Specific Reaction of 9-*cis*-Retinoyl Fluoride with Bovine Opsin†

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ABSTRACT: Opsin readily undergoes Schiff base formation between an active site lysine and 9-*cis*- or 11-*cis*-retinaldehyde to form the visual pigments isorhodopsin (λ_{max} = 487 nm) and rhodopsin (λ_{max} = 500 nm), respectively (Dratz, 1977). It would be predicted that 9-*cis*-retinoyl fluoride (**1**), an isostere of 9-*cis*-retinal, should be an active site directed, mechanism-based labeling agent of opsin, since a stable peptide bond should be formed instead of a Schiff base. It is shown here that 9-*cis*-retinoyl fluoride (**1**) reacts with opsin in a time-dependent fashion ($t_{1/2}$ = 9 min at 25 μM **1**) to form a new, nonbleachable pigment with a λ_{max} of approximately 365 nm. β-Ionone competitively slows down the rate of the reaction. The absorbance of the new pigment at approximately 365 nm is similar to that of model amide compounds. This result is consistent in a general and qualitative way with the Nakanishi–Honig point-charge model for visual pigments which requires that the chromophore be charged, a situation not possible when the retinoid is linked to opsin via a peptide bond rather than a protonated Schiff base [Honig, B., Dinur, U., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979) *J. Am. Chem. Soc. 101*, 7084–7086]. 9-*cis*-Retinoyl fluoride (**1**) is approximately 4-fold more potent than *all-trans*-retinoyl fluoride (**2**) as an inactivator of bovine opsin. Importantly, 13-*cis*-retinoyl fluoride (**3**) is inactive, and no new absorption band at 365 nm is observed. These and other experiments demonstrate the first specific active site directed inactivation of opsin. The labeling agents are potentially of great use in photochemical, mechanistic, and structural studies on the role(s) of rhodopsin in visual transduction and adaptation. This paper also introduces the use of acyl fluorides as mechanism-based inactivators of proteins able to form Schiff bases with aldehydes. Their potential great specificity is demonstrated here by the fact that of the 11 potentially acylatable primary amino groups of opsin, only an active site residue is susceptible to reaction with the retinoyl fluorides.

The absorbance of a photon of light by rhodopsin is the initial event in vision (Wald, 1968; Hubbell & Bownds, 1979). This event causes the isomerization of the 11-*cis*-retinal Schiff base of rhodopsin to the all-trans form, which then triggers a series of conformational changes in the protein. The photochemical process occurs in picoseconds, and the protein conformational changes occur in the microsecond, millisecond, and second time scale (Uhl & Abrahamson, 1981). Metastable intermediates prior to the decay of metarhodopsin II are thought to activate transducin or the G protein, which in turn activates a phosphodiesterase (O'Brien, 1981). These steps are assumed to be necessary in the visual transduction mechanism whose immediate goal is the hyperpolarization of the rod outer segment membranes (Uhl & Abrahamson, 1981). Although the *all-trans*-retinal remains bound to the opsin via a Schiff base, it soon is hydrolyzed to afford *all-trans*-retinal and opsin (Uhl & Abrahamson, 1981). At high light intensities, much of the rhodopsin is bleached in this manner and remains as inactivated opsin. At low light intensities, most of the opsin is combined with 11-*cis*-retinal to form rhodopsin. Thus bleaching plays an important role in visual adaptation and allows vertebrates to adapt to background light over a scale of the order of 10 logs of intensity (Dowling, 1967).

Despite the obvious importance of rhodopsin, specific irreversible inactivators have yet to be designed for it. Specific active site directed irreversible inhibitors of rhodopsin, or perhaps more precisely opsin, would be of great interest for several reasons. For one, a vitamin A based inactivator would allow for the correlation between rhodopsin conformation and its ability to activate transducin and in turn be phosphorylated (Yoshizawa & Fukada, 1983). Secondly, if the inactivators were to function in the retina, the resultant pigment would be nonbleachable, and hence, the relationships between normal photochemical bleaching and visual adaption could be directly determined. In addition, a radiolabeled specific inactivator would be useful for protein structural studies on the various rod and cone opsins. Finally, the UV/visible spectra of the inactivator adduct could yield important information on the role of opsin–ligand interactions in the fine tuning of the absorption spectra of the visual pigments, a topic of great interest now, due to the pioneering studies of Nakanishi, Honig, and their co-workers (Honig et al., 1979).

As is well-known, opsin undergoes ready Schiff base formation with an active site lysine by the mechanism shown in Scheme I with 9-*cis*-retinal (Henselman & Cusanovich, 1976). 9-*cis*-Retinoyl fluoride, a close isostere of 9-*cis*-retinal (**1**), should behave similarly except that it possesses a leaving group (F^-) and should undergo peptide bond formation rather than Schiff base formation (Scheme I). Hence, **1** should be a specific mechanism-based inactivator of opsin and should form a nonbleachable pigment (Rando, 1974; Abeles & Maycock, 1976).[1] This prediction has been realized and is reported in this paper. *all-trans*-Retinoyl fluoride (**2**), as reported before, also inactivates opsin but is approximately 4-fold less potent than **1** (Wong & Rando, 1982). Since 13-*cis*-retinaldehyde does not form a Schiff base with opsin, 13-*cis*-retinoyl fluoride (**3**) should be inert in these studies (Hubbard & Wald, 1952). This is shown to be the case. Hence, the acyl fluorides function as aldehyde equivalents in the studies reported herein.

Materials and Methods

Chemicals. 9-*cis*-Retinoic acid, 13-*cis*-retinoic acid, and *N,N*-diethyl-*all-trans*-retinamide were provided by Hoffmann-La Roche Inc. of Nutley, NJ, and Basel, Switzerland.

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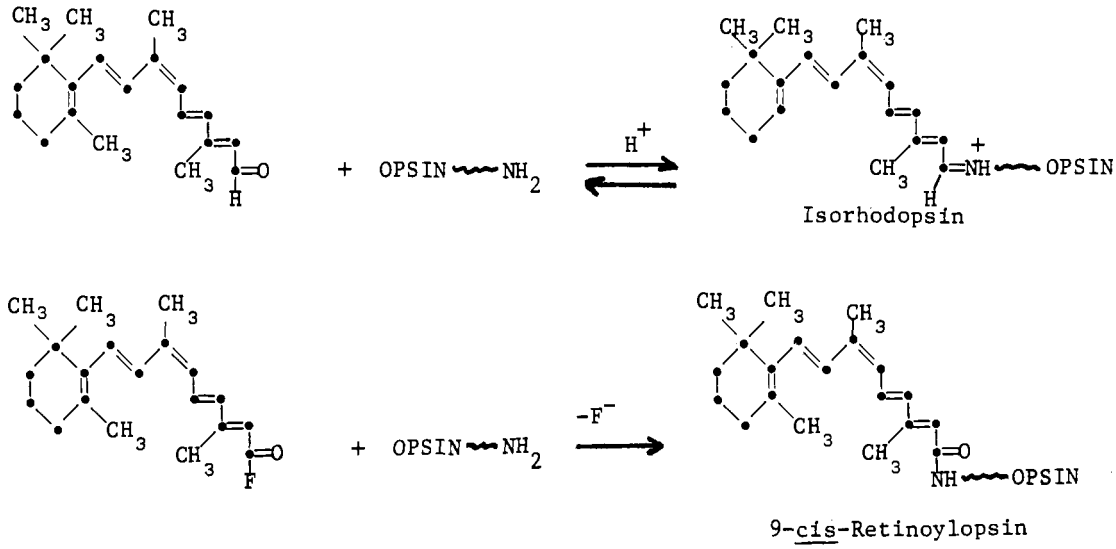
[1] The term inactivator is used here in the sense of irreversibly preventing opsin treated with **1** from combining with 11-*cis*-retinal to form rhodopsin. We as yet do not know whether or not the new pigment formed from opsin and **1** is physiologically and biochemically active in and of itself.

0006-2960/84/0423-0020$01.50/0

Scheme I



All additional reagents were at least reagent grade while all solvents utilized for high-pressure liquid chromatography (HPLC) were UV grade.

Synthesis of all-trans-, 9-cis-, and 13-cis-Retinoyl Fluorides. All retinoyl fluorides are synthesized from the corresponding retinoic acids (*all-trans*-retinoic acid from Sigma) by the following procedure, which was done totally in the dark or under a lamp with a Kodak safelight filter, No. 1. A 0.1–0.2-mmol aliquot of the appropriate retinoic acid was suspended in 2–4 mL of diethyl ether freshly distilled over lithium aluminum hydride (Alfa Products). Then under nitrogen, a 5-fold excess of the fluoridating agent prepared from the condensation of diethylamine (Aldrich) with hexafluoropropene (PCR Research Chemicals) according to the published procedure was added slowly to the suspension with stirring in an ice/water bath (Takaoka et al., 1979). The reaction was normally completed within 1 h, as monitored by thin-layer chromatography in 7:3 diethyl ether/hexane, but is allowed to warm to room temperature if not completed within that time.

Purification of the resulting retinoyl fluorides as yellow powders was accomplished via preparative silica gel (Analtech Inc. silica gel G, without organic binders, 500 or 1000 μm, 20 × 20 cm) chromatography that utilized as solvent a 7:3 mixture of diethyl ether/hexane (Burdick and Jackson Laboratories, UV grade). The R_f for the retinoyl fluorides in this system was 0.75 while the R_f for the starting retinoic acids was 0.50. The yields for the three fluorides were between 50 and 70%.

The structures of the retinoyl fluorides were ascertained by both chemical and spectroscopic means. *all-trans*-Retinoyl fluoride was reacted with methanol and water/2-propanol to afford methyl *all-trans*-retinoate and *all-trans*-retinoic acid, respectively, as shown by comparison of infrared spectra, proton nuclear magnetic resonance (NMR), and chromatographic coelution. Furthermore, all three retinoyl fluorides were reduced by lithium aluminum hydride to their respective alcohols, and the resulting retinols were shown to be isomerically pure. This was not true, however, for the unstable 11-cis compounds, which yielded a mixture of the retinoids. The procedure for the reduction and analyses of the retinoyl fluorides was as follows: After 2–3 mg of the appropriate retinoyl fluoride was evacuated under high vacuum in the dark for 90 min, the flask containing the sample was cooled on an ice/water bath, and then a 200-fold excess of 1 N lithium aluminum hydride in diethyl ether (Aldrich) was added. After 10 min, the reaction was terminated with 300 μL of 50% methanol in water. The resulting solution was extracted 4 times with hexane, and the combined solutions were evaporated to dryness. The product was redissolved in 250 μL of hexane, and 5 μL was injected onto a 5 μm particle size 25-cm size Hibar-II, Lichrosorb silica column (Merck, Inc.) with either 20% ether/hexane or 5% dioxane/hexane (Groenendijk et al., 1980) as eluants at a flow rate of 2 mL/min. A Waters Model M-6000A HPLC pump was used with either a Model 440 UV detector containing a 365-nm filter or a Model 480 variable-wavelength detector. Under these chromatographic conditions, the various isomers were clearly separated. The *all-trans*, 9-*cis*-, and 13-*cis*-retinoyl fluorides gave >95% of the corresponding retinol under these conditions, thus demonstrating that the fluorination reactions proceeded with stereochemical integrity.

Spectroscopic Data on Retinoyl Fluorides. Due to the limited supply of 9-*cis*-retinoic acid and 13-*cis*-retinoic acid, C^{13} NMR spectra of the corresponding acyl fluorides were not taken. ^{19}F NMR samples were dissolved in $CDCl_3$/1% Me_4Si (Aldrich) and sealed in 5-mm amber Pyrex tubes (Norell, Inc.), and spectra were obtained on a Bruker WM 300 WB spectrometer. Trifluoroacetic acid in $CDCl_3$/1% Me_4Si was used as an external standard, and ppm are downfield from it. The ^{13}C NMR spectrum of the *all-trans*-retinoyl fluoride was taken on a JEOL FX-270 spectrophotometer. Infrared spectra were obtained on a Perkin-Elmer 710 B spectrophotometer while ultraviolet data were acquired on a Perkin-Elmer 552A UV/vis spectrophotometer with quartz microcuvettes (Beckman). Spectral data for the retinoyl fluorides were as follows. For *all-trans*-retinoyl fluoride: IR (CCl_4) 1780 (C=O) cm^{-1}; ^{19}F NMR 118.797 ppm; ^{13}C NMR 179.794 (J = 38.43 Hz) 180.361 ppm; UV max (CH_3CN) 376 nm (ϵ 24 440). For 9-*cis*-retinoyl fluoride: IR (CCl_4) 1780 (C=O) cm^{-1}; ^{19}F NMR 118.059 ppm; UV max (CH_3CN) 358 nm (ϵ 20 500). For 13-*cis*-retinoyl fluoride: IR (CCl_4) 1780 (C=O) cm^{-1}; ^{19}F NMR 118.072 ppm; UV max (CH_3CN) 362 nm (ϵ 22 000).

Finally, the ultraviolet spectra of two model retinamides were determined. *N,N*-Diethyl-*all-trans*-retinamide (Hoffmann-La Roche Inc.) had a λ_{max} = 345 nm with an ϵ = 23 000 in 4 mM dodecyl maltoside/20 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5, and *N*-butyl-9-*cis*-retinamide had a λ_{max} = 343 in the same buffer. This compound was

prepared in situ by adding an excess of *n*-butylamine to a known concentration of 9-*cis*-retinoyl fluoride.

Isolation of Bovine Rod Outer Segments and Purification of Rhodopsin. Isolation of rod outer segments and discs followed the sucrose flotation method of McCaslin & Tanford (1981). Purified solubilized rhodopsin was obtained by affinity chromatography on a Con A–Sepharose 4B (Sigma) column by the procedure of DeGrip et al. (1980), except in the last step. During dialysis, the 20 mM nonylglucose (Calbiochem) was exchanged with 4 mM dodecyl maltoside (Calbiochem), keeping the buffer constant, which consists of 20 mM Pipes (Calbiochem), pH 6.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. We found the rhodopsin to be much more stable to bleaching and regeneration in the dodecyl maltoside. The purified protein had an A_{280}/A_{500} = 1.7–1.8 and was stored in 5% sucrose and quickly frozen by being dropped into liquid nitrogen (200–800-μL aliquots) stored in polymer tubes and maintained at −70 °C. There was a slow loss of regeneration capacity over time even though the integrity of rhodopsin as measured by its spectrum was maintained indefinitely (Radding & Wald, 1956). All experiments were done on purified solubilized rhodopsin with A_{280}/A_{500} = 1.7–1.8. The detergent dodecylmaltose proved to be ideal for rhodopsin purification since both rhodopsin and opsin are stable in this detergent as shown by maximal regeneration of opsin (DeGrip, 1982).

The extents of regeneration were determined in the following manner. Approximately 3.2 μM purified rhodopsin in 20 mM Pipes, pH 6.5, 150 mM NaCl, 1 mM $MnCl_2$, $MgCl_2$, and $CaCl_2$, and 4 mM dodecyl maltoside was bleached under a table lamp on ice in the presence of 33 mM NH_2OH for 30–45 min. Then, a 7-fold excess of 11-*cis*-retinal dissolved in absolute ethanol was added, and the resulting solution was allowed to incubate at room temperature in the dark for 1 h before the change in absorbance at 500 nm upon bleaching was measured (Hubbard et al., 1971).

Kinetics of Inactivation and Competition Experiments. To ascertain the inhibitory effects of the various isomers of retinoyl fluoride on the regeneration of bleached bovine rhodopsin, the appropriate retinoyl fluoride dissolved in 2-propanol was added to the rhodopsin bleached as previously described after dialysis and then incubated for the appropriate time in the dark at 25 °C. The reaction was quenched by the addition of 2 N NH_2OH, pH 6.5, to a final concentration of 33 mM. The sample was bleached again for 10 min, then a 7-fold excess of 11-*cis*-retinal dissolved in absolute ethanol was added, and the resulting solution was incubated in the dark for 1 h at room temperature before the ΔA_{500} upon bleaching was measured.

For the kinetic experiments, bleached bovine rhodopsin in 10 mM dodecylmaltose and 66 mM KH_2PO_4, pH 6.5, was incubated with 9-*cis*-retinoyl fluoride at various times and concentrations and then 2 N NH_2OH, pH 6.5, was added to a final concentration of 33 mM to quench the reaction. As described previously, the extent of regeneration was determined by adding 11-*cis*-retinal and then bleaching to ascertain the ΔA_{500}. All points given were done in duplicate. Once rhodopsin was inhibited by 9-*cis*-retinoyl fluoride, neither extended dialysis nor extended incubations with 11-*cis*-retinal led to further regeneration.

In the competition experiment with freshly distilled β-ionone, (Aldrich), 4.0 μM bleached bovine opsin was incubated at the appropriate times with 9-*cis*-retinoyl fluoride and then quenched by the addition of 2 N NH_2OH, pH 6.5 (final concentration 33 mM). The extent of regeneration, as measured by the change at A_{500} upon bleaching, was determined and compared to the extent of regeneration of bleached bovine rhodopsin preincubated with β-ionone (final concentration 383 μM), according to the method of Yoshizawa & Matsumoto (1975), prior to addition of 9-*cis*-retinoyl fluoride. Stock solutions of β-ionone were prepared mixed with 2-propanol following the procedure of Yoshizawa & Matsumoto (1975).

Covalent Modification of Purified Rhodopsin with Methyl Acetamidate. Purified rhodopsin (3.2–6.2 μM, 1-mL volume) was amidinated with methyl acetimidate (Pierce Chemical) at pH 7.2 following the published procedures of Nemes et al. (1980) and DeGrip et al. (1973a,b). Excess reagent and reaction byproducts were removed by dialysis against four 300-mL changes of buffer containing 20 mM Pipes, pH 6.5, 150 mM NaCl, 1 mM $MnCl_2$, $MgCl_2$, and $CaCl_2$, and 4 mM dodecylmaltose at 20-min intervals. The regeneration capacity of this amidinated rhodopsin was approximately 70% of non-amidinated rhodopsin after bleaching and addition of 11-*cis*-retinal. Then the amidinated protein was bleached under conditions previously described except in the absence of NH_2OH followed by the addition of a 10-fold excess of 9-*cis*-retinoyl fluoride. After incubation for 60 min in the dark at room temperature, 2 N NH_2OH, pH 6.5, was added to a final concentration of 33 mM, an aliquot of the solution was bleached again for 10 min on ice under a table lamp, and then 11-*cis*-retinal was added to check for inhibition of regeneration. Complete inhibition had occurred.

After bleaching of the amidinated rhodopsin and inhibition of its regeneration capacity with 9-*cis*-retinoyl fluoride, the modified protein was purified by affinity chromatography by the following procedure. A 1.6-nmol sample of the protein (~3.2 μM) in 0.5 mL is loaded at a flow rate of 1 mL/h onto a small column (0.8 × 9 cm, Bio-Rad, Richmond, CA) packed with 0.5 mL of Con A–Sepharose 4B (Pharmacia Co.) that had been washed with 20 volumes of buffer containing 20 mM Pipes, pH 6.5, 150 mM NaCl, 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$, and 1 N NaCl (buffer A), followed by a 10-volume wash with buffer containing everything as before except for the 1 N NaCl (buffer B). The column was washed finally with four column volumes in the same buffer as the protein solution, which consists of 4 mM dodecyl maltoside/20 mM Pipes, pH 6.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$ (buffer C).

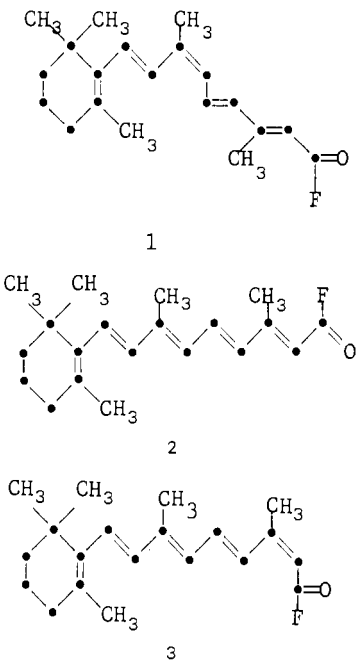
To make sure all the protein binds to the column, the eluant was recycled 2 or 3 more times at the same flow as before. Fractions were collected on a Gilson fraction collector in 0.6-mL aliquots.

The Con A column was washed with 30 volumes of buffer C and the modified protein was eluted with 200 mM methyl α-mannoside (grade III, Sigma) dissolved in buffer C.

Ultraviolet Spectra of Modified Opsins. The ultraviolet spectra of all the purified modified proteins eluted with 200 mM methyl α-mannoside were taken on a Perkin-Elmer 552A UV/vis spectrophotometer between 600 and 250 nm at a slit width of 0.25 nm in quartz microcuvettes. The blank consists of 20 mM Pipes, pH 6.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ in 4 mM dodecylmaltose.

To ensure that the 365-nm absorption that was recorded for the modified opsin was not due to *all-trans*-retinal, retinoic acid, or retinal oxime, the following controls were performed. (a) Purified rhodopsin (6.2 μM), 800 μL, was bleached on ice for 45 min under a table lamp, and then 20 μL of 2 N NH_2OH, pH 6.5, was added to the solution, which is bleached again for another 30 min. UV spectra of the bleached solution before and after addition of hydroxylamine are taken. The solution then was purified by Con A–Sepharose 4B affinity

Chart I



chromatography. No discrete absorption was found at 365 nm. (b) Purified rhodopsin (3.0 μM), 1.2 mL, was bleached, and 5 μL of 2 N NH_2OH was added (8.3 mM final concentration). Then, 4 μL of a 30 mM ethanolic solution of *all-trans*-retinoic acid (Sigma) was added (25 μM final concentration) to the bleached rhodopsin, and the solution was incubated at room temperature in the dark for 60 min. An aliquot was checked at this point for regeneration capacity, which turned out to possess 85% capacity. The remainder of the solution was passed through a column of Con A–Sepharose 4B with a bed volume of 0.5 mL, which was equilibrated with buffer C as described previously and washed with 30 bed volumes of buffer C. The protein then was eluted with 200 mM methyl α-mannoside in buffer C. No discrete absorption was found at 365 nm.

Results

Specific Labeling of Bovine Opsin by 9-cis-Retinoyl Fluoride. The retinoyl fluoride derivatives of 9-*cis*-retinoic acid, *all-trans*-retinoic acid, and 13-*cis*-retinoic acid were prepared by using the fluoridation method described in the literature (Takaoka et al., 1979). The fluorination reactions occurred in fairly high yield to afford 9-*cis*-retinoyl fluoride (**1**), *all-trans*-retinoyl fluoride (**2**), and 13-*cis*-retinoyl fluoride (**3**) (Chart I). In these instances, the fluorination reactions proceeded with high stereochemical integrity (see Materials and Methods). Under the same conditions, however, the relatively unstable 11-*cis*-retinoic acid was converted to a mixture of isomeric retinoyl fluorides and, hence, was not further investigated.

Incubation of purified bovine opsin with **1** at varying concentrations led to the time-dependent irreversible "inactivation" of the protein as shown in Figure 1. These experiments were performed in dodecyl maltoside as detergent. In order to reliably achieve complete inactivation of opsin within 1 h, an 8–10-fold excess of **1** over opsin was required. A similar situation is found with regeneration, where an approximately 7-fold excess of 11-*cis*-retinal is required for complete regeneration under the same circumstances. It should also be noted that the rates of inactivation with **1**, as well as the rates of regeneration with 11-*cis*-retinal, are dependent on the nature

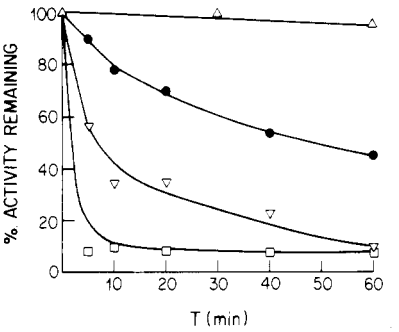


FIGURE 1: Time-dependent inactivation of opsin with 9-*cis*-retinoyl fluoride. 2.4 μM opsin was incubated alone (△) and with (●) 9.5, (▽) 25.5, and (□) 51 μM **1** for varying periods of time in the dark at 25 °C as indicated under Materials and Methods. Aliquots were removed, and the extent of rhodopsin regeneration was determined.

Table I: Opsin Regeneration after Treatment with 9-*cis*-Retinoyl Fluoride under Varying Conditions[a]

experiment	% regeneration
opsin + 2-propanol	95
rhodopsin + 9-*cis*-retinoyl fluoride (10-fold excess)	100
opsin + NH_2OH + 9-*cis*-retinoyl fluoride	90
opsin + 9-*cis*-retinoyl fluoride (10-fold excess)	5
opsin + *all-trans*-retinoic acid	92

[a] Opsin or rhodopsin (3.2 μM) in the usual buffer was incubated at room temperature (25 °C) for 1 h each with either 2-propanol, a 10-fold excess of **1** in 2-propanol, or a 20-fold excess of *all-trans*-retinoic acid in 2-propanol. In a separate experiment, opsin (3.2 μM) was pretreated with 2 N hydroxylamine before the addition of **1** in 2-propanol. The samples were then treated with 11-*cis*-retinal, and the regeneration of rhodopsin was followed as described under Materials and Methods. In the case of rhodopsin, 2 N hydroxylamine was added, followed by bleaching and the addition of 11-*cis*-retinal. In final controls, rhodopsin was boiled for 5 min, whereupon it thermally bleached, and was then treated with 11-*cis*-retinal as above.

of the detergent. Both rates are at least several-fold slower in dodecyl maltoside vs. digitonin. The activity of opsin modified by **1** cannot be restored in the least either by continual dialysis or by chromatography on Con A–Sepharose columns. The remaining activity of opsin was determined by measuring the extent of its specific combination (regeneration) in the presence of 11-*cis*-retinal (McCaslin & Tanford, 1981; Henselman & Cusanovich, 1976). In this assay, the visible absorbance increase at approximately 500 nm is measured. It is clear from Figure 1 that time-dependent inactivation occurs; however, even boiled, denatured rhodopsin yields a small ΔOD at 500 nm when treated with 11-*cis*-retinal (Table I). This is reflected in the curves shown in Figure 1, where it appears that a residual activity of opsin remains after incubation with 9-*cis*-retinoyl fluoride. We believe that complete active site directed modification has occurred. However, the assay utilized here does not distinguish between 0 and 5–8% activity remaining (Table I).

The results shown in Figure 1 are not immediately informative of the fact that the mode of inactivation is active site directed and, hence, specific. It is possible that **1** could acylate non active site lysines or other nucleophiles, either of which reaction could abolish opsin's activity by this nonspecific mechanism. Further controls were performed as indicated in Table I. First, pretreatment of bleached opsin with hydroxylamine before the addition of **1** eliminates the inhibition. Hydroxylamine rapidly forms a hydroxymate with **1** and, hence, quenches its activity. Second, treatment of unbleached

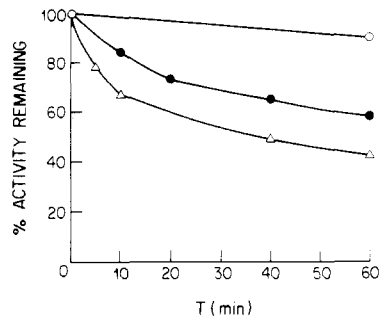


FIGURE 2: Competitive protection by β-ionone. As in the experiment performed in Figure 1, 4.0 μM opsin was incubated alone (○) and with (●) 30 μM **1** plus 383 μM β-ionone and with (△) 30 μM **1** at 25 °C. The remaining activity of the opsin was determined as in Figure 1 and under Materials and Methods.

Table II: Comparison of *all-trans*-, 9-*cis*-, and 13-*cis*-Retinoyl Fluorides as Inactivators of Opsin Regeneration[a]

isomer	concn of isomers and extents of regeneration
9-*cis*-retinoyl fluoride	4-fold, 50%; 10.5-fold, 5%; 21-fold, 8%
all-trans-retinoyl fluoride	8-fold, 100%; 16-fold, 36%; 32-fold, 12%
13-*cis*-retinoyl fluoride	10-fold, 100%; 20-fold, 100%; 40-fold, 100%

[a] Opsin (3.2 μM) was incubated with the indicated concentrations of the retinoyl fluorides for 1 h under the usual conditions. The extent of regeneration with 11-*cis*-retinal was determined as discussed under Materials and Methods. It should be noted that 5–8% "regeneration activity" can be obtained with boiled rhodopsin. Thus, the assay does not distinguish 0% activity from 8%.

rhodopsin with **1** has no effect on the ability of the rhodopsin to be bleached and regenerated in the presence of 11-*cis*-retinal. This is to be expected because the chromophore of rhodopsin blocks access of **1** to its active site. Finally, since some retinoic acid would be expected to arise spontaneously during the hydrolysis of **1** by water, retinoic acid was tested as a possible inhibitor. Retinoic acid (all-trans) has no effect on the activity of the opsin. These results, taken together, make it unlikely that the observed inactivation of opsin by **1** is nonspecific in nature. If **1** is a specific active site directed inactivator of opsin, it would be further predicted that reversible competitive antagonists of opsin's regeneration should also slow down the rate of inactivation or modification by **1**. This is shown in Figure 2, where it is demonstrated that β-ionone, a somewhat weak competitive inhibitor of opsin regeneration, decreases the rate of opsin modification by **1** (Yoshizawa & Matsumoto, 1975).

It was of further interest to compare the extents of inactivation observed with **1** to those of its all-trans and 13-cis isomers, **2** and **3**. Although all three compounds possess virtually identical chemical reactivities, they should markedly differ in their abilities to react with an active site residue of opsin. This expectation was realized as shown in Table II. Interestingly, even at a 40-fold excess of 13-*cis*-retinoyl fluoride (**3**), essentially no inhibition of regeneration could be measured. Higher concentrations of **3** were not possible to achieve due to solubility limitations.

It should be noted that although we have no direct evidence at this point as to which residue of opsin is modified, it is likely to be the active site lysine. Consistent with this idea is the observation that extended incubation of the inactivated and modified opsin with hydroxylamine does not lead to reactivation. Had a cysteine, for example, been derivatized, the resultant thio ester would have been unstable to hydroxylamine.

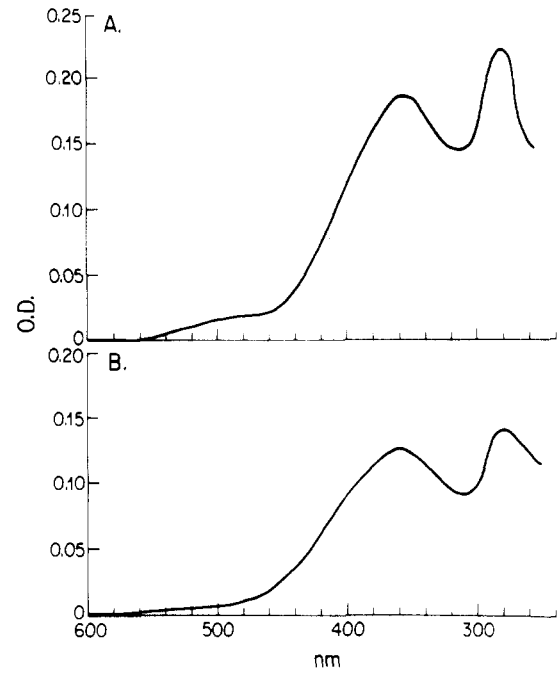


FIGURE 3: Ultraviolet spectrum of opsin modified with 9-*cis*-retinoyl fluoride after treatment with methyl acetimidate. (A) 3.2 μM opsin previously amidinated as rhodopsin, bleached, and processed as recorded under Materials and Methods was treated with 33 μM **1** for 1 h at 25 °C until essentially no regeneration activity remained. The opsin was applied to a Con A–Sepharose 4B column as described under Materials and Methods. Fractions containing protein elutable with methyl α-mannoside were pooled, and the ultraviolet spectra were recorded. Approximately 70% of the protein applied to the column was specifically eluted. (B) 3.2 μM opsin was treated with 33 μM **1** for 1 h at 25 °C until no regeneration activity remained. The opsin was applied to a Con A–Sepharose column as described under Materials and Methods. Fractions containing protein elutable with methyl α-mannoside were pooled, and the ultraviolet spectra was recorded. The yield of protein specifically eluted was 70–80% of that applied.

Ultraviolet Spectra of Retinoyl Fluoride Modified Opsins. Opsin by itself has a typical protein ultraviolet spectrum with a λ_{max} at approximately 280 nm (Dratz, 1977). When opsin was completely inactivated with **1**, a new absorption band appeared at approximately 365 nm (Figure 3B), after removal of all the excess retinoids by careful chromatography on a Con A–Sepharose column with extensive washing with buffer followed by opsin elution with methyl α-mannoside. Under the chromatographic conditions chosen, noncovalently bound retinoids such as the retinoic acids, the retinal oximes, and the retinoyl hydroxamate were eluted in the wash. This is important here because many of these compounds themselves absorb in the 365-nm range. The chromophore shown in Figure 3 was stable to extensive incubation with hydroxylamine and extensive dialysis, again strongly suggesting an amide linkage at the active site (i.e., lysine). In order to ensure that the spectrum shown in Figure 3B was that of active site derivatized opsin, rhodopsin was first reacted with methyl acetamidate before bleaching and reaction with **1**. Methyl acetamidate blocks 10 of the 11 primary amino groups of rhodopsin without much effecting subsequent bleaching and regeneration (DeGrip et al., 1973a,b). As can be seen in Figure 3A, the resulting ultraviolet spectrum of the opsin pretreated with methyl acetamidate followed by bleaching and reaction with **1**, which leads to the inactivation of the modified opsin with a time course similar to that of opsin, was quite similar to the spectrum in Figure 3B after chromatography. These data are again consistent with a specific mode of de-

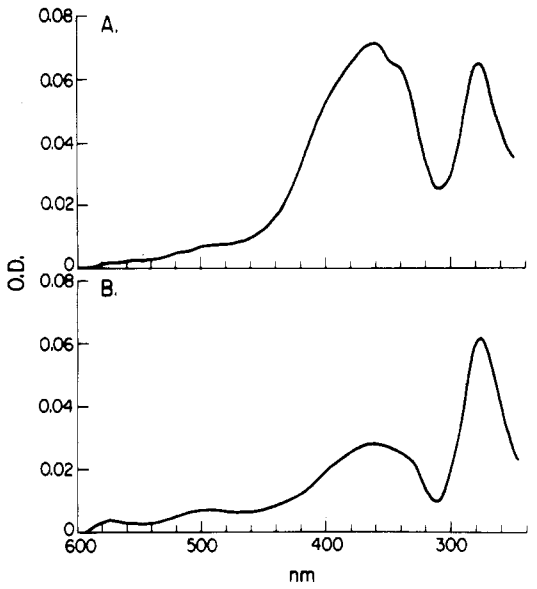


FIGURE 4: Ultraviolet spectra of bleached rhodopsin treated with *all-trans*-retinoyl fluoride and 13-*cis*-retinoyl fluoride. (A) 6.2 μM bleached rhodopsin was incubated with a 30-fold excess of **2** (187 μM) followed by purification via affinity chromatography as described under Materials and Methods. (B) 6.2 μM bleached rhodopsin was incubated with a 30-fold excess of **3** (186 μM final concentration), and the protein spectrum was obtained after the solution was passed through a Con A–Sepharose 4B column and specifically eluted with methyl α-mannoside.

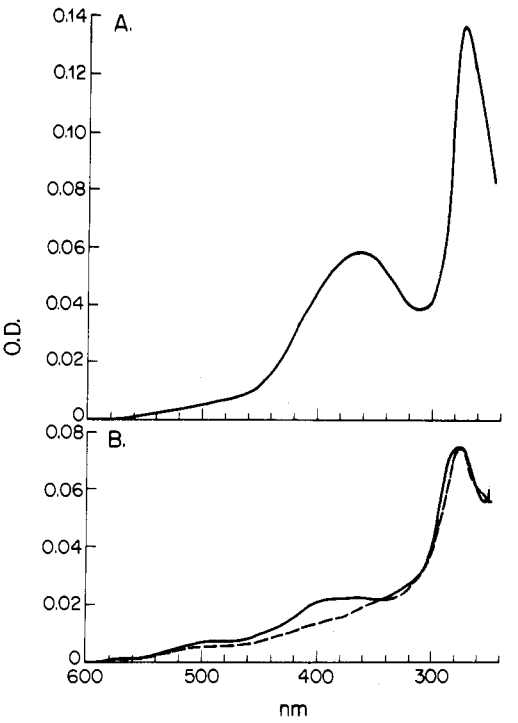


FIGURE 5: Ultraviolet spectra of opsin modified with 9-*cis*-retinoyl fluoride and 13-*cis*-retinoyl fluoride. (A) 6.2 μM opsin was treated with 33 μM **1** at 25 °C for 80 s. Regeneration of the resulting opsin was inhibited to the extent of approximately 50% under these conditions. The opsin was applied to a Con A–Sepharose column and thoroughly washed with buffer and eluted with methyl α-mannoside. The ultraviolet spectrum of the product is shown. (B) Under the same conditions as (A), opsin was treated with 33 μM **3** and worked up as in (A), and the ultraviolet spectrum of the modified protein was recorded. The dotted curve is the ultraviolet spectrum of opsin pretreated with an 8-fold excess of *all-trans*-retinoic acid before processing as before.

rivatization. The chromophores exhibited in Figure 3 are not bleached even under conditions similar to those that totally bleach rhodopsin. This was shown by exposing the new pigment to fluorescent light and rechromatographing it on Con A–Sepharose. The relative intensities of the 365/280-nm peaks were the same, within experimental error.

The λ_{max} of the new pigment shown in Figure 3 is approximately 365 nm (**1**). This can be compared to model retinamides. *N*-Butyl-9-*cis*-retinamide has a λ_{max} at 343 nm, and *N*,*N*-diethyl-*all-trans*-retinamide has a λ_{max} = 345, both in the buffer system used in the protein modification experiments.

The ultraviolet spectrum of opsin derivatized with an excess of *all-trans*-retinoyl fluoride (**2**) is shown in Figure 4A. Again a new chromophore at approximately 365 nm developed, although its shape is somewhat different to that previously described in Figure 3. Since a relatively large excess of **2** had to be used to effect full inhibition of regeneration, the possibility of nonspecific acylation by **2** must be entertained. That this is probably not very important could be shown by incubation of opsin with an excess of **3**, which, as already mentioned, did not significantly block regeneration. As can be seen in Figure 4B, opsin treated in this manner shows only relatively minor absorbance at 365 nm, possibly due to nonspecific acylation, compared to opsin treated with **2**. That the absorbance at 365 nm following the incubation of opsin with **1** is correlated with active site derivatization is further shown in Figure 5. In this experiment, opsin was treated with a 5-fold excess of either 9-*cis*-retinoyl fluoride (A) or 13-*cis*-retinoyl fluoride (B) for 80 min at room temperature. Both samples were then chromatographed on Con A–Sepharose in the usual way, and their regenerative capacities in the presence of 11-*cis*-retinal and their ultraviolet spectra were recorded. The sample treated with **1** was approximately 50% inhibited. As can be seen here, opsin treated with **3** showed virtually no absorption at 365 nm (B), whereas substantial absorption at 365 nm is found with the opsin treated with **1**. The dotted line in Figure 5B gives the ultraviolet absorption spectrum of opsin treated with an excess of *all-trans*-retinoic acid after chromatography. The minor absorption in the 365-nm region is similar to that observed in the 13-*cis*-retinoyl fluoride treated case.

Discussion

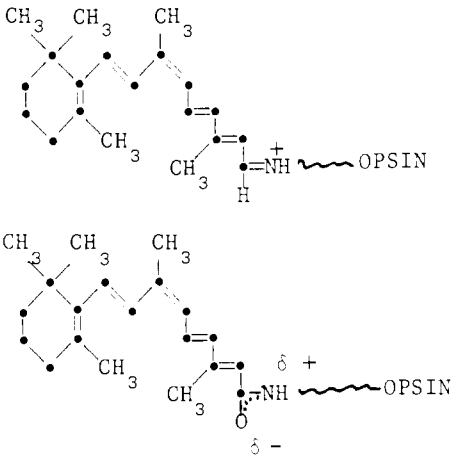
The studies reported here demonstrate the specific nature of the inactivation of opsin by **1**. The inactivation is time dependent and slowed down by β-ionone, a competitive inhibitor of the regeneration of rhodopsin from opsin and 11-*cis*-retinal. Pretreatment of the inhibitor, **1**, with hydroxylamine destroyed its activity consistent with the expected mode of inactivation. The supposition that inactivation of opsin by **1** did not occur because of nonspecific acylation reactions of non active site nucleophilic amino acid groups is, of course, consistent with the β-ionone experiments. Also consistent with this idea are the following facts. (1) Rhodopsin itself is not affected by **1** since it has its active site blocked. (2) The blockage of non active site nucleophiles of rhodopsin by amidination led to a bleachable and regeneratable protein in the presence of 11-*cis*-retinal. This amidinated opsin proved to be readily inactivated by **1**, which is again suggestive that it is a reaction with an active site nucleophile that results in the inactivation of the opsin. (3) *all-trans*-Retinoyl fluoride (**2**) also inactivates opsin but is, as expected, less potent than **1**. **2** should be less potent than **1** because the Schiff base equilibrium between *all-trans*-retinal and opsin is not very favorable compared to that with 9-*cis*-retinal. (4) 13-*cis*-Retinoyl fluoride, an isostere of 13-*cis*-retinal, is inert as an inactivator of opsin although it is chemically as reactive as **1**. 13-*cis*-Retinal (**3**) of course does not form a Schiff base with opsin

(Hubbard & Wald, 1952). The large difference in the activity of **2** vs. **3** is likely due to the fact that the 13-cis isomer is denied access to the active site, whereas the all-trans isomer **2** must be able to gain access to it. This is because the *all-trans*-retinal Schiff base is formed during the bleaching of rhodopsin, and consequently, *all-trans*-retinal, and its close isotere **2**, must be able to gain access to the active site of opsin by the principle of microscopic reversibility.

The spectral studies are also consistent with a specific mode of inactivation. Treatment of opsin with **1** led to a new nonbleachable pigment with a $\lambda_{max} \sim 365$ nm after removal of all noncovalently bound retinoids by Con A–Sepharose chromatography. Treatment of rhodopsin with methyl acetamidate, a reagent that is known to block all accessable primary amino groups (i.e., 10 lysine residues) of rhodopsin, leads to a modified pigment still bleachable and subsequently regenerable in the presence of 11-*cis*-retinal to the extent of approximately 70% (DeGrip et al., 1973a). Rhodopsin modified in this manner was bleached and treated with **1** to afford a pigment with approximately the same spectrum as in the untreated case (Figure 3). The optical density of the 365-nm peak relative to that of the 280-nm peak was somewhat greater in the unamidinated case (Figure 3B) than in the amidinated case (Figure 3A). This probably reflects the fact that amidination of rhodopsin decreases the activity of the untreated control by approximately 30%. If **1** were reacting substantially with non active site nucleophiles, a more intense or more complicated absorption spectrum would be expected in the pigment generated from the untreated (methyl acetamidate) case. What is striking here is the fact that 10 of the 11 primary amino groups of opsin are not at the active site, yet **1** does not appear to react in any substantial way with them. It must be remembered that opsin possesses, of course, many other nucleophilic amino acids on other than its primary amino groups. In fact, it contains a total of 47 charged amino acids in addition to 10 cysteines (Hargrave et al., 1983). These also are susceptible, in principle, to acylation by the acyl fluorides. Considering this, the specificity of **1** for the active site of opsin is striking and probably reflects the relatively low chemical reactivity of **1** due to its extended conjugation. It must also be kept in mind, however, that the pK_a of the active site lysine of opsin may be much lower than that of its other primary amino groups (lysines), which would certainly markedly increase its chemical reactivity. Since the active site lysine forms Schiff bases with 11-*cis*- and 9-*cis*-retinal at pHs even below 7, it would seem reasonable that its $pK_a(NH_3^+)$ is unusually low for a lysine (Wald & Hubbard, 1952). As a further point concerning the specificity of the inactivation process, it is clear that at least qualitatively there is a direct relationship between the extent of inactivation with **1** and the intensity of the new chromophore at 365 nm (Figures 3 and 5).

Interestingly, the new pigment created with **2**, the *all-trans*-retinoyl fluoride, exhibited a similar absorption spectrum as that created with **1**. This suggests that there are no large perturbations of the chromophore's absorption spectrum by the protein. The fact that the chromophore produced from **2** and opsin had an increased OD vs. that of the chromophore from **1** and opsin could reflect the fact that the extinction coefficients of *all-trans*-retinamides are greater than those of the 9-*cis*-retinamides. It is well-known that the 9-cis and 11-cis isomers of the retinoids have lower extinction coefficients than the all-trans isomers (Kropf & Hubbard, 1970). The above conjecture, of course, assumes an active site lysine is labeled by **1** and **2**. Further studies with radiolabeled retinoyl fluorides will be of substantial interest here. Finally, the treatment of opsin with **3** did not lead to inactivation nor, importantly, to the generation of any substantial visible/ultraviolet absorption bands beyond those attributable to the protein backbone.

Scheme II



Given the plausible, but as yet unproven, assumption that a retinamide is generated at opsin's active site, it is of interest to compare the λ_{max} of model amides and the adducts of opsin with those of **1** and **2**. In the same buffer as used in the opsin derivatization experiments, *N*-butyl-9-*cis*-retinamide shows a λ_{max} of 343 nm whereas the 9-*cis*-retinoyl fluoride–opsin adduct shows a λ_{max} of 365 nm. A similar λ_{max} was also found for the *all-trans*-retinoyl–opsin adduct, although the shape of the absorption spectrum was somewhat different here. Rhodopsin itself absorbs at 500 nm and isorhodopsin at 487 nm (Dratz, 1977). These λ_{max} values are far-red-shifted over the retinoids themselves, which absorb maximally at approximately 387 nm, and their Schiff bases, which absorb even lower at approximately 365 nm (Blatz et al., 1972). Certainly, a large part of the rhodopsin shift is attributable to the formation of protonated Schiff bases (Dratz, 1977). In the case of model Schiff bases, this shift is approximately 100 nm to the red (Blatz et al., 1972). The further red shift of 40 nm or so is presumably due to a point-charge effect as proposed by Nakanishi & Honig (Honig et al., 1979). In this model, the absorption spectrum of rhodopsin is due to a negatively charged residue placed at set distance away from the protonated Schiff base head group. In a qualitative way, our results are in agreement with this model in the sense that a large red shift does not occur in the opsin-bound chomophore vs. the model compounds. This is to be expected because although the retinamide is sterically bound at the active site quite similarly to the Schiff base, the situation is quite different electronically since the peptide cannot be protonated (Scheme II). Nevertheless, we do see an approximately 20-nm red shift between model amides and the putative opsin-bound amides, the origen of which remains to be determined. In the main, however, the work reported here is certainly consistent with the idea that Schiff base protonation is critical for the mechanism of the rhodopsin red shift as formulated by Nakanishi & Honig (Honig et al., 1977).

The specificity of the inactivation process with **1** can be partly attributed to the fact that this compound strongly resembles 9-*cis*-retinal, a normal ligand for opsin, since the atomic radius of fluorine is very close to that of hydrogen. The acyl fluorides, such as the type introduced here for biochemical mechanistic studies, may then be assumed to be aldehyde equivalents and may be generally useful in studying the mechanistic enzymology of aldehydes. It is important to note

that the retinoyl fluorides reported here are also relatively stable to hydrolysis. For example, incubation of **2** for 23 h in 10% water/2-propanol resulted in only approximately 40% hydrolysis to retinoic acid. It is assumed that this stability is a reflection of the extended conjugation present in the vitamin A backbone of these molecules. Although part of the specificity of modification of inactivation of opsin reported here is due to the isosterism of **1** and 9-*cis*-retinal, other factors must be involved. Certainly, the fact that opsin catalyzes chemical reactions with certain specific aldehydes to form specific, active site directed, Schiff bases is likely to be quite important. The acyl fluorides undergo the initial phase of these transformations, but the reactions are subverted from their normal course because of the fact that fluoride is a good leaving group. Thus, these acyl fluorides are in fact mechanism-based inactivators and as mentioned before may take advantage of the presumably low pK_a of the active site lysine. As a group, these molecules are highly specific enzyme inactivators requiring the catalytic participation of the target enzyme for specificity (Rando, 1974; Abeles & Maycock, 1976). The use of these inactivators can then be extended to opsin even though opsin is not an enzyme. However, opsin is the agent of chemical as well as photochemical reactions, and hence, the principle of mechanism-based inactivators applies. The work reported here then introduces the use of acyl fluorides as aldehyde equivalents for use in mechanistic and inhibition studies. As the acyl fluorides include retinoyl fluorides, using them in this way may also be helpful in the specific derivatization and labeling of retinoid binding proteins other than the visual pigments (Gawinowicz & Goodman, 1982; Pepe et al., 1982).

The new pigments generated from opsin and either **1** or **2** should be of considerable immediate interest in studies in the role(s) of ligand configuration and rhodopsin action. It is already clear that these pigments are, as expected, unbleachable. It is of interest to determine whether they will behave biochemically and physiologically like rhodopsin in an intact system and what the role of opsin conformation is in visual transduction and adaptation.

Acknowledgments

We thank Dr. William Scott and Hoffmann-La Roche Inc. for their generous supply of 11-*cis*-retinal, 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, and 11-*cis*-retinoic acid.

Registry No. **1**, 87760-33-6; **2**, 83802-77-1; **3**, 87760-34-7; 9-*cis*-retinoic acid, 5300-03-8; 13-*cis*-retinoic acid, 4759-48-2; *all-trans*-retinoic acid, 302-79-4; *N*,*N*-diethyl-*all-trans*-retinamide, 33631-53-7; *N*-butyl-9-*cis*-retinamide, 87760-35-8; β-ionone, 79-77-6; methyl acetamidate, 14777-29-8.

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