E*)-12-Fluororetinal (1). To a magnetically stirred mixture of nitrile **5** (2.85 g, 9.53 mmol) in hexanes (40 mL) cooled to −78 °C was added over ca. 5 min a hexane solution of diisobutylaluminum hydride (1 M, 14.3 mL, 14.3 mmol). After being stirred for 3 h at 0 °C the yellow solution was cooled to −78 °C and hydrolyzed by the slow addition of a slurry of silica gel (20 g, 40–63 μm) deactivated with water (4 mL) in 50% ether–hexanes (50 mL). The resultant mixture was stirred at 0 °C for 1 h and filtered. The filter cake was washed with ether (0.1 L) and the combined filtrates concentrated in vacuo on a rotary evaporator to give 2.8 g of crude fluororetinal **1** as a red oil. Column chromatography of the crude product through a short column of silica gel with use of 5% ether–hexanes as eluant gave 2.47 g (86%) of pure **1** as an orange oil: UV (hexanes see Figure 2); ^1H NMR (acetone-d_6) δ 1.01 (s, 6 H), 1.70 (s, C_5-CH_3), 1.96 (s, C_9-CH_3), 2.40 (s, C_{13}-CH_3), 6.10 (d, C_8-H, J = 16 Hz), 6.15 (br d, C_{10}-H, J = 12 Hz), 6.20 (d, C_{14}-H, J = 7 Hz), 6.32 (br d, C_7-H, J = 16 Hz), 6.46 (d of d, C_{11}-H, J = 12 and 21 Hz), and 10.10 (d, C_{15}-H, J = 7.2 Hz); ^{19}F NMR ($CFCl_3$) −22.1 (d, J = 21 Hz).

Schiff Bases of *all-trans*- and 11-*cis*-Fluororetinal. To an ethanolic solution of 12-fluororetinal (ca. 0.5 absorbance units in 2 mL of volume) 50 μL of aqueous methylamine (40%, Eastman) was added. The Schiff base formation was monitored by measuring absorbance increase around 370 nm at room temperature. The reaction was completed within 1 h. The absorption spectrum of the Schiff base was recorded followed by the addition of 1 drop of concentrated HCl to the sample to protonate the Schiff base. Under these conditions the spectrum of the protonated Schiff base was unchanged at least 20 min after the addition of HCl. Volume changes caused by the addition of a small amount of methyl-

(19) See: Honig, B.; Dinur, U.; Nakanishi, K.; Balogh-Nair, V.; Gawinowicz, M. A.; Arnaboldi, M.; Motto, M. G. *J. Am. Chem. Soc.* **1979**, *101*, 7086–8.

(20) For the controversy on this issue as a result of ^{13}C NMR studies of ^{13}C enriched rhodopsin see: (a) Shriver, J.; Mateescu, G.; Fager, R.; Torchia, D.; Abramson, E. W. *Nature (London)* **1977**, *270*, 273–4. (b) Reference 19. (c) Yamaguchi, A.; Unemoto, T.; Ikegami, A. *Photochem. Photobiol.* **1981**, *33*, 511–16.

(21) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923–5.

amine (2.5%) or HCl (less than 1%) were not compensated. The molar extinction coefficients of 12-fluororetinal isomers were determined by weighing.

Cattle Opsin and 12-Fluororhodopsin. ROS membranes and extracted opsin in 1% digitonin (10 mM Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, pH 7.0) were prepared according to the method previously described.[11,13] In the experiments as shown in Figures 3 and 4 the digitonin extract was used for an opsin source. 12-Fluororhodopsin was formed in ROS membranes and extracted with 1% Ammonyx LO (10 mM Hepes, pH 7.0) for further purification.

Formation Kinetics of 12-Fluororhodopsin and Determination of Its Molar Extinction Coefficient. Opsin extracted with digitonin was mixed with 11-*cis*-12-fluororetinal dissolved in a small amount of ethanol. In all the pigment formation experiments the total amount of ethanol did not exceed 2%. The reaction mixture contained ca. 0.5 A units rhodopsin equivalent of opsin and 0.54 A units of 11-*cis*-12-fluororetinal in 1% digitonin, 10 mM Hepes, pH 7.0. Under this condition the chromophore is about 2.6 times in excess over opsin. The reaction was carried out at 25 °C and the absorbance increase at 510 nm was monitored by a recording spectrophotometer (Figure 3). The second-order rate constant (k_2) of the formation of 12-fluororhodopsin was estimated from the initial phase of the pseudo-first-order plot (not shown) taken from Figure 3.

$$\text{opsin} + \text{11-}cis\text{-12-fluororetinal} \xrightarrow{k_2} \text{12-fluororhodopsin}$$

The rate constant, k_2, is calculated to be 3×10^2 M^{-1} s^{-1} for the 12-fluororhodopsin formation in 1% digitonin, 10 mM Hepes, pH 7.0 at 25 °C. The rate constant of rhodopsin formation is largely dependent on the concentration of digitonin.[11] Under similar conditions k_2 of rhodopsin formation was reported to be about 3×10^2 M^{-1} s^{-1}.[11]

In order to determine the molar extinction coefficient (ϵ) of 12-fluororhodopsin an excess of the aldehyde (11-*cis*-12-fluororetinal or 11-*cis*-retinal) was incubated with increasing amounts of opsin. At the highest concentration of opsin the ratio between the aldehyde and opsin was about 3. The total volume of the reaction mixtures was adjusted to 2.0 mL by changing the amount of 1% digitonin (10 mM Hepes, pH 7.0) added. The reaction mixtures were incubated in the dark for 2 h at 25 °C, which assured the formation of both 12-fluororhodopsin and rhodopsin. Then, 1 M hydroxylamine was added to the reaction mixtures to a final concentration of 50 mM to remove free aldehyde and random Schiff bases which may interfere with further experiments. The amount of pigment formed was determined by bleaching the samples with orange light. Both pigments were stable to hydroxylamine under these conditions. The results are shown in Figure 4. The extinction coefficient of 12-fluororhodopsin was calculated from the ratio of tangents of curves in the figure and ϵ of rhodopsin, i.e. 42 000 at 498 nm.[15]

Purification of 12-Fluororhodopsin. 12-Fluororhodopsin, regenerated in ROS membranes, was extracted with 1% Ammonyx LO buffered in 10 mM Hepes, pH 7.0. Chromatographic separation was carried out on a hydroxylapatite (Bio-Gel HTP, Bio-Rad) column at 4 °C in the dark. A solution of 10 mL of 12-fluororhodopsin (ca. 24 A units) was placed on a 1.5 cm × 20 cm column buffered with 10 mM Hepes, pH 7.0, containing 1% Ammonyx LO. The column was eluted with discontinuous gradients with use of 200 mL 1% Ammonyx LO-Hepes (arrow A in Figure 8a), followed by 100 mL of 0.25 M NaCl–1% Ammonyx LO-Hepes (arrow B), and 100 mL of 0.15 M sodium phosphate (pH 6.5)–1% Ammonyx LO-Hepes (arrow C). The sodium chloride elution (arrow B) is not essential, and can be eliminated. The absorption spectra of fractions a, b, and c were measured and shown in Figure 8b. These spectra show that peak a and peak b contain protein impurities and unreacted 12-fluororetinal and that peak c is purified 12-fluororhodopsin.

The γ-band extinction coefficient (ϵ_{280}) of purified 12-fluororhodopsin was estimated from that of the α band (ϵ_{507} = 35 000, Figure 4); ϵ_{280} = 104 000. This value is about 40% larger than that of rhodopsin (ϵ_{278} = 74 000).[15] We do not know the contribution of retinal chromophore to the γ-band absorption of visual pigments, but it is reasonable to assume that the chromophore contribution to γ-band is similar in 12-fluororhodopsin and in rhodopsin. Therefore, the difference in the γ-band absorption is likely to be explained by contamination of denatured opsin from which the chromophore was removed. Rechromatography attempting to remove the denatured opsin contaminant did not improve the purity.

Spectrophotometry at Low Temperatures. For low-temperature spectrophotometry, a specially designed glass cryostat with quartz windows[22] was used. The temperature of the sample was monitored with a copper–constantan thermocouple attached to the sample cell which had a light path of 2 mm. The sample was irradiated with a 2 kW xenon lamp whose light passed through a glass cutoff filter with or without an interference filter. Absorption spectra were measured with a Hitachi 323 recording spectrophotometer. For correction of scattering of the sample, ground glass plates were placed in the reference and sample beams.

For low-temperature experiments, glycerol was added to 12-fluororhodopsin extract to yield a final glycerol concentration of 66%.

Acknowledgment. The work done at the University of Hawaii was supported by a grant from the U.S. Public Health Services (AM-17806). The work done at Kyoto was supported by a grant from the Japanese Ministry of Education (No. 548017). The NMR spectrometer at the University of Oregon was purchased with grants from Murdoch Charitable Trust and the National Science Foundation.

(22) Yoshizawa, T. "Handbook of Sensory Physiology", Dartnell, H. J. A., Ed.: Springer-Verlag, West Berlin, **1972**; Vol. VII/I, pp 146–79.