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all-trans-Retinoids and Dihydroretinoids as Probes of the Role of Chromophore Structure in Rhodopsin Activation†

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ABSTRACT: The absorption of a photon of light by rhodopsin results in the cis to trans isomerization of the 11-*cis*-retinal Schiff base chromophore. In the studies reported here, an attempt is made to determine the mechanism of the energization of rhodopsin as it relates to the chemistry of the isomerization process and the geometrical state of the chromophore. Studies were performed with vitamin A analogues to probe this mechanism. Both 11-*cis*-7,8-dihydroretinal and 9-*cis*-7,8-dihydroretinal form bleachable pigments when combined with opsin. Photolysis of these pigments in the presence of G-protein results in the activation of the latter as revealed by its GTPase activity. Phosphodiesterase is also activated when it is included in the incubation. Therefore, the possibility that rhodopsin is energized by mechanisms involving photochemically induced charge transfer from the protonated Schiff base to the β-ionone ring can be discarded. Further studies were conducted with *all-trans*-vitamin A derivatives to determine if these compounds can form the GTPase-activating state R*, a situation that is possible, in principle, by microscopic reversibility. Neither *all-trans*-retinal nor its oxime, when incubated with bovine opsin in the dark, caused activation of the GTPase, requiring at least a 5 kcal/mol energy gap between them. Furthermore, stoichiometric adducts of *all-trans*-retinoids and opsin were also unable to mediate activation of the GTPase. Since both *all-trans*-15,16-dihydroretinylopsin and *all-trans*-retinoylopsin possess an *all-trans*-retinoid permanently adducted to opsin, it can be concluded that the *all-trans*-retinoid chromophore–opsin linkage may be necessary but not sufficient to achieve activation of the visual pigment.

The absorption of a photon of light by rhodopsin results in the isomerization of the 11-*cis*-retinal protonated Schiff base chromophore to its all-trans congener (Hubbard & Kropf, 1958). Concomitant with this isomerization, conformational changes in the protein take place resulting eventually in the hydrolysis of the protonated Schiff base of *all-trans*-retinal generating the aldehyde and opsin (Wald, 1968). On the way to opsin formation, one of the rhodopsin conformers, R*,[1] probably identical with or arising from the spectroscopically defined state metarhodopsin II (Parkes et al., 1979; Calhoon et al., 1981), causes the exchange of GTP for GDP in the G-protein (Fung & Stryer, 1980), which in turn activates a cyclic GMP phosphodiesterase (Fung et al., 1981). The G-protein eventually hydrolyzes the GTP to GDP, returning the G-protein to an inactive state with respect to phosphodiesterase activation. Since enzymatic activity of GTP hydrolysis is the activity measured in the work reported here, we refer to the enzyme as "GTPase" rather than emphasizing the protein's role as mediator of activation of the phosphodiesterase, as implied by the term "G-protein". The hydrolysis of cGMP is the only metabolically significant event known to

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[1] Abbreviations: CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Meta, metarhodopsin; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); Rh, rhodopsin; ROS, rod outer segments; R*, GTPase-activating state of rhodopsin; Tris, tris(hydroxymethyl)aminomethane.

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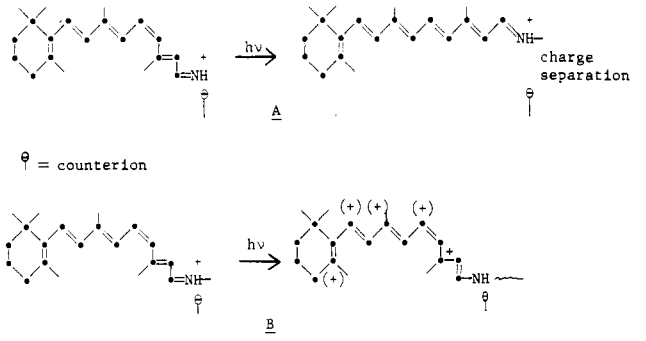
Scheme I



be directly linked to rhodopsin photolysis. Of great interest is the fact that similar, or the same, biochemical sequelae also follow the interaction of certain neurotransmitters and hormones with their receptors (Shinozawa et al., 1979; Gilman, 1984). In these latter cases, the binding of the ligand to its membrane-bound receptor triggers conformational changes in the receptor required to catalyze the GTP for GDP exchange in the informational transducing protein. Thus, the presumed biochemical mechanism of rhodopsin activation appears to be quite general in nature and is shared by certain drugs, neurotransmitters, and hormones acting on their receptors. The major difference between rhodopsin and these other receptors is that, in the case of rhodopsin, the ligand is incorporated into the receptor and triggers conformational changes in the protein subsequent to the absorption of light and double-bond isomerization of the chromophore.

An important aspect of the photochemical activation of rhodopsin by light, and by analogy of drug–receptor interactions, is the energetics and mechanism of the process. The conformational states of rhodopsin initiated by light absorption would be expected to be higher in energy than those of opsin plus *all-trans*-retinal in order for complete bleaching to occur. Indeed, it has been shown that bathorhodopsin, the first distinct intermediate formed after rhodopsin photolysis, is 35 kcal/mol higher in energy than is rhodopsin (Cooper, 1979a). From this energetic maximum, succeeding conformers, including metarhodopsin II, form and decay spontaneously, thus ensuring that the bleaching process spontaneously goes to completion.

Although many different mechanisms may be considered for the energization step, the most attractive ones mechanistically appear to involve some form of photochemically induced charge separation in a milieu of low microscopic dielectric constant. Energization by charge separation can basically take two forms here. In one model the actual isomerization of the double bond at the bathorhodopsin state could separate the positively charged Schiff base head group from its protein-bound counterion (Scheme IA) (Honig et al., 1979). A second possibility involves photochemically induced intramolecular charge redistribution whereby the positive charge is moved away from its counterion to the β-ionone ring (Scheme IB). In this paper, it is shown that the obligate transfer of charge from the protonated Schiff base to the β-ionone ring cannot be important in the transduction process (Scheme IB). It had previously been shown that simple photochemically induced double-bond isomerization in and of itself, without the movement of charge, is also not sufficient to activate rhodopsin (Calhoon & Rando, 1985). In further consideration of the energetics of rhodopsin activation, the possible activation of opsin by *all-trans*-retinoids was studied. Studies of this type can yield information on the energy difference between *all-trans*-retinal plus opsin and R*, the conformer that activates the GTPase. It is shown here that *all-trans*-retinal and derivatives thereof cannot drive opsin into R*, requiring that there is at least a 3.1 kmol/mol difference between R* and opsin plus *all-trans*-retinal. The mechanistic implications of these findings are discussed.

Materials and Methods

Materials. [γ-^{32}P]GTP, [^{3}H]cGMP, and [^{14}C]5′GMP were obtained from Amersham Inc. Bovine retinas were obtained from Hormel Inc. PIPES was obtained from Aldrich Chemical Co. Dodecyl maltoside and CHAPSO were obtained from Behring Diagnostics. Pure egg phosphatidylcholine was obtained from Avanti Inc. Concanavalin A–Sepharose 4B and methyl α-mannopyranoside were obtained from Sigma Chemical Co. Ammonyx LO was provided by Onyx Chemical Co. 11-*cis*- and 9-*cis*-7,8-dihydroretinal were generous gifts of Professor K. Nakanishi of Columbia University (Arnaboldi et al., 1979).

Preparation of Proteins. All procedures involving rhodopsin or modified rhodopsins were carried out in the absence of light or under dim red light. All samples were kept wrapped in foil when not being handled.

Rod outer segments were prepared by 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. (1977). Rod outer segments were solubilized in the detergent CHAPSO (27 mM) in 10 mM PIPES (pH 6.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. 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. The criterion 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 GTPase (G-protein) and phosphodiesterase, 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. Rhodopsin/egg phosphatidylcholine vesicles were prepared for use with the GTPase and phosphodiesterase assays. The concentration and volume of the rhodopsins varied with the different preparations and ranged from 10 to 50 μM and from 1 to 5 mL. The lipid in chloroform was dried with N_2 in 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 10 mM Tris, pH 8. A ratio of about 100 lipid molecules per protein molecule was obtained.

The vesicles obtained in this manner contained functional or potentially functional protein as shown by the following criteria. An electron micrograph of rhodopsin vesicles appeared characteristic of uniform unilamellar vesicles. All samples prepared in this manner retained their characteristic λ_{max} and OD when allowance was made for vesicle-induced light scattering or when the samples were resolubilized with Ammonyx LO, implying the absence of denaturation or precipitation of the protein. The least stable of the preparations, opsin with no retinoid added, was competent to activate GTPase when regenerated.

Preparation of Chromophore-Modified Rhodopsin. The pigments were prepared essentially as in Wong & Rando (1984). Rhodopsin in dodecyl maltoside was bleached in the presence of 20 mM NH_2OH for 30–60 min with intense light filtered through running water and a yellow filter (Corning

3-68, cutoff of 540 nm). Excess NH_2OH was removed via gel filtration on G-50 Sephadex. The appropriate retinoid was added and the mixture agitated on a Nutator for 30–60 min at 25 °C. An aliquot was then removed and checked for uninhibited opsin by incubation with 11-*cis*-retinal. The reaction was quenched by the addition of 1 M NH_2OH to a final concentration of 20 mM. Excess retinoids were removed by affinity chromatography on a small concanavalin A column. The *all-trans*-retinal (oxime) plus opsin samples were made by the addition of equimolar amounts of chromophore to opsin in lipid vesicles.

Formation of 15,16-Dihydrorhodopsin by Reduction of the Schiff Base Linkage of Rhodopsin. *all-trans*-15,16-Dihydrorhodopsin (*all-trans*-retinylopsin) was prepared by irradiation of rhodopsin in the presence of 100 mM $NaBH_4$. A yellow filter (Corning 3-68, cutoff of 540 nm) was used to minimize photoisomerization of the all-trans product back to cis. Excess $NaBH_4$ was removed by dialysis at 4 °C.

11-*cis*-15,16-Dihydrorhodopsin was prepared by reaction with $NaCNBH_3$ in the dark. 0.75 M $NaCNBH_3$ was incubated with 26 μM rhodopsin for 3 h at 27 °C. The pigment was then dialyzed at 4 °C.

As a control, rhodopsin was reacted with 100 mM $NaBH_4$ in the dark and treated otherwise as with the $NaCNBH_3$ preparation. The spectrum and GTPase activation of the resulting pigment was unchanged.

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 semimicrocuvettes.

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 by using activated charcoal to separate the unhydrolyzed GTP from the labeled free phosphate. The 0.5-mL assay mixture included 80 mM Tris (pH 8), 4 mM $MgCl_2$, 0.5 μM GTP, and the enzyme and rhodopsin samples.

Assay of cGMP Phosphodiesterase Activity. The ability of the modified rhodopsins to activate phosphodiesterase was measured by assaying a mixture of the modified rhodopsin in lipid vesicles and a crude rod outer segment preparation. This approach was intended to follow that of Yoshizawa & coworkers (Fukada et al., 1981, 1982; Yoshizawa et al., 1983), who reported phosphodiesterase activation with some of these pigments. Rod outer segments were prepared by a single flotation of 50 retinae in 34% sucrose. The supernatant was diluted 3-fold with 10 mM Tris, pH 8, and pelleted. The pellet was brought up in 50 mL of 10 mM Tris and frozen in that form. This preparation was used as the source of phosphodiesterase.

The phosphodiesterase assay was a modification from Thompson et al. (1974). Following incubation, 5'GMP production was measured by hydrolysis with snake venom (*Ophiophagus hannah*) and followed by separation of guanosine from cGMP on ion-exchange columns. The reaction mixture of 0.5 mL contained 80 mM Tris (pH 8), 4 mM $MgCl_2$, 100 μM GTP, 1 mM cGMP, 25 μL of the ROS, and 0.75 μM pigment in lipid vesicles.

RESULTS

Dose–Response Curves for GTPase and Phosphodiesterase Activation. In order to study the abilities of various retinal analogues, when combined with opsin and photolyzed, to activate the GTPase or the cGMP phosphodiesterase, it was important to first determine the relationship between the concentration of photochemically activated rhodopsin and GTPase or phosphodiesterase activation in the assay system used. In the experiments shown in Figure 1, known amounts of irradiated rhodopsin were mixed with unexposed rhodopsin and incubated with GTPase and [γ-^{32}P]GTP under the assay conditions described under Materials and Methods. The total amount of rhodopsin was constant. The curve in Figure 1 shows that, although the curve is rather steep, there is still a linear range between approximately 2% and 50% of photolyzed rhodopsin where partial activation of GTPase could be measured. A curve was generated for cGMP phosphodiesterase activation by using the same procedure. Again, as can be seen in Figure 2, an activation curve exhibiting a considerable linear segment between 10% and 50% photolyzed rhodopsin was observed. Thus analogues that behave comparably to partial agonists should be discernible, in addition to those analogues that simply do not function.

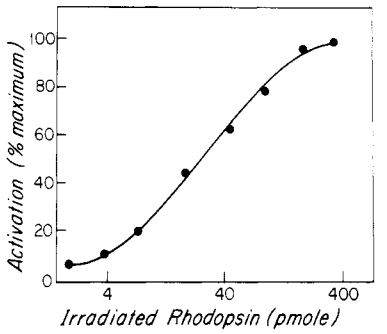


FIGURE 1: Effect of partial irradiation of rhodopsin upon GTPase activation. Rhodopsin in egg phosphatidylcholine vesicles was irradiated and mixed with varying percentages of unirradiated vesicles. Total rhodopsin content was maintained constant at 0.73 μM. GTPase activation relative to the activation obtained with irradiation of 100% of the rhodopsin was calculated as follows: [(GTPase activity with given percent irradiation of rhodopsin) − (GTPase activity with unirradiated rhodopsin)]/[(GTPase activity with irradiated rhodopsin) − (GTPase activity with unirradiated rhodopsin)] × 100. In this assay the ratio of GTPase activity with irradiated rhodopsin to that with unirradiated rhodopsin is 7.4.

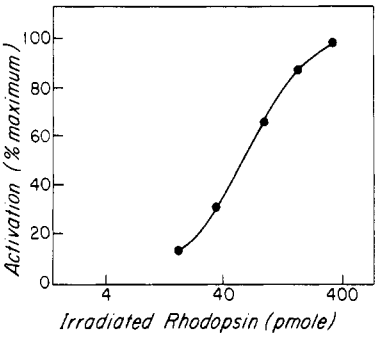
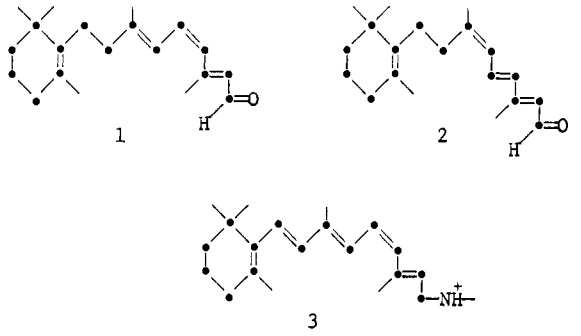


FIGURE 2: Effect of partial irradiation of rhodopsin upon phosphodiesterase activation. Rhodopsin in egg phosphatidylcholine vesicles was irradiated and mixed with varying percentages of unirradiated vesicles. Total rhodopsin content was maintained constant at 0.73 μM. Phosphodiesterase activation relative to the activation obtained with irradiation of 100% of the rhodopsin was calculated as follows: [(phosphodiesterase activity with given percent irradiation of rhodopsin) − (phosphodiesterase activity with unirradiated rhodopsin)]/[(phosphodiesterase activity with irradiated rhodopsin) − (phosphodiesterase activity with unirradiated rhodopsin)] × 100.

Retinal Analogue Incorporation and GTPase Activation. In the initial studies, pigments were prepared from the synthetic analogues 11-*cis*-7,8-dihydroretinal (**1**) and 9-*cis*-7,8-dihydroretinal (**2**). The pigments formed from these analogues maximally absorbed at 425 and 427 nm, respectively, in dodecyl maltoside, as compared to a previously reported value of 420 nm for 9-*cis*-7,8-dihydrorhodopsin in 0.5% digitonin (Arnaboldi et al., 1979). Both pigments bleach upon irra-



diation and hence undergo a photochemical cis to trans isomerization (Arnaboldi et al., 1979). It was of interest to determine whether these pigments could activate GTPase. Since both analogues contain a single bond at the 7,8-position, photochemically induced charge transfer from the protonated Schiff base to the β-ionone ring cannot occur (Scheme IB). Thus, mechanisms requiring this type of energy storage process can be ruled in or out depending on the activity of these pigments. In the manner already published, the pigments were incorporated into egg yolk phosphatidylcholine vesicles and tested for their abilities to activate GTPase under standard assay conditions (Table I). As can be seen in Table I, the pigments formed from **1** and **2** produced approximately 52% and 43%, respectively, of the activation afforded by the rhodopsin controls.

A second experiment along the lines described above was performed to study whether or not the 11-*cis*-15,16-dihydro analogue **3** can activate GTPase when photolyzed. The pigment was formed by the sodium cyanoborohydride reduction of rhodopsin (Fager, 1982). As a control, rhodopsin was reduced with sodium borohydride in the dark, which, although a more powerful reducing agent than sodium cyanoborohydride, does not reduce the protonated Schiff base of rhodopsin when the reaction mixture is kept dark (Bownds & Wald, 1965). The rhodopsin samples, reduced with either cyanoborohydride or borohydride, were incorporated into phosphatidylcholine vesicles, and their abilities to activate GTPase when irradiated were determined, as can be seen from Table I. The 15,16-dihydro chromophore was not able to activate GTPase; however, the borohydride-treated control was able to activate GTPase, to about the same extent as rhodopsin itself.

A separate set of analogue studies was conceived with the question in mind of whether *all-trans*-retinal and analogues could combine with opsin to form metarhodopsin II or some similar state able to activate GTPase. These experiments were performed to further define the internal thermodynamics of rhodopsin activation. In principle, this could occur by microscopic reversibility, and indeed, a series of published papers makes this claim (Fukada et al., 1981, 1982; Yoshizawa & Fukada, 1983). To this end, four compounds of opsin and *all-trans*-retinoid were prepared. Opsin was incubated with *all-trans*-retinal and *all-trans*-retinal oxime. Neither of these compounds was able to activate opsin toward GTPase activation in the slightest (Table II). Large excesses of *all-trans*-retinal (oxime) could not be added due to solubility limitations. To avoid the complication of the unknown degree of association of *all-trans*-retinal or *all-trans*-retinal oxime with opsin, two stoichiometric adducts were formed, *all-trans*-retinoylopsin, which forms an irreversible peptide bond with opsin (Scheme II) (Wong & Rando, 1984), and *all-trans*-15,16-dihydrorhodopsin, formed by reduction with $NaBH_4$ under illumination (Bownds & Wald, 1965). Neither *all-trans*-15,16-dihydrorhodopsin (Table II) nor *all-trans*-

Table I: 9-*cis*-7,8-Dihydro- and 11-*cis*-7,8-Dihydroretinoid Chromophores and Rhodopsin-Mediated Activation of GTPase (G-Protein or Transducin)

modified rhodopsin	GTPase activation[a] (%)
9-*cis*-7,8-dihydro-Rh	−4
9-*cis*-7,8-dihydro-Rh + light	43
11-*cis*-7,8-dihydro-Rh	8
11-*cis*-7,8-dihydro-Rh + light	52
Rh (reacted with $NaBH_4$ in dark)	3
Rh (reacted with $NaBH_4$ in dark) + light	98
11-*cis*-15,16-dihydro-Rh (retinylopsin)	−1
11-*cis*-15,16-dihydro-Rh + light	2

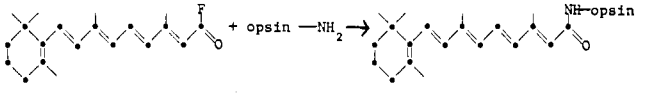
[a] GTPase activation is presented relative to GTPase activation by native rhodopsin. All pigments were incorporated in egg phosphatidylcholine vesicles. The activity of GTPase was measured as production of free ^{32}P in the presence of approximately 0.75 μM modified rhodopsin in the dark or under room lights as discussed under Materials and Methods. The relative GTPase activity was calculated as follows: [(GTPase activity in the presence of the modified rhodopsin) − (activity with rhodopsin in dark)]/[(activity with rhodopsin in light) − (activity with rhodopsin in dark)] × 100.

Table II: *all-trans*-Retinoid Chromophores and Rhodopsin-Mediated Activation of GTPase (G-Protein or Transducin)

modified rhodopsin	GTPase activation[a] (%)
opsin + *all-trans*-retinal	2
opsin + *all-trans*-retinal + light	69[b]
opsin + *all-trans*-retinal oxime	4
all-trans-15,16-dihydro-Rh (retinylopsin)	−5
all-trans-15,16-dihydro-Rh + light	−2

[a] GTPase activation is presented relative to GTPase activation by native rhodopsin. All pigments were incorporated in egg phosphatidylcholine vesicles. The activity of GTPase was measured as production of free ^{32}P in the presence of approximately 0.75 μM modified rhodopsin in the dark or under room lights as discussed under Materials and Methods. The relative GTPase activity was calculated as follows: [(GTPase activity in the presence of the modified rhodopsin) − (activity with rhodopsin in dark)]/[(activity with rhodopsin in light) − (activity with rhodopsin in dark)] × 100. [b] This assay demonstrates that the integrity of opsin was maintained with the procedures used. Irradiation of a mixture of opsin in egg phosphatidylcholine vesicles and equimolar *all-trans*-retinal leads to isomerization of all-trans to 11-*cis*-retinal, regeneration of rhodopsin, and subsequent photoexcitation of rhodopsin and activation of GTPase activity. Under these conditions, one would not expect activation equivalent to that obtained with rhodopsin.

Scheme II



retinoylopsin (Calhoon & Rando, 1985) was able to activate the GTPase, either in light or in dark. As a further control, in a separate experiment *all-trans*-15,16-dihydrorhodopsin was produced by reducing rhodopsin in lipid vesicles during irradiation (100 mM $NaBH_4$, 20 min, 4 °C) followed by dilution and two washes with 10 mM Tris (pH 8). This sample also showed no activation of GTPase in a similar experiment. It can be concluded that *all-trans*-retinoids by themselves cannot drive opsin to the R* state and hence cannot cause the activation of GTPase. In addition, it can be noted that 9-*cis*-retinoylopsin is also incapable of activation of the GTPase upon irradiation (Calhoon & Rando, 1985), which leads to the conclusion that a cis to trans photoisomerization is not in and of itself sufficient to drive GTPase activation.

Retinal Analogue Incorporation and cGMP Phosphodiesterase Activation. Since the relevant biochemical end point

Table III: Effect of Chromophore Modification on Rhodopsin Activation of Phosphodiesterase

modified rhodopsin	phosphodiesterase activation[a] (%)
11-*cis*-7,8-dihydrorhodopsin	9
11-*cis*-7,8-dihydrorhodopsin + light	80
opsin + *all-trans*-retinal	−5
opsin + *all-trans*-retinal + light	62[b]
opsin + *all-trans*-retinal oxime	10
all-trans-15,16-dihydro rhodopsin	−11

[a] Phosphodiesterase activation is presented relative to phosphodiesterase activation by native rhodopsin. All pigments were incorporated in egg phosphatidylcholine vesicles. The activation of phosphodiesterase was measured as production of [^{3}H]5′GMP as discussed under Materials and Methods. A sample of modified rhodopsin in vesicles was mixed under dim red light with rod outer segments containing phosphodiesterase. Some vesicles were irradiated prior to mixing with the rod outer segments. The relative phosphodiesterase activity was calculated as follows: [(phosphodiesterase activity in the presence of modified rhodopsin) − (activity with rhodopsin in dark)]/[(activity with rhodopsin in light) − (activity with rhodopsin in dark)] × 100. [b] This assay demonstrates that the integrity of opsin was maintained with the procedures used. Irradiation of a mixture of opsin in egg phosphatidylcholine vesicles and equimolar *all-trans*-retinal leads to isomerization of *all-trans* to 11-*cis*-retinal, retinal, regeneration of rhodopsin, and subsequent photoexcitation of rhodopsin and activation of phosphodiesterase activity. Under these conditions, one would not expect activation equivalent to that obtained with rhodopsin.

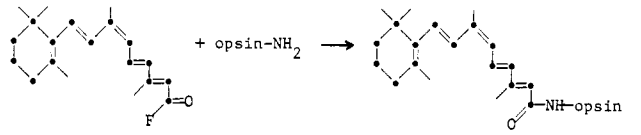
of rhodopsin photolysis is activation of the cGMP phosphodiesterase, it was of interest to confirm here the expected parallelism between GTPase and phosphodiesterase activation. To these ends, several of the rhodopsin analogues studied in Table I and II were assayed for their abilities to activate the phosphodiesterase under conditions more closely approaching those of Fukada et al. (1982). It is clear that, as expected, only those rhodopsin derivatives capable of activating the GTPase can activate the cGMP phosphodiesterase (Table III).

Discussion

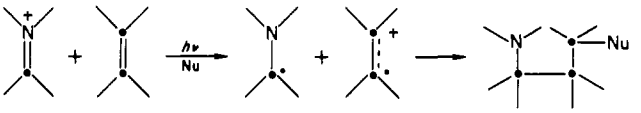
Light of 500 nm, the λ_{max} for rhodopsin, carries with it 57 kcal/mol of photons. Since bathorhodopsin is 35 kcal/mol higher in energy than rhodopsin (Cooper, 1979a), and since *all-trans*-retinal is 4.1 kcal/mol more stable than 11-*cis*-retinal (Rando & Chang, 1983), the efficiency of conversion of photochemical energy to the potential energy of bathorhodopsin is approximately 68%. It is assumed that this high initial energy of bathorhodopsin is critical for the spontaneous and exergonic formation of the remaining intermediates in the cascade.

Given the reasonable assumption that at least partial isomerization of the 11-cis to all-trans double bond has occurred by the time bathorhodopsin has formed, many different mechanisms of energy storage could be constructed in addition to hypotheses concerning charge movement. For example, strain energy induced in the chromophore could account for an increase in bathorhodopsin energy content. However, there would be no clear way of transmitting this energy to the protein backbone of opsin. Furthermore, photochemically induced double-bond isomerization of retinoids in and of itself was shown not to be important in studies with 9-*cis*-retinoyl fluoride, **4**, an isostere of 9-*cis*-retinal, which forms a peptide bond rather than a Schiff base with the active site lysine of opsin (Scheme III) (Calhoon & Rando, 1985). The peptide bond of 9-*cis*-retinoylopsin is uncharged, unlike the Schiff base, but the two pigments are otherwise similar, so if GTPase activation involves only strain and not charge movement, the two pigments should give similar activations. Although 9-*cis*-retinoylopsin will photoisomerize when irradiated, it cannot

Scheme III



Scheme IV[a]



[a] Nu = nucleophile.

activate the GTPase, suggesting involvement of charge movement in the activation. At least two different classes of mechanisms involving charge movement can be suggested for energy storage. The first suggestion is that the chemical isomerization of the double bond moves the positively charged Schiff base head group from its negatively charged counterion in a low dielectric medium (Scheme IA). If the β-ionone ring is held in place, then the partial or full isomerization of the double bond can separate charge in bathorhodopsin (Honig et al., 1979). Coulomb's law calculations predict that a considerable amount of energy could be stored by the protein in this manner, assuming a locally low dielectric medium (Honig et al., 1979). In a very simplified scheme, the charges would be brought closer together again in later conformational states. A second mechanism, or class of mechanisms, also needs to be considered in this context (Scheme IB). Photochemical excitation leads to a redistribution of charge in which the positive charge in the Schiff base is transferred to the carbon backbone of vitamin A through the β-ionone ring. Here again, charge would be separated in a low dielectric medium. This process could occur by an excited state redistribution of charge (Mathies & Stryer, 1976; Lewis, 1978; Warshel, 1978) or by an actual intramolecular single-electron transfer. The latter would be an intramolecular analogy of the well-known photochemical adduction of olefins to Schiff bases, which involves the intermediacy of a radical cation (Scheme IV) (Mariano, 1983). It is noteworthy that calculations on analogues of protonated Schiff bases predict the excited state to be similar to that shown in Scheme IB with charge transferred to the carbon skeleton (Salem & Bruckman, 1975). Furthermore, dipole measurements of photochemically excited protonated Schiff bases of *all-trans*-retinal are in accord with this kind of charge redistribution (Mathies & Stryer, 1976). Although eventually an *all-trans*-retinoid product would form as a result of the absorption of a photon in this model, the complete 11-cis to trans isomerization would not be obligate at very early times (i.e., by the formation of bathorhodopsin). One of the attractive features of a mechanism of this second type is that it does not require substantial atomic motion in the apparently miniscule time (<6ps) required for energization to occur (Busch et al., 1972).

As a partial test of these models, synthetic pigments containing retinal analogues **1** and **2** were prepared and tested for their ability to activate the GTPase activity of G-protein. In addition, the reduced Schiff base of rhodopsin was also studied for its ability to activate GTPase. The 7,8-dihydro bond in **1** and **2** prevents migration of the positive charge into the ionone ring through carbon 8, although they both can photochemically isomerize and bleach. Rhodopsin analogues prepared from **1** and **2** should be able to activate GTPase when exposed to light if the mechanism in Scheme IA were necessary and sufficient, but it should not if the mechanism shown in

Scheme IB were obligate. The results were unequivocal; both pigments formed from **1** and **2** were able to activate GTPase when exposed to light, although less well than rhodopsin itself. These results all but eliminate any argument for a mechanism of the second type (Scheme IB), an intramolecular charge redistribution mechanism where the positive charge is delocalized into the β-ionone ring, but are consistent with the mechanism shown in Scheme IA. A charge redistribution model in which the positive charge in the Schiff base head group need only migrate as far as the 9,10 carbon–carbon bond of the vitamin A backbone is not ruled out by the experiments reported here. Further studies using suitable 9,10- and 13,14-dihydroretinal analogues will be required to address this question.

Curiously, though, the reduced Schiff base of rhodopsin was ineffective at activating GTPase when exposed to light (Table I). Since reduction converts the Schiff base to an amine, it would have perhaps been predicted that the pigment formed from this analogue would photochemically activate the GTPase if the Scheme IA mechanism but not the Scheme IB mechanism was operative. There are several reasons why a negative result might have been obtained here. First, the reduction of the Schiff base to an amine markedly alters the pK_a of its conjugate acid, with an amine being at least 2–3 pH units more basic than its corresponding imine. Since proton transfers involving the protonated Schiff base may be important for metarhodopsin II formation, the substitution of an amine for an imine could have profound effects on the activation process. Second, although controls were performed, it is not possible to rule out the chemical denaturation of rhodopsin and its reduced analogue by the relatively harsh conditions required in the reduction process. Finally, SP^3 rather than SP^2 hybridization at carbon atom 15 would also affect the charge movement mechanism shown in Scheme IA because of the different conformations taken by the Schiff base and amine. Further studies with other retinoid analogues will be required to determine which, if any, of these conditions prevail.

The finding that the synthetic pigments formed from **1** and **2** only partially activate the GTPase and the phosphodiesterase requires comment. The partial activation could be due to several factors. The quantum yield for the photolysis of the synthetic pigments could be much lower than that for rhodopsin photolysis. Moreover, the amount and longevity of the R* intermediate formed could also be much lower than with rhodopsin. This could reflect the possibility of that the synthetic pigments are much less efficient at converting light energy into potential energy than in rhodopsin, although they absorb light of higher energy.

The fact that bathorhodopsin is relatively high in energy compared to the subsequent intermediates in the bleaching process is an important one. This means that the formation of conformational intermediates past bathorhodopsin will occur spontaneously, and indeed the active conformation(s) of rhodopsin will spontaneously decay to form any inactive conformers, prior to hydrolysis of the chromophore to form opsin and *all-trans*-retinal. Of great interest is the question of whether *all-trans*-retinal, or derivatives thereof, can activate opsin, because this would yield information on the internal thermodynamics of opsin activation. In principle, the addition of *all-trans*-retinal to opsin could generate some R* and yield a chemically rather than photochemically activated species. Although this is true, in principle, by microscopic reversibility, such an occurrence is dependent on the thermodynamic and kinetic barriers placed between *all-trans*-retinal plus opsin and R* (Scheme V). For the sake of argument, here R* is considered to be a conformer of the spectroscopically defined state metarhodopsin II. If the system is allowed to come to "equilibrium", then it might be possible to estimate the $\Delta G°$ difference between opsin plus *all-trans*-retinal and metarhodopsin II. Indeed, in a current mechanical model for rhodopsin activation it is considered that an "open" rhodopsin conformer produced by the cis to trans isomerization of the chromophore is all that is required to begin the biochemical cascade (Yoshizawa & Fukada, 1983). However, neither *all-trans*-retinal nor *all-trans*-retinal oxime formed significant R* as measured by its ability to stimulate the GTPase activity in the dark. Due to the limited solubilities of the retinoids, high concentrations of these substances relative to opsin were not attempted to try to force the equilibrium to the left (Scheme V). Nevertheless, an upper limit of 0.5% R* could have been formed without detection on the basis of the assay system shown in Figure 1, which requires that the $\Delta G°$ between opsin plus *all-trans*-retinal (oxime) and R* must be at least 3.1 kcal/mol, a value consistent with the microcalorimetric studies of Cooper (Cooper, 1979a,b, 1981; Cooper & Converse, 1976). However, these latter measurements were of enthalpy charges rather than free energy, so that direct quantitative comparisons cannot be made. Nevertheless, at pH 8.0 Cooper reports that metarhodopsin II is 5.3 kcal/mol higher in energy than opsin and *all-trans*-retinal. The difference is even more exaggerated at pH 5.4, being 10.4 kcal/mol. It could be argued that the hydrolysis of the Schiff base of retinylidene opsin is highly exothermic, masking a favorable internal equilibrium. This is rendered less likely by experiments with *all-trans*-retinoyl fluoride, a close isostere of *all-trans*-retinal (Calhoon & Rando, 1985). This molecule can react stoichiometrically with opsin, almost certainly at the active site lysine residue, to form an amide, retinoylopsin, rather than a Schiff base, which will not be susceptible to hydrolysis under the conditions of the experiments (Scheme II) (Wong & Rando, 1984). The fact that *all-trans*-retinoylopsin was also not capable of activating GTPase in the dark is consistent with the previous results with the all-trans analogues and, in addition, makes it unlikely that these results were obtained because of the unfavorable equilibrium position between *all-trans*-retinal plus opsin and the corresponding Schiff base. This latter point was also consistent with the lack of activity of *all-trans*-15,16-retinylopsin (Table II). Thus, it is shown here that activation of opsin fails to occur with four different *all-trans*-retinoid analogues.

Scheme V

$$\text{Meta II} \rightleftharpoons \text{Meta III} \overset{H_2O}{\rightleftharpoons} \text{opsin} + \textit{all-trans}\text{-retinal}$$

The results with the *all-trans*-retinoids are further consistent with physiological experiments performed with isolated frog, rabbit, and skate retinae (Perlman et al., 1982; Pepperberg & Masland, 1978; Pepperberg et al., 1978). Freshly bleached retinae were studied electrophysiologically and spectrophotometrically. It was found that adding 11-*cis*-retinal would characteristically lower the light threshold as measured electrophysiologically. However, *all-trans*-retinal and other *all-trans*-retinoids, as well as 13-*cis*-retinal, had no effect over controls in sensitizing retinae to low light levels. In some of these experiments, *all-trans*-retinal was incubated with bleached retinae for hours with no change in the measured light sensitivity (Pepperberg & Masland, 1978). However, upon the addition of 11-*cis*-retinal to these retinae, the log threshold values immediately decreased by at least 3 orders of magnitude. These results show that *all-trans*-retinal cannot activate under these conditions, nor is it an inhibitor of ac-

tivation. These results, however, along with ours, must be contrasted to previously published studies that presented data showing that frog opsin activates retinal phosphodiesterase in the dark in the presence of 1 equiv of either *all-trans*-retinal or *all-trans*-retinal oxime (Fukada et al., 1981, 1982; Yoshizawa & Fukada, 1983). As our results were obtained with bovine opsin, a species difference between the bovine and frog photoreceptors could possibly explain the differences in the biochemical results. However, this does not explain the dichotomy between the physiological and biochemical results obtained with the frog. We have no explanation for these discrepancies at this time.

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Registry No. GTPase, 9059-32-9; cGMP phosphodiesterase, 9068-52-4; 9-*cis*-7,8-dihydroretinal, 72535-16-1; 11-*cis*-7,8-dihydroretinal, 98574-24-4; retinal, 116-31-4.

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