Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981a) *Biochemistry 20*, 2110–2120.
Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981b) *Nature (London) 292*, 301–306.
Mornet, D., Pantel, P., Bertrand, R., Audemard, E., & Kassab, R. (1981c) *FEBS Lett. 125*, 54–58.
Okamoto, Y., & Sekine, T. (1978) *J. Biochem. (Tokyo) 83*, 1375–1379.
Okamoto, Y., Okamoto, M., & Sekine, T. (1980) *J. Biochem. (Tokyo) 88*, 361–371.
Seidel, J. C. (1980) *J. Biol. Chem. 255*, 4355–4361.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4876.
Strzelecka-Golaszewska, M., & Sobieszek, A. (1981) *FEBS Lett. 134*, 197–202.
Takeuchi, K. (1982) *J. Biochem. (Tokyo) 91*, 1001–1007.
Webb, M. R., & Trentham, D. R. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 6702.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
West, J. J., Nagy, B., & Gergely, J. (1967) *J. Biol. Chem. 242*, 1140–1145.
Yamamoto, K., & Sekine T. (1979) *J. Biochem. (Tokyo) 86*, 1855–1862.

Biochemical Properties of 9-*cis*- and *all-trans*-Retinoylopsins†

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ABSTRACT: The stoichiometry of the reaction between [^{14}C]-9-*cis*-retinoyl fluoride, a close isostere of 9-*cis*-retinal, and bovine opsin and the biochemical and spectral properties of this new pigment were investigated. The stoichiometry of retinoid incorporation is approximately one in dodecyl maltoside, a detergent in which opsin is capable of regeneration with 11-*cis*-retinal. Interestingly, in Ammonyx LO, a detergent that does not permit rhodopsin regeneration, the stoichiometry of binding is still approximately one. By contrast, heat-denatured opsin does not irreversibly bind substantial [^{14}C]retinoyl fluoride. This result strongly suggests that the nucleophilicity of the active site lysine is retained in Ammonyx LO but that further conformational changes in the protein, required to form rhodopsin, are not possible. These results are all consistent with an active site directed mechanism for the irreversible reaction of 9-*cis*-retinoyl fluoride with opsin probably at the active site lysine residue. The ultraviolet spectra of 9-*cis*-retinoylopsin and its all-trans congener show λ_{max}'s at 373 and 380 nm, respectively, somewhat bathochromically shifted from their respective model *N*-butylretinamides which absorb at 347 and 351 nm. Photolysis of both 9-*cis*- and *all-trans*-retinoylopsins leads to the same photostationary state. This shows that, as expected, photoisomerization without bleaching occurs. The photolysis of either 9-*cis*- or *all-trans*-retinoylopsin in the presence of the G protein (transducin) does not lead to the activation of the latter. This is consistent with the notion that a protonated Schiff base is critical for the function of rhodopsin.

Vertebrate scotopic vision begins with absorption of light by rhodopsin, the major membrane-bound protein in rod outer segments (Hubbell & Bownds, 1979). Bovine rhodopsin is a 39-kDa protein comprised of a single polypeptide chain linked via a protonated Schiff base between lysine-296 and the chromophore 11-*cis*-retinal (Hargrave et al., 1983). Absorption of light by this holoprotein results in the isomerization of the chromophore to its all-trans congener (Hubbard & Kropf, 1958) followed by a series of conformational changes in the protein, which eventually results in the hydrolysis of the *all-trans*-retinal–opsin Schiff base (Wald, 1968). This process is called bleaching, because without the chromophore opsin does not absorb in the visible range. One of the conformers (probably metarhodopsin II) (Parkes et al., 1979; Calhoon et al., 1981) on the way to Schiff-base hydrolysis activates the G protein (transducin), a biochemical step proposed to be of importance in the cascade of events that eventually leads to closing of sodium channels with subsequent hyperpolarization of the rod outer segments (Shinozawa et al., 1979).

The state of protonation of the Schiff base of rhodopsin is thought to be of prime importance for its functioning. The ability of rhodopsin to efficiently transduce light energy into the chemical potential energy of the intermediates is almost certainly related to charge movement caused by light absorption (Honig et al., 1979). This necessitates that the Schiff base be protonated. Furthermore, in the critical metarhodopsin I → metarhodopsin II conversion, a proton is gained by the protein (Cooper & Converse, 1976) at the same time as the Schiff base is probably deprotonated (Doukas et al., 1978), suggesting that proton translocations are important in this step. Finally, the protonated Schiff base is also important in determining the λ_{max}'s for the various visual pigments (Honig et al., 1976).

On the basis of what has been stated concerning the importance of protonated Schiff base formation, it would be of some interest to study the function of an opsin containing a vitamin A analogue not capable of being protonated but, nevertheless, a close isostere of the chromophore of rhodopsin or isorhodopsin. To these ends we have developed 9-*cis*-retinoyl

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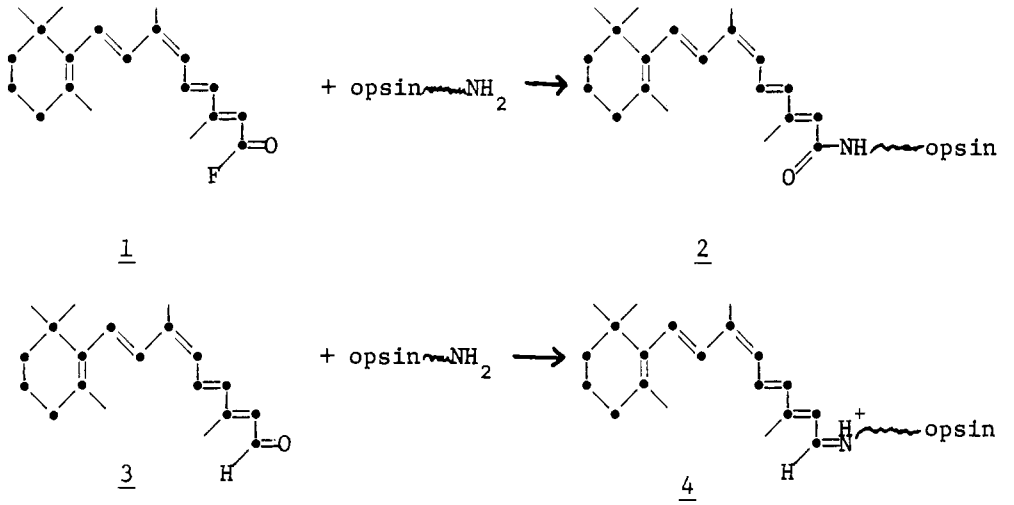
Scheme I



fluoride (**1**) as a specific active site directed inactivator of opsin (Wong & Rando, 1982, 1984). The molecule, a close isostere of 9-*cis*-retinal (**3**), inactivates opsin towards regeneration with 11-*cis*-retinal and forms a new pigment (Scheme I) (Wong & Rando, 1984). The inactivator was presumed to acylate the active site lysine of opsin to form a chromophore (**2**) similar in structure to that found in isorhodopsin (**4**). Since I is assumed to form a peptide with opsin (**2**) rather than a Schiff base, the resultant chromophore could not be protonated at physiological pHs. Hence, a study of the biochemical function of this new pigment should help define the role of the protonated Schiff base in visual transduction. It is the purpose of this paper to further characterize the nature of the interaction of **1** with opsin and define the biochemical and spectral properties of the new pigment.

Materials and Methods

Materials. [γ-^{32}P]GTP was obtained from Amersham; bovine retinas were obtained from Hormel. *N*-butylamine, dicyclohexylcarbodiimide, and 1,4-piperazinediethanesulfonic acid (PIPES) were obtained from Aldrich Chemical Co. Dodecyl maltoside and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) were obtained from Behring Diagnostics. Concanavalin A–Sepharose 4B was obtained from Sigma Chemical Co. Ammonyx LO was provided by Onyx Chemical Co. 9-*cis*-Retinoic acid, 11-*cis*-retinoic acid, and [15-^{14}C]-9-*cis*-retinoic acid were provided by Hoffman-La Roche Inc. of Nutley, NJ.

Preparation of Proteins. Rod outer segments were prepared following the procedure of Papermaster & Dryer (1974). Rhodopsin was purified on a refrigerated Concanavalin A–Sepharose 4B column as modified from van Breugel et al. (1975). Rod outer segments were solubilized in the detergent 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate [10 mM 1,4-piperazinediethanesulfonic acid, pH 6.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 27 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate]. The solubilized rhodopsin was loaded onto the concanavalin A column, and the lipids and other proteins were eluted with the same buffer. The detergent was then exchanged to 6 mM dodecyl maltoside, and the rhodopsin was eluted with 0.5 M methyl α-mannopyranoside in the dodecyl maltoside buffer. The sample was then dialyzed to remove the methyl α-mannopyranoside. The criteria for rhodopsin purity used was the ratio of optical density at 280 nm to that at 500 nm. The ratio for most preparations fell between 1.9 and 2.0. An extract of ROS containing the soluble proteins, including the G protein, was prepared as in Hurley et al. (1981). The samples were frozen at −70 °C in small (1–2-mL) aliquots.

Preparation of Protein/Lipid Vesicles. Detergent interferes with the GTPase assay, so vesicles were made with egg phosphatidylcholine and the desired modified rhodopsins. The lipid in chloroform was dried with N_2 into a conical tube. The protein in detergent buffer was added and the tube vortexed to suspend the lipid. After 1 h at 25 °C, the suspension was dialyzed at 4 °C over 3 days with three exchanges of 1 L of nondetergent buffer. A ratio of about 100 lipid molecules per protein molecule was desired.

Syntheses of all-trans- and 9-cis-Retinoyl Fluorides. The retinoyl fluorides were prepared from retinoic acid as in Wong & Rando (1984). [15-^{14}C]-9-*cis*-Retinoyl fluoride was prepared from [15-^{14}C]-9-*cis*-retinoic acid diluted with cold 9-*cis*-retinoic acid to a specific activity of ca. 1000 dpm/nmol.

Syntheses of all-trans- and 9-cis-N-Butylretinamides. One equivalent of the appropriate retinoic acid was dissolved in dry methylene chloride along with 1.1 equiv of dry *n*-butylamine and 1 equiv of dicyclohexylcarbodiimide. The solution was stirred in the dark under nitrogen for 24 h at room temperature. Thin-layer chromatography on silica (0.2-mm silica gel 60 sheets from EM reagents) with chloroform as the eluant showed the formation of single new spot running with an R_f of approximately 0.5. The retinoic acid remained at the origin. The *N*-butyl amides were purified by silica chromatography again with chloroform as the eluant. The NMR, ultraviolet, and infrared spectra of these compounds were wholly in accord with the assigned structures.

Preparation of all-trans- and 9-cis-Retinoylopsins. The *all-trans*- and 9-*cis*-retinoylopsins were prepared essentially as in Wong & Rando (1984). Rhodopsin in dodecyl maltoside was bleached for 30 min with intense light filtered through running water and a yellow filter (Corning 3-68, cutoff of 540 nm). The appropriate retinoyl fluoride was added and the mixture agitated on a Nutator for 30 min at 25 °C. An aliquot was then removed and checked for uninhibited opsin by incubation with 11-*cis*-retinal. The inhibition reaction was quenched by the addition of 1 N NH_2OH to a final concentration of 20 mM. Excess retinoids were removed by affinity chromatography on a small concanavalin A column.

Permethylated Rhodopsin. In some cases rhodopsin was methylated before bleaching and regenerating with retinoyl fluoride. The permethylation of rhodopsin in this fashion

eliminates the possibility of nonspecific reaction of the retinoyl fluorides with the 10 non-active-site lysine residues. The methylation procedure (10–15 μM rhodopsin, 4 mM formaldehyde, and 20 mM $NaCNBH_3$, 2 h at room temperature) followed Jentoft & Dearborn (1979). The excess reagents were removed by gel filtration on Sephadex G-50. Approximately 20 methyl groups were taken up by this method as determined with [^{14}C]formaldehyde (C. Longstaff, unpublished results). Rhodopsin methylated in this fashion does not react with sodium trinitrobenzenesulfonic acid (C. Longstaff, unpublished results). The reaction of the lysine residues of rhodopsin with sodium trinitrobenzenesulfonic acid gives rise to a change of absorption that can be monitored at 420 nm (DeGrip et al., 1973). These results suggest the non-active-site lysines have been dimethylated. However, permethylated rhodopsin can be bleached and regenerated with 11-*cis*-retinal. Furthermore, upon irradiation, the pigment will activate the G protein (R. Calhoon, unpublished results). Complete details of the preparation and properties of permethylated rhodopsin will be published separately.

Ultraviolet Spectra of Modified Opsins and Model Compounds. All ultraviolet spectra were taken on a Perkin-Elmer 552A UV/vis spectrophotometer between 600 and 250 nm in quartz semi-microcuvettes.

Assay of the G Protein GTPase Activity. The assay of GTPase activity was a modification from Thacher (1978). The assay involved measuring the release of ^{32}P from [γ-^{32}P]GTP with activated charcoal to separate the unhydrolyzed GTP from the labeled free phosphate. The 0.5-mL assay mixture included 8 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8), 4 mM $MgCl_2$, 0.5 μM GTP, and the enzyme and rhodopsin samples.

RESULTS

Stoichiometry of [15-^{14}C]-9-cis-Retinoyl Fluoride Binding to Bovine Opsin. Previous studies had strongly suggested a specific reaction between bovine opsin and **1** (Wong & Rando, 1984). Since bovine opsin contains 11 lysine residues, only one of which, lysine-296, engages in Schiff-base formation with 11-*cis*- or 9-*cis*-retinal, it was important to determine the stoichiometry of the irreversible reaction between **1** and the protein. To these ends, [^{14}C]-9-*cis*-retinoyl fluoride was prepared, and the stoichiometry of its irreversible binding to bovine opsin was measured. Since **1** could in principle react with all 11 lysine residues, it was important to differentiate between specific (meaning active site directed) and nonspecific reactions. [^{14}C]-9-*cis*-Retinoyl fluoride was incubated with (1) opsin, in dodecyl maltoside, a detergent that allows for regeneration with 11-*cis*-retinal, (2) non-active-site lysine N-dimethylated opsin derived from N-dimethylated rhodopsin [the latter modified protein was prepared by completely methylated rhodopsin with formaldehyde and sodium cyanoborohydride (see Materials and Methods)], and (3) heat-denatured opsin, and (4) opsin in a detergent (Ammonyx LO) that does not allow for regeneration. As seen in Table I, the stoichiometry of ^{14}C binding to freshly prepared opsin is approximately one, strongly suggesting an active site directed mechanism. The calculation for the stoichiometry of binding was done in two ways. In column 2 it was assumed that all of the purified protein was susceptible to derivatization with [^{14}C]-**1**. As rhodopsin of A_{280}/A_{500} = 1.9–2.0 was used in the experiments, a certain proportion of this material (~20%) did not regenerate with 11-*cis*-retinal after bleaching. In column 3 it was assumed that only that proportion of the opsin able to regenerate with 11-*cis*-retinal was susceptible to derivatization with [^{14}C]-**1**. Since, as will be discussed shortly, non-

Table I: Stoichiometry of 9-*cis*-Retinoyl Fluoride Binding to Opsin

protein modified	(mol of retinoid incorporated)/(mol of total protein) × % inhibition[a]	(mol of retinoid incorporated)/(mol of regenerable opsin) × % inhibition[b]
opsin in dodecyl maltoside	1.03	1.39
permethylated opsin	0.69	0.89
opsin in dodecyl maltoside boiled for 10 s[c]	0.27	0.36
opsin in Ammonyx LO[d]	1.13	1.4

[a] Percent inhibition is calculated from the regeneration with 11-*cis*-retinal before and after treatment with the retinoyl fluoride. [b] Regenerable opsin is calculated from the regeneration of freshly bleached opsin with 11-*cis*-retinal prior to further treatment. [c] A 1.3-mL aliquot of the rhodopsin sample in a small glass test tube was shaken in a boiling water bath until the red color was gone. An aliquot of this sample showed no regeneration upon addition of 11-*cis*-retinal. [d] Rhodopsin in dodecyl maltoside (1.5 mL) was dialyzed overnight against 1 L of the same buffer substituting 2% Ammonyx LO. An aliquot of this sample showed no regeneration upon addition of 11-*cis*-retinal.

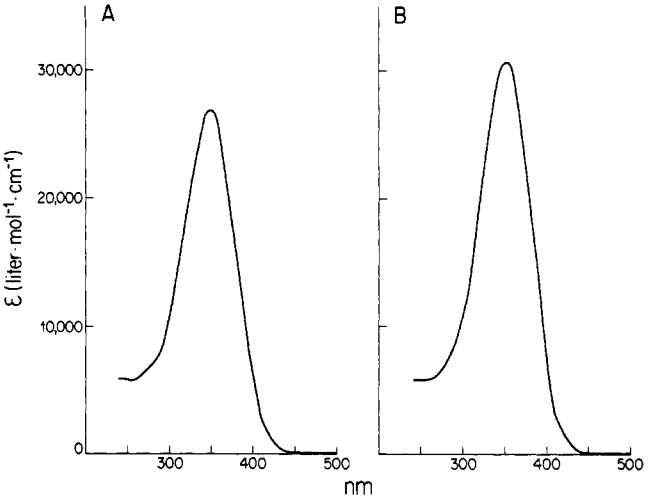


FIGURE 1: Ultraviolet spectra of the *N*-butylretinamide: (A) 66.2 μM *N*-butyl-9-*cis*-retinamide in 2-propanol; (B) 61.6 μM *N*-butyl-*all-trans*-retinamide in 2-propanol. Extinction coefficients are based upon the measured weight of the samples.

regenerable opsin can still be derivatized with **1**, it is likely that the true stoichiometry is closer to 1.03 than to 1.39. Somewhat less than stoichiometric binding was found with permethylated opsin, perhaps because some denaturation accompanies the methylation process. Heat-denatured opsin does not bind substantial [^{14}C]-**1**, as would be predicted, since those structural features of opsin that make the active site lysine readily reactive toward vitamin A like carbonyl-containing compounds are destroyed by heating. Unanticipated, however, are the results with Ammonyx LO. Although rhodopsin is stable in this detergent, it will not regenerate with 11-*cis*- or 9-*cis*-retinal after bleaching (DeGrip, 1982). Nevertheless, the active site lysine still appears to be reactive toward vitamin A like aldehydes as revealed by its stoichiometric reactions with [^{14}C]-9-*cis*-retinoyl fluoride.

Spectra of Model Compounds and Bleaching of Retinoylopsin. From the studies described above it seemed highly likely that the active site lysine-296 was acylated by **1**. It was then of interest to compare the ultraviolet spectra of retinoylopsin with model amides and study the photochemistry of the former. In Figure 1 the ultraviolet spectra in 2-propanol of the *N*-butyl amides of 9-*cis*- and *all-trans*-retinoic acids are given. In Figure 2 the ultraviolet spectra of 9-*cis*-retinoylopsin and

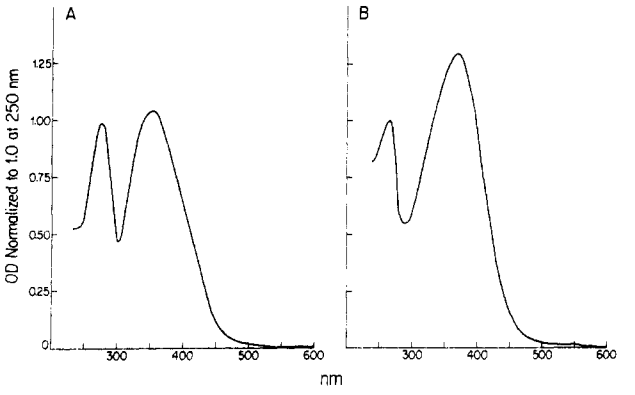


FIGURE 2: Ultraviolet spectra of retinoylopsin: (A) 12 μM 9-*cis*-retinoylopsin in dodecyl maltose buffer; (B) 1.9 μM *all-trans*-retinoylopsin in dodecyl maltoside buffer. The optical density scales are normalized so that the OD at 280 nm is 1, so that the retinoyl absorption peaks may be compared. Concentrations are based upon rhodopsin concentration of the starting material as measured at 500 nm.

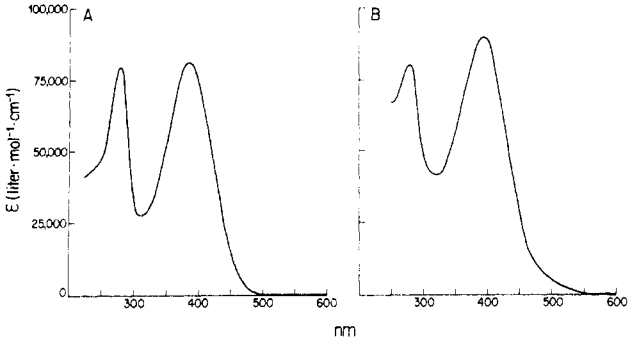


FIGURE 3: Ultraviolet spectra of permethylated retinoylopsin. (A) Rhodopsin was N-dimethylated, bleached, and reacted with 9-*cis*-retinoyl fluoride as described in Table II. Extinction coefficients are based upon the extinction coefficient at 280 nm for that sample as determined from the OD of the original rhodopsin measured at 500 nm. (B) *all-trans*-Retinoylopsin produced in the same manner. The values of extinction given in Table II are calculated from this figure.

all-trans-retinoylopsin, generated by the reaction of **1** and *all-trans*-retinoyl fluoride with opsin, are presented. Previous spectra on these adducts suffered from excessive light scattering and, hence, precise λ_{max}'s could not be obtained (Wong & Rando, 1984). In order to be sure that none of the chromophore in Figure 2 is due to nonspecific acylation of opsin by **1**, the acylation reaction was repeated with opsin prepared from permethylated rhodopsin. Permethylated opsin was used here to ensure that all of the ultraviolet absorption observed was due only to active site derivatized material. The resulting spectrum of the permethylated 9-*cis*-retinoylopsin is shown in Figure 3A and that of permethylated *all-trans*-retinoylopsin in Figures 3B. Figures 2 and 3A are quite similar, which is consistent with the known stoichiometry of the reaction of the retinoyl fluorides with opsin. The λ_{max}'s of the two pigments are similar as are their ϵ's. The spectroscopic data for the model compounds and the retinoyl opsins are listed in Table II.

On the basis of the expected mode of interaction of **1** with opsin, the resulting retinoylopsin would not be expected to bleach, although the λ_{max}'s and ϵ's should change as the result of forming a new steady-state mixture upon illumination. Photolysis of permethylated 9-*cis*- and *all-trans*-retinoylopsins gave rise to the results shown in panels A and B of Figure 4, respectively. Approximately the same steady-state spectra were obtained starting from either the permethylated 9-*cis*-

Table II: Spectroscopic Data on Retinoylopsins and *N*-Butylretinamides

	λ_{max}	$\epsilon\lambda_{max}$
N-butylretinamide[a]		
9-cis	347	26 800
all-trans	351	30 300
retinoylopsin[b]		
9-cis	373	
all-trans	380	
methylated retinoylopsin[c]		
9-cis	386	80 800
all-trans	393	90 100

[a] Spectra were taken in 2-propanol. [b] Samples were prepared as under Materials and Methods. No calculation of ϵ was made for these samples due to the possibility of nonspecific reaction of the retinoyl fluorides with non-active-site lysine residues. [c] A 5.0-mL aliquot of 12.5 μM rhodopsin (62.5 nmol) was methylated as described under Materials and Methods. Of this, 28 nmol was recovered from the gel filtration column (3 mL of 9.34 μM). A total of 2.8 mL of the sample was bleached, and the sample divided into two equal aliquots, giving 13 nmol of opsin in each sample. A total of 25 μL of 5.1 μM (128 nmol) 9-*cis*-retinoyl fluoride in 2-propanol was added to one sample; 25 μL of 10.3 mM (258 nmol) *all-trans*-retinoyl fluoride was added to the other. Both samples were cleaned up as described under Materials and Methods. Concentration of the final product was based upon the ϵ at 280 of the particular sample as determined from the OD at 500 of the starting material. Extinction values at the λ_{max} were determined from the spectra of Figure 3.

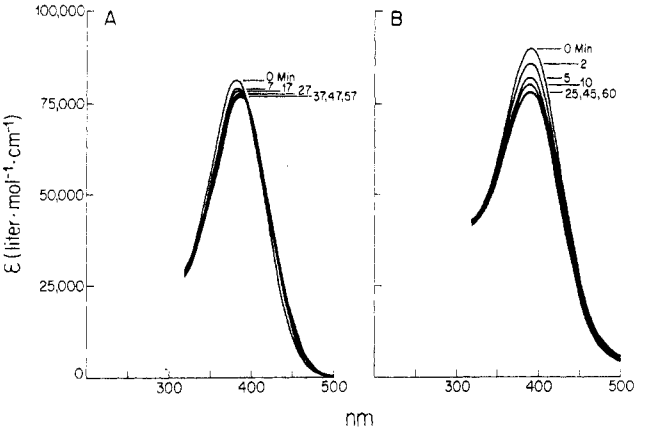


FIGURE 4: Effect of irradiation upon the absorption spectra of retinoylopsins: (A) 9-*cis*-retinoylopsin; (B) *all-trans*-retinoylopsin. The retinoylopsin was irradiated until three spectra superimposed, indicating a photoequilibrium state possibly containing 11-cis, 9-cis, and all-trans isomers. The spectrum labeled 0 (zero) is the spectrum of unirradiated retinoylopsin and is the same as that in Figure 3. The other spectra are labeled by the cumulative duration of illumination in minutes. The cuvette was illuminated from above with a 100-W incandescent bulb at a distance of 20 cm to the center of the buffer in the cuvette. The light intensity at that distance was 9000 lumens.

retinoylopsin or the permethylated *all-trans*-retinoylopsins. As expected, bleaching did not occur, but a new photostationary state was reached. The final λ_{max} was 390 nm, and the final ϵ was approximately 77 000.

Interaction of Photolyzed 9-cis-Retinoylopsin with the G Protein. Given that 9-*cis*-retinoylopsin undergoes a photochemical isomerization process, it was of interest to determine if this isomerization would lead to activation of G protein, the only biochemical entity whose activity has been shown to be directly modified by rhodopsin photolysis (Wheeler & Bitensky, 1977). The activity of the G protein was measured by following its GTPase activity as shown in Table III. Photolysis of 9-*cis*-retinoylopsin in the presence of the G protein did not lead to the activation of the latter within experimental error. Interestingly, *all-trans*-retinoylopsin did not activate the G protein in the light or in the dark.

Table III: Effect of Retinoylopsin on G Protein GTPase Activity[a]

opsin derivative	GTPase activity[b] in dark	GTPase activity[b] in light	% GTPase activation[c]
rhodopsin	9.0	45.7	100.0
opsin	7.7	8.7	2.7
9-*cis*-retinoylopsin	7.3	8.8	4.1
all-trans-retinoylopsin	7.5	8.4	2.4

[a] Each condition was repeated 6 times. Opsin derivatives are in egg phosphatidylcholine vesicles (see Materials and Methods). [b] GTPase activity is given in picomoles of GTP consumed per minute. Dark means dim red light. Light means room lights. [c] GTPase activation is given relative to the full activation by rhodopsin, calculated as the difference in light and dark activities divided by the same difference in the presence of rhodopsin.

Discussion

It had previously been shown that 9-*cis*-retinoyl fluoride (**1**) is an active site directed, irreversible inactivator of opsin (Wong & Rando, 1984). The molecule, which is isosteric with 9-*cis*-retinal, probably interacts with opsin by a mechanism similar to that found in Schiff-base formation but, because of the substitution of a fluorine atom for a hydrogen atom, forms a peptide with the active site lysine rather than a reversible Schiff base (Scheme I). A specific mode of reaction between **1** and opsin requires that only one out of a total of 11 lysine residues be acylated by the analogue (Hargrave et al., 1983). The N terminus of rhodopsin is already acylated. Amino acid residues, other than lysine, that might be sites of reaction between **1** and opsin can be ruled out on the basis of previous data and the fact that the retinoylopsin chromophore is quite stable to hydroxylamine treatment (Wong & Rando, 1982). Esters or thio esters would be cleaved under these conditions. Using ^{14}C-labeled **1**, it could be shown that the stoichiometry of binding of **1** to opsin is approximately one molecule of **1** per opsin molecule. If none of the nonregenerable protein could bind **1**, then the stoichiometry is 1.39. On the other hand, if the nonregenerable protein could bind **1**, the ratio becomes 1.03. Results with the detergent Ammonyx LO suggests the latter to be the case. Thus, the stoichiometry of 1.39 must be taken as an upper limit and would require that the remaining "denatured" opsin that cannot regenerate with 11-*cis*-retinal also cannot react with **1**. Opsin, containing its non-active-site lysines blocked by dimethylation, incorporated up to 0.9 molecule of **1** per opsin molecule. However, here some of the active site lysines were probably also methylated during the methylation procedure, which would decrease the extent of incorporation of **1**. Heat-denatured opsin only took up approximately 0.3 equiv of **1**, which is consistent with the idea that the three-dimensional structure of the protein must be maintained in order for the active site lysine to engage in a reaction with **1**. Since **1** is an acyl fluoride, it could, in principle, react with all of the lysines of opsin and probably would, at high enough concentrations. Nevertheless, at relatively low concentrations of **1** (8–10-fold **1** per opsin) a selective active site directed reactions certainly occurs. It should be noted that in order to achieve full regeneration with 11-*cis*-retinal within the same time scale as in retinoylopsin formation, roughly the same fold excess of the chromophore is required. Slow hydrolysis of **1** precludes a long incubation period with a smaller excess. The basis for the selectivity of labeling largely resides in the close structural similarity between **1** and 9-*cis*-retinal. Thus, **1** can engage in the acid–base-catalyzed reactions, which occur at the active site of opsin and can readily react with an active site moiety (almost certainly the lysine-296). In addition, the experiments were performed at pH 6.5, which is about 3 orders of magnitude lower than the normal pK_A of ϵ-amino groups of lysine. This also favors reaction at the active site lysine, which presumably must not be protonated at pH 6.5 or else it could not engage in Schiff-base formation.

Of the many detergents available for solubilization of rhodopsin, it is clear that only a few, such as dodecyl maltoside and digitonin, will allow for regeneration with 11-*cis*- or 9-*cis*-retinal after bleaching (De Grip, 1982). Although rhodopsin is stable in many detergents, including the often used Ammonyx LO, regeneration is not possible, meaning that the opsin is subtly and, in some cases, reversibly denatured (De Grip, 1982). In this context, the fact that [^{14}C]-9-*cis*-retinoyl fluoride titrates opsin solubilized with Ammonyx LO is quite interesting and was unanticipated. This means that the active site lysine is still capable of selectively engaging in nucleophilic attack on vitamin A like carbonyl-containing compounds but that the conformational changes in the opsin molecule required in forming rhodopsin are no longer possible in detergents such as Ammonyx LO. This suggests at least a two conformational step model for rhodopsin regeneration, in which Schiff-base formation between opsin and retinal precedes a second folding step whereby the vitamin A backbone is sequestered by the active site of opsin to form rhodopsin. The notion that rhodopsin is a chelatelike complex, with the vitamin A moiety deeply buried in the protein, has been recognized for many years (Kropf & Hubbard, 1958). The minimal two-step nature of the regeneration process is revealed by the use of **1** because the interaction between the lysine and the acyl fluoride moiety is irreversible in nature and does not require an additional protein folding step to secure it. When the interactive partners are the active site lysine and a retinal capable of forming a pigment, further protein folding is required to stabilize the Schiff base to rapid hydrolysis. However, whatever the initial conformational state of the opsin is, it must still recognize the vitamin A derivative to which it is to be bound. It had previously been shown that 13-*cis*-retinoyl fluoride will not irreversibly bind to opsin (Wong & Rando, 1984), which is in line with the fact that 13-*cis*-retinal will not form a pigment with opsin (Hubbard & Wald, 1952). In addition, *all-trans*-retinoyl fluoride is approximately 4-fold less potent as an inactivator of opsin than is **1** (Wong & Rando, 1982, 1984).

Although definitive active site mapping studies on 9-*cis*-retinoylopsin have yet to be performed, it is fairly certain that the active site lysine has been labeled. As mentioned above, 13-*cis*-retinoyl fluoride does not inactivate opsin, and *all-trans*-retinoyl fluoride does so but much less potently than **1**. The bond formed between **1** and opsin is stable to boiling and hydroxylamine treatment, which eliminates the esters, thio esters and other labile linkages. Furthermore, the ultraviolet spectra of the retinoylopsins are consistent with the idea that the active site lysine residue is acylated. Permethylation of the non-active-site lysine residues of rhodopsin with formaldehyde and sodium cyanoborohydride, followed by bleaching and reaction with **1**, gives rise to an ultraviolet spectrum similar to that found in the reaction of **1** with unmodified opsin. The slight differences in the λ_{max}'s could easily be due to small conformational shifts in the highly methylated protein. The λ_{max}'s of the retinoylopsins are red-shifted approximately 25 nm compared to model amides in 2-propanol. This small shift could easily be due to hydrophobic interactions between the protein and chromophore. It is known that a hydrophobic environment can cause powerful bathochromic shifts on retinoids (Tabushi et al., 1979). The effect of protein binding on the ϵ of the retinamides is rather more remarkable and unanticipated. For example, in the case of 9-*cis*-retinoylopsin the measured ϵ was 80 760 whereas that for *N*-butyl-9-*cis*-

retinamide was 26 800. This increase, at least qualitatively, finds analogy in the enhanced absorbance of the protonated Schiff base of 11-*cis*-retinal in rhodopsin over that of model compounds.

As expected from what was stated above, the irradiation of permethylated 9-*cis*-retinoylopsin or permethylated *all-trans*-retinoylopsin did not lead to their bleaching. Both pigments reached approximately the same photostationary state, suggesting that they contain the same isomeric composition. The λ_{max} for the 9-cis shifted from 375 to 377 nm and that for the all-trans from 380 to 378 nm. The overall ϵ of the mixture decreased slightly when starting from the permethylated 9-*cis*-retinoylopsin, and decreased to a greater extent in the case of the *all-trans*-retinoylopsin. This finding suggests that there is substantial 11-*cis*-retinoylopsin in the steady-state mixture. Starting from 9-*cis*-retinoylopsin, only a small decrease in ϵ would be observed, since 9-cis possesses an ϵ intermediate between its all-trans and 11-cis congeners. In the case of the *N*-butylretinamides as is the case with retinaldehydes, the 11-cis isomers show lower ϵ's at their λ_{max}'s than do their all-trans or 9-cis congeners. The ϵ for the *N*-butyl amide of 11-*cis*-retinoic acid is 19 200 at its λ_{max} compared to 26 800 and 30 300 for the 9-cis and all-trans compounds, respectively. The *all-trans*-retinoids, having the largest ϵ's, would of course suffer the greatest decrease upon photolysis.

It is well-known that a significant fraction of the energy absorbed by rhodopsin is converted into the potential energy of the protein (Cooper, 1979). Light of 500 nm imparts 57 kcal/mol energy into rhodopsin, and of this energy 35 kcal/mol is stored in bathorhodopsin and 22 kcal is lost as heat. Given that 11-*cis*-retinal is 4.1 kcal/mol less stable than the all-trans form, the fraction of the photochemical energy stored by bathorhodopsin, assuming that isomerization about the C_{11}–C_{12} bond has already occurred, is approximately 66%. A possible mechanism presented for energy storage involves the separation of the positively charged Schiff-base head group from the negatively charged fixed amino acid gegenion as a consequence of the isomerization reaction (Honig et al., 1979). This transduction of light energy into the chemical potential energy of the protein is likely to be important in signal transduction, because it allows for the spontaneous formation of metarhodopsin II and its subsequent relaxation to opsin and *all-trans*-retinal. Given the presumed primary importance of the protonated Schiff-base head group in this mechanism, it was of interest to study a vitamin A conjugate that could not bear a positive charge at physiological pHs. As mentioned before, 9-*cis*-retinoylopsin should be sterically similar to isorhodopsin save for the fact that the former does not possess a positively charged Schiff base. On the basis of this, it would be predicted that although 9-*cis*-retinoylopsin could undergo photochemical isomerization, it should not be able to form a bathorhodopsin-like intermediate due to its inability to store sufficient light energy, even though the pigment absorbs light far to the blue of rhodopsin. Indeed, photolysis of 9-*cis*-retinoylopsin in the presence of the G protein did not measurably activate this protein toward the hydrolysis of GTP. This observation is consistent with the idea that charge separation and energy conversion contribute to the functioning of rhodopsin.

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Registry No. **1**, 87760-33-6; 9-*cis*-retinoic acid, 5300-03-8; *all-trans*-retinoic acid, 302-79-4; *N*-butyl-9-*cis*-retinamide, 87760-35-8; *N*-butyl-*all-trans*-retinamide, 33631-44-6; *n*-butylamine, 109-73-9; *all-trans*-retinoyl fluoride, 83802-77-1; lysine, 56-87-1.

REFERENCES

Calhoon, R. D., Ebrey, T. G., & Tsuda, M. (1981) *Biophys. J. 33*, 290a.

Cooper, A. (1979) *Nature (London) 282*, 531–533.

Cooper, A., & Converse, C. A. (1976) *Biochemistry 15*, 2970–2978.

De Grip, W. J. (1982) *Methods Enzymol. 81*, 256–265.

De Grip, W. J., Daemen, F. J. M., & Bonting, S. L. (1973) *Biochim. Biophys. Acta 323*, 125–142.

Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Rao, J. K. M., & Argos, P. (1983) *Biophys. Struct. Mech. 9*, 235–244.

Honig, B., Greenberg, A. D., Dinur, U., & Ebrey, T. G. (1976) *Biochemistry 15*, 4593–4599.

Honig, B., Ebrey, T., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.

Hubbard, R., & Wald, G. (1952) *J. Gen. Physiol. 36*, 264–307.

Hubbard, R., & Kropf, A. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 130–139.

Hubbell, W. L., & Bownds, M. D. (1979) *Annu. Rev. Neurosci. 2*, 17–34.

Hurley, J. B., Barry, B., & Ebrey, T. G. (1981) *Biochim. Biophys. Acta 675*, 359–365.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4359–4365.

Kropf, A., & Hubbard, R. (1958) *Ann. N.Y. Acad. Sci. 74*, 266–280.

Ovchinnikov, Y. A. (1982) *FEBS Lett. 148*, 179–191.

Papermaster, D. S., & Dreyer, W. J. (1974) *Biochemistry 13*, 2438–2444.

Parkes, J. H., Liebman, P. H., & Pugh, E. N. (1979) *Invest. Ophthalmol. Visual Sci.*, 22 (ARVO Abstr.).

Shinozawa, T., Sen, I., Wheeler, G., & Bitensky, M. (1979) *J. Supramol. Struct. 10*, 185–190.

Tabushi, I., Kuroda, Y., & Shimosawa, K. (1979) *J. Am. Chem. Soc. 101*, 4759–4760.

Thacher, S. M. (1978) *Biochemistry 17*, 3005–3011.

van Breugel, P. J. G. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Exp. Eye Res. 24*, 581–584.

Wald, G. (1968) *Science (Washington, D.C.) 162*, 230–239.

Wheeler, G. L., & Bitensky, M. W. (1977) *Biochemistry 16*, 4238–4242.

Wong, C. G., & Rando, R. R. (1982) *J. Am. Chem. Soc. 104*, 7374–7375.

Wong, C. G., & Rando, R. R. (1984) *Biochemistry 23*, 20–27.