Light-Induced Dephosphorylation of Two Proteins in Frog Rod Outer Segments

Influence of Cyclic Nucleotides and Calcium

ARTHUR S. POLANS, JOSEPH HERMOLIN, and M. DERIC BOWNDS

From the Laboratory of Molecular Biology, the Department of Zoology and the Neurosciences Training Program, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Two minor proteins of frog rod outer segments become phosphorylated when retinas are incubated in the dark with $^{32}P_i$. The proteins, designated component I (13,000 daltons) and component II (12,000 daltons), are dephosphorylated when retinas are illuminated. The dephosphorylation is reversible; the two proteins are rephosphorylated when illumination ceases. Each outer segment contains ~ 10^6 molecules of components I and II. These remain associated with both fragmented and intact outer segments but dissociate from the outer segment membranes under hypoosmotic conditions. The extent of the light-induced dephosphorylation increases with higher intensities of illumination and is maximal with continuous illumination which bleaches 5.0×10^5 rhodopsin molecules/outer segment per second. Light which bleaches 5.0×10^3 rhodopsin molecules/outer segment per second causes approximately half-maximal dephosphorylation. This same intermediate level of illumination causes half-suppression of the light-sensitive permeability mechanism in isolated outer segments (Brodie and Bownds. 1976. *J. Gen. Physiol.* **68:**1–11) and also induces a half-maximal decrease in their cyclic GMP content (Woodruff et al. 1977. *J. Gen. Physiol.* **69:**667–679). The phosphorylation of components I and II is enhanced by the addition of cyclic GMP or cyclic AMP to either retinas or isolated rod outer segments maintained in the dark. Several pharmacological agents which influence cyclic GMP levels in outer segments, including calcium, cause similar effects on the phosphorylation of components I and II and outer segment permeability. Although the cyclic nucleotide-stimulated phosphorylation can be observed either in retinas or isolated rod outer segments, the light-induced dephosphorylation is observed only in intact retinas.

INTRODUCTION

In vertebrate rod outer segments several light-dependent reactions have been described which may be components of the pathway(s) linking photon ab-

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sorption by rhodopsin to the permeability decrease of the plasma membrane. Light initiates the phosphorylation of rhodopsin (Bownds et al., 1972; Kühn and Dryer, 1972) and activates a GTPase (Carretta and Cavaggioni, 1976; Robinson and Hagins, 1977; Wheeler and Bitensky, 1977).[1] Bleaching rhodopsin also activates a cyclic GMP phosphodiesterase which lowers the level of endogenous cyclic GMP (Keirns et al., 1975; Brodie and Bownds, 1976; Fletcher and Chader, 1976; Orr et al., 1976; Goridis et al., 1977; Woodruff et al., 1977). This activation occurs rapidly (Yee and Liebman, 1979). Woodruff and Bownds (1979) recently have demonstrated in isolated outer segments that the $t_{\frac{1}{2}}$ of the cyclic GMP decrease is ~ 125 ms, and that bleaching one rhodopsin molecule can lead to the loss of ~ 10^5 molecules of cyclic GMP. The extent of the cyclic GMP decrease becomes larger as illumination is increased over four log units of light intensity, and outer segment permeability assayed by an in vitro technique is suppressed by this same range of light intensity (Brodie and Bownds, 1976; Woodruff et al., 1977).

This paper reports a further reaction: the light-initiated dephosphorylation of two proteins associated with frog rod outer segments. The phosphorylation level of these proteins is influenced by calcium ions and cyclic nucleotides and thus may be linked to light-induced changes in cyclic GMP and permeability of the outer segment. It has been suggested in other systems that cyclic nucleotides may influence ion permeability by controlling the phosphorylation or dephosphorylation of proteins (Greengard, 1976), and that calcium ions might be involved in such control (Rasmussen and Goodman, 1977).

In the experiments reported here frog retinas or isolated outer segments are incubated in Ringer's solution with $^{32}P_i$. Proteins then are analyzed by sodium dodecyl sulfate gradient–polyacrylamide gel electrophoresis and autoradiography to measure any changes in the level of protein phosphorylation which occur in response to light or alterations in the concentrations of cyclic nucleotides or calcium. The gradient gel technique employed in this work has permitted clear separation of proteins in the molecular weight range from 5,000 to 250,000 daltons and is especially useful in resolving low molecular weight proteins. The electrophoretic analysis has been performed on crude rather than purified outer segment preparations to prevent loss of any easily eluted proteins. The conditions employed in these experiments have been found to maintain the functional integrity of isolated rod outer segments (Bownds and Brodie, 1975). The levels of illumination used are those which have been shown to affect permeability and induce cyclic GMP changes in suspensions of isolated outer segments and are comparable to the intensities which elicit responses from living amphibian rod cells (Fain, 1976).

MATERIALS AND METHODS

Bullfrogs (*Rana catesbeiana*) were kept in holding tanks 2–4 wk prior to use and fed ~ 5 g of Purina Dog Chow (Ralston Purina Co., St. Louis, Mo.) with vitamin supplements three times a week. During the holding period frogs were kept on the photoperiod described by Woodruff and Bownds (1979). Animals were removed from

[1] Biernbaum, M., and D. Bownds. Influence of light and calcium on guanosine 5′-triphosphate in isolated frog rod outer segments. *J. Gen. Physiol.* In press.

the holding tanks 3–4 h before the end of the 12-h dark period and kept in the dark 1–2 h before sacrifice. All darkroom manipulations were performed at room temperature and under infrared illumination using an image converter (FJW Industries, Mount Prospect, Ill.). Animals were sacrificed and the retinas removed as described by Woodruff et al. (1977). Retinas were gently rinsed in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$ and 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-enthanesulfonic acid], pH 7.5) and then transferred to the solutions described below.

Phosphorylation in Intact Retina

Each retina was incubated in the dark in 200 μl Ringer's solution containing ~ 1 mCi of carrier-free $^{32}P_i$. The time of incubation for most experiments was 8 min. (This resulted in a stable level of ^{32}P incorporation into components I and II [see Results]). After incubation retinas were transferred into 300 μl Ringer's solution without the isotope. Details of illumination, drug additions, etc. are given in Results. Light intensity was calibrated as described by Brodie and Bownds (1976). In most experiments retinas were agitated gently for 1–2 min in this Ringer's solution to detach rod outer segments. Portions (100 μl) of the suspension were precipitated by the addition of 200 μl 10% trichloroacetic acid (TCA) in preparation for electrophoresis. Treatment of acid-precipitated rod outer segments is described below. Other portions (50 μl) of the rod outer segment suspension were used to determine rhodopsin concentration by difference spectroscopy after dissolving the outer segments in 200 μl of 0.04 M hexadecyltrimethyl ammonium chloride (Bownds et al., 1971).

In some experiments the intact retina, in 200 μl of Ringer's solution, was quenched by the addition of 200 μl of TCA after exposure to differing conditions of illumination or drug additions and then disrupted in a glass homogenizer. The resultant suspension was centrifuged at 1,500 *g* for 30 min and the pellet was resuspended in 600 μl of 10 mM sodium phosphate buffer, pH 7.5. Portions (10 and 20 μl) were solubilized in a final concentration of 2% sodium dodecyl sulfate and heated at 100°C for 2 min. Protein determinations were made according to the methods of Lowry et al. (1951) using as a standard bovine serum albumin which had been treated like the retinal samples. The assay proved to be linear between 10 and 80 μg protein under these procedures. Samples from the remaining retinal homogenate were prepared for electrophoresis by centrifuging at 1,500 *g* for 30 min and by resuspending the pellet to give a final protein concentration of 0.5–1.0 mg/ml in the following solubilization solution: 0.0625 M Tris-Cl, pH 6.8, 2.3% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and 2% bromphenol blue. To demonstrate that no protein was lost preferentially during the washing procedures, some of the homogenized retinal suspension was dissolved directly into the solubilization solution and subjected to electrophoresis.

In most of the experiments of this paper, outer segments were shaken from the retina for electrophoretic analysis. The intact retina preparation was not used generally because the ratio of components I and II to total protein was much more variable in this preparation than the ratio of components I and II to rhodopsin concentration in outer segment preparations. This may have been due to greater variability in non-outer segment proteins in the retina preparation, as well as unequal generation of nonproteinaceous Lowry positive material in the two preparations.

Phosphorylation in Isolated Rod Outer Segments

Portions (200 μl) of crude rod outer segment suspensions were incubated for 8–10 min in the dark with 1 mCi of carrier-free $^{32}P_i$ under various conditions and quenched

with TCA as already described. Acid-precipitated outer segments then were centrifuged at 1,500 *g* for 30 min and the pellet was washed in 300 μl Ringer's solution. The final pellet was dissolved in solubilization solution to yield a rhodopsin concentration of 0.5–1.0 mg/ml. Some rod outer segment samples also were dissolved directly in the solubilization solution to demonstrate that no proteins were preferentially lost during the washing procedure.

Sodium Dodecyl Sulfate Gradient–Polyacrylamide Gel Electrophoresis

The methods employed were derived from O'Farrell (1975) based upon the procedures of Davis (1964), Ornstein (1964), and Laemmli (1970). The following solutions were used:

(*a*) 6% acrylamide solution: 0.375 M Tris-Cl, pH 8.8, 6% total acrylamide,[2] 10% glycerol, 0.2% sodium dodecyl sulfate, 0.024% TEMED (*N,N,N′,N′*-tetramethylethylenediamine), and 0.0045% ammonium persulfate

(*b*) 20% acrylamide solution: 0.375 M Tris-Cl, pH 8.8, 20% total acrylamide, 25% glycerol, 0.2% sodium dodecyl sulfate, 0.024% TEMED, and 0.0016% ammonium persulfate

(*c*) stacking gel solution: 0.125 M Tris-Cl, pH 6.8, 4.5% total acrylamide, 0.2% sodium dodecyl sulfate, 0.084% TEMED, and 0.025% ammonium persulfate

(*d*) electrode buffer: 0.025 M Tris, 0.192 M glycine, 0.2% sodium dodecyl sulfate, pH 8.6.

GEL PREPARATION After degassing and addition of ammonium persulfate, 33 ml of both the 6% and 20% acrylamide solutions were mixed by a linear gradient maker (Bio-Rad Laboratories, Richmond, Calif. or Instrumentation Specialities Co., Lincoln, Nebraska) and pumped (Stalprodukter peristaltic pump, Uppsala, Sweden) from the bottom into a 28 × 14-cm slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) with 1.5-mm gel spacers. The gradient was pumped at ~ 2 ml/min, and once prepared, the gel was overlaid with a saturated solution of isobutanol. Complete polymerization occurred 3 h after the addition of catalyst, at which time the gel surface was washed several times with 0.375 M Tris-Cl, pH 8.8 buffer. The gel was allowed to stand overnight, and the stacking gel was layered no more than 3–4 h before use to avoid diffusion of the pH gradient. The gels were run with water cooling at constant current, using initially a 10-mA current per gel until samples entered the stacking gel and then 17.5 mA per gel to complete the run. An average run took ~ 17 h before the tracking dye left the gel. The tracking dye was allowed to leave the gel and the migration of dansylated cytochrome *c* (13,300 daltons) (Inouye, 1971) was observed under ultraviolet irradiation during the run to determine when maximum separation of the low molecular weight region had occurred. Molecular weight determinations were made with reference to the mobility in polyacrylamide gel of standards (Dunker and Rueckert, 1969): myosin (220,000 daltons), lactoperoxidase (92,600), bovine serum albumin (62,000), ovalbumin (46,400), RNase A (15,800), lysozyme (13,600), cytochrome *c* (13,300) and selected peptides of myoglobin (myoglobin [16,900], myoglobin I - II [14,400], myoglobin I [8,100], myoglobin II [6,200] and myoglobin III [2,500]) purchased from BDH Chemicals Ltd., Poole, England. Gels were stained with Coomassie Brilliant Blue according to the methods of Fairbanks et al. (1971).

AUTORADIOGRAPHY Gels were dried with a Hoefer slab gel dryer and exposed with X-ray film (Kodak X-omat R, Eastman Kodak Co., Rochester, N.Y.). Isolated rod outer segment samples, containing 15 μg rhodopsin, were exposed with film for 3½

[2] In all gel solutions, bisacrylamide is 2.66% of the total acrylamide.

d, while retinal samples, containing roughly 5–7 μg rhodopsin, were exposed for 7 d. The film was developed (Kodak liquid X-ray developer) for 6 min, stopped in water for 30 s, and fixed (Kodak rapid fixer) for 6 min. Band densities from the autoradiograph were determined with a Joyce-Loebel & Co. microdensitometer (Gateshead-upon-Tyne, England). The level of protein phosphorylation was determined by integrating the area under the peaks in the autoradiograph with a Hewlett-Packard Co., 9820 calculator (Palo Alto, Calif.) interfaced with a Numonics Corp. model 264 electonics graphic calculator (Lansdale, Pa.). Measurements were made in the range in which optical density was linear with radioactivity.

Acrylamide, *N,N'*-methylenebisacrylamide, sodium dodecyl sulfate and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories. Carrier-free $^{32}P_i$ was obtained from New England Nuclear, Boston, Mass. The remaining chemicals were purchased from either Sigma Chemical Co., St. Louis, Mo. or Mallinckrodt, Inc., St. Louis, Mo.

RESULTS

The electrophoretic separation of proteins from a crude suspension of isolated rod outer segments and from an intact retina is illustrated in Fig. 1. Several proteins become radioactive when the retinas are incubated with $^{32}P_i$ in the dark, and the detection of these proteins by autoradiography of the acrylamide gels also is shown. The two low molecular weight proteins that are the subject of this paper are designated as component I (apparent molecular weight 13,000 daltons) and component II (12,000 daltons). They are barely detectable with Coomassie stain, but their relatively high incorporation of $^{32}P_i$ makes them easily identifiable using autoradiographic procedures. Fig. 1 demonstrates that these two proteins can be separated easily from other proteins whether electrophoresis is performed using samples from intact retinas or suspensions of outer segments.

Several observations suggest that components I and II are associated with outer segments, rather than with possible contaminants. (*a*) In different preparations in which 2–20% of the outer segments retain their mitochondria-rich inner segments after detaching from the retina, the amounts of components I and II remain proportional to the rhodopsin content. (*b*) Addition of pigment epithelium, a nonretinal contaminant, does not alter the Coomassie staining intensity or the autoradiographic intensity of these components. (*c*) The ratio between rhodopsin content and components I and II remains the same when either retina or isolated rod outer segments are analyzed. (*d*) Components I and II remain associated with the rod outer segment fraction after purification by ficoll flotation (Bownds et al., 1971). (*e*) In different retinas the dephosphorylation of the two proteins has proven to be proportional to the amount of rhodopsin bleached regardless of variation in contaminants. Immunohistochemical studies should more definitively localize components I and II, and such work is underway.

Components I and II appear to be proteins, for they are susceptible to protease digestion while remaining insensitive to lipases and RNases. Treatment of rod outer segment samples with either trypsin (5 μg/ml) or pronase (5 μg/ml) for 30 min at 37°C abolishes their bands whereas similar treatments with lipase (5 μg/ml) or RNase (5 μg/ml) have no effect.

An estimate, within an order of magnitude, of the number of molecules of components I and II present per outer segment can be obtained from the following considerations: electrophoresis of an outer segment suspension containing 15 μg rhodopsin (3.8×10^{-10} mol) results in band intensities in the

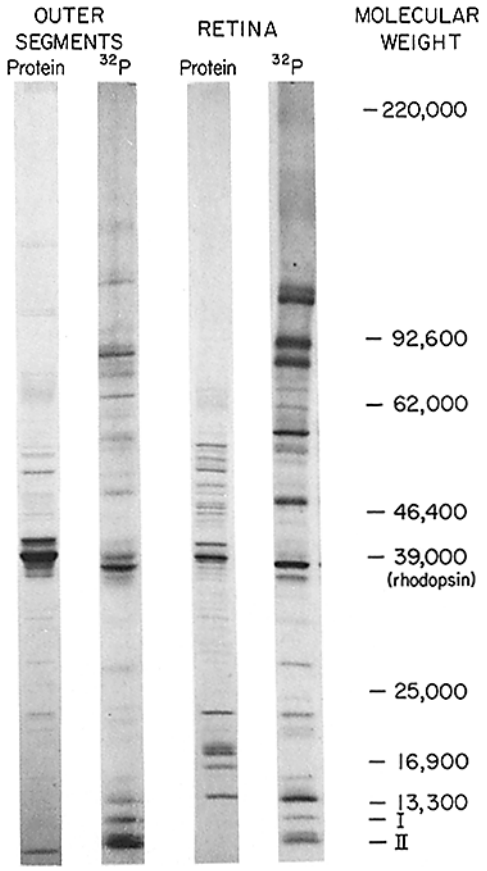

FIGURE 1. Electrophoretic separation of proteins from isolated rod outer segments and retina. Retinas, incubated in the dark for 8 min with $^{32}P_i$, either were quenched with TCA, or the rod outer segments were detached from the retina and subsequently treated with TCA. Proteins from the crude suspension of isolated rod outer segments and retina prepared as described in Materials and Methods were analyzed on a 6–20% linear gradient sodium dodecyl sulfate–polyacrylamide gel. Samples of isolated rod outer segments contain 15 μg of rhodopsin, corresponding to ~ 30 μg total protein. Retinal samples contain 30 μg total protein, corresponding to ~ 5 μg rhodopsin. Protein is stained with Coomassie Brilliant Blue, and phosphorylation of proteins is detected by autoradiography. Molecular weight standards are listed in Materials and Methods and the positions of components I and II are indicated.

Coomassie stain for components I and II barely detectable by densitometry and comparable to that observed for 0.01 μg of cytochrome *c* (a protein with electrophoretic mobility similar to components I and II). The assumption then is made that these bands contain 0.01 μg each of component I and II (mol wt

13,000 and 12,000, or 8×10^{-13} mol is present for every 3.8×10^{-10} mol of rhodopsin. Since there are $\sim 3 \times 10^9$ rhodopsin molecules in an outer segment, this corresponds to approximately 10^6 molecules each of component I and II per outer segment.

Both the protein staining and autoradiographic patterns of Fig. 1 indicate bands in the retinal sample that are not present in the crude outer segment suspension. These proteins most likely are associated with parts of the retina other than the outer segments. Similarly, some bands seen in the protein staining pattern from the outer segment suspension are absent in the retinal sample. These are probably outer segment proteins present in the retinal sample at a concentration too low for detection (see Fig. 1 legend). A detailed comparison of the patterns is not appropriate, for differing amounts of nonretinal contamination are found in different preparations, so that the banding pattern shown in Fig. 1 changes slightly between experiments. It is for this reason that attention is directed at proteins such as components I and II, which clearly can be associated with outer segments using the criteria just listed, and which undergo light-sensitive transformations. Work of the sort being done by Godchaux and Zimmerman (1978), Kühn (1978), and Papermaster et al. (1978) is necessary to establish the list of proteins actually associated with the outer segment.

Phosphorylation and Dephosphorylation in Retina

Fig. 2 demonstrates that components I and II are phosphorylated to a stable value in ~ 8 min when retinas are incubated in the dark with $^{32}P_i$ and outer segments are then detached for electrophoretic analysis. Retinas routinely were not incubated for more than 8 min in order to avoid possible deterioration. The level of phosphorylation obtained after 8 min of incubation with the isotope is relatively stable but does not reflect saturation of the phosphate binding sites, since ^{32}P incorporation can be enhanced or depressed by the pharmacological perturbations discussed below.

If retinas are transferred after 8 min to Ringer's solution without the isotope, the phosphorylation level of components I and II is maintained over the next 20 min. (The phosphorylation levels of components I and II 17 min after transfer are, respectively, 81 ± 12 and 106 ± 13% of the level at the end of 8 min incubation with the isotope [mean values ± SEM for three determinations]). To measure the effects of light and drug additions retinas were incubated with the isotope for 8 min and variations involving light and drug additions were completed within 20 min after transferring the retinas to Ringer's solution without the isotope. The stable level of phosphorylation for the 20-min period following transfer does not reflect irreversibly bound ^{32}P, since ^{32}P can be displaced partially within 10–20 min if the retinas are incubated in the dark with 1 mM P_i which is not radioactive.

Light-induced loss of ^{32}P which had been bound to components I and II in the dark is shown in Fig. 3. (This illustrates one of the 22 separate experiments in which the phosphorylation/dephosphorylation sequence was observed.) Electrophoresis was performed on the outer segments from two retinas which, after incubation with $^{32}P_i$, either were kept dark or illuminated. The autora-

diograph of the lower portion of a polyacrylamide gel and the densitometric scan of the autoradiograph demonstrate the phosphorylation of components I and II in the dark and their dephosphorylation in the light. The light-activated phosphorylation of rhodopsin (39,000 daltons) and a fourth protein (apparent mol wt, 12,500 daltons) also are shown. No reproducible effect of light was found on the phosphorylation level of other proteins. A phosphorylated band migrating just beneath rhodopsin (shown in Fig. 3) can be resolved

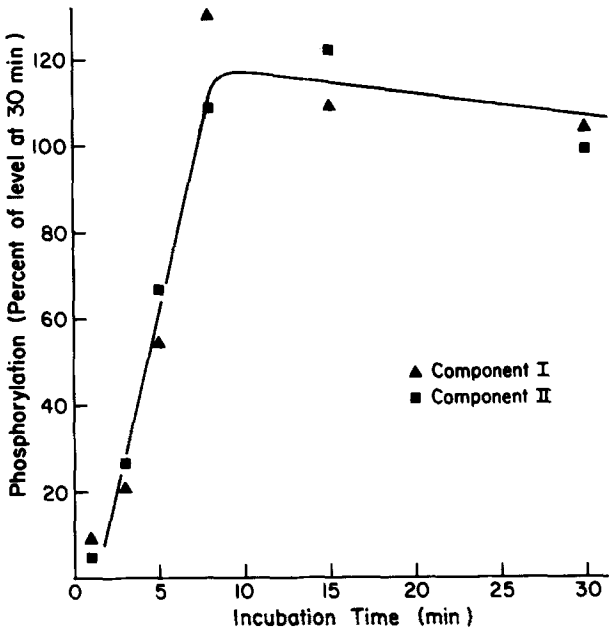

FIGURE 2. Phosphorylation level of components I and II as a function of incubation time. Dark-adapted retinas are incubated each in 200 μl Ringer's solution with ~ 1 mCi carrier-free $^{32}P_i$. At the times indicated, a retina is transferred to 300 μl Ringer's solution without the isotope and the retina is shaken gently for 2 min. An aliquot of the outer segment suspension is used to determine rhodopsin content and two samples are quenched with TCA. The samples are processed for electrophoresis and autoradiography as described in Materials and Methods, and samples containing 15 μg rhodopsin are applied to the gels. The phosphorylation level of the retina incubated for 30 min arbitrarily is defined as 100%, and all other values are expressed as a percent of this value. The data are from one of three experiments with similar results.

completely from the highly radioactive rhodopsin region in the light sample by allowing electrophoresis to continue several hours after the dansylated cytochrome *c* has left the gel. It then can be shown that this phosphorylated band does not alter its phosphate content upon illumination.

Comparisons of electrophoretic bands from isolated rod outer segment suspensions obtained by shaking different retinas must be made cautiously, for the protein pattern can vary from retina to retina depending on contamination from non-outer segment protein. The protein band (13,500 daltons)

shown in Fig. 3 which appears to dephosphorylate upon illumination can serve as an example. The light effect is not reproducible and appears to be due to variation in the concentration of contaminating phosphorylated protein

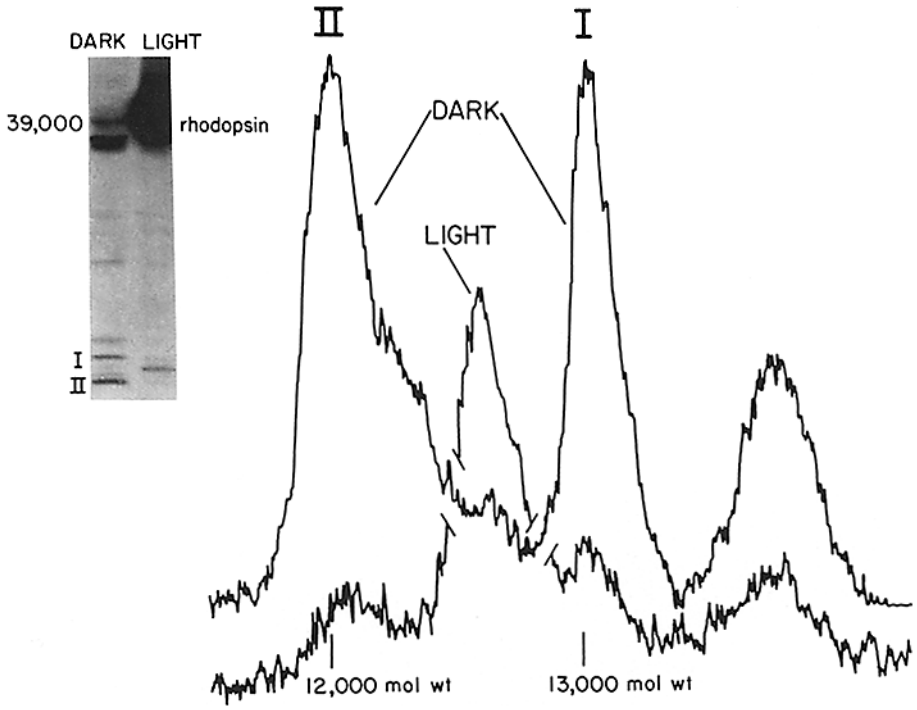

FIGURE 3. Dephosphorylation and phosphorylation of frog rod outer segment proteins caused by illumination. Two retinas are incubated in the dark for 8 min each in 200 μl Ringer's solution with 1 mCi carrier-free $^{32}P_i$. Each retina then is transferred to 300 μl Ringer's solution without isotope. One retina is kept dark while the other is exposed for 3 min to a light source bleaching 5×10^5 rhodopsin molecules/outer segment per second. The two retinas then are shaken gently and simultaneously for 2 min to detach rod outer segments. Samples from each suspension of outer segments are quenched with TCA and processed for electrophoresis and autoradiography as described in Materials and Methods. The autoradiograph for the lower portion of the polyacrylamide gel is shown in the inset. Depicted is the dephosphorylation of components I (13,000 daltons) and II (12,000 daltons) and the light-activated phosphorylation of rhodopsin (39,000 daltons) and a fourth protein (12,500 daltons). No light-sensitive changes in phosphorylation levels of other proteins were observed. The densitometric scan for the dark condition has been displaced upward slightly for clarity. The phosphorylation/dephosphorylation sequences shown here were observed in 22 separate experiments.

present in the outer segment suspensions from different retinas. This is suggested by examination of Fig. 1 which reveals that the Coomassie staining intensity and autoradiographic intensity of this protein band is higher in the electrophoretic pattern obtained from intact retina than in the pattern ob-

tained from outer segment suspensions, in spite of the fact that the retinal sample that was analyzed contained less rhodopsin (fewer outer segments) than the outer segment sample.

The magnitude of the dephosphorylation of components I and II varies

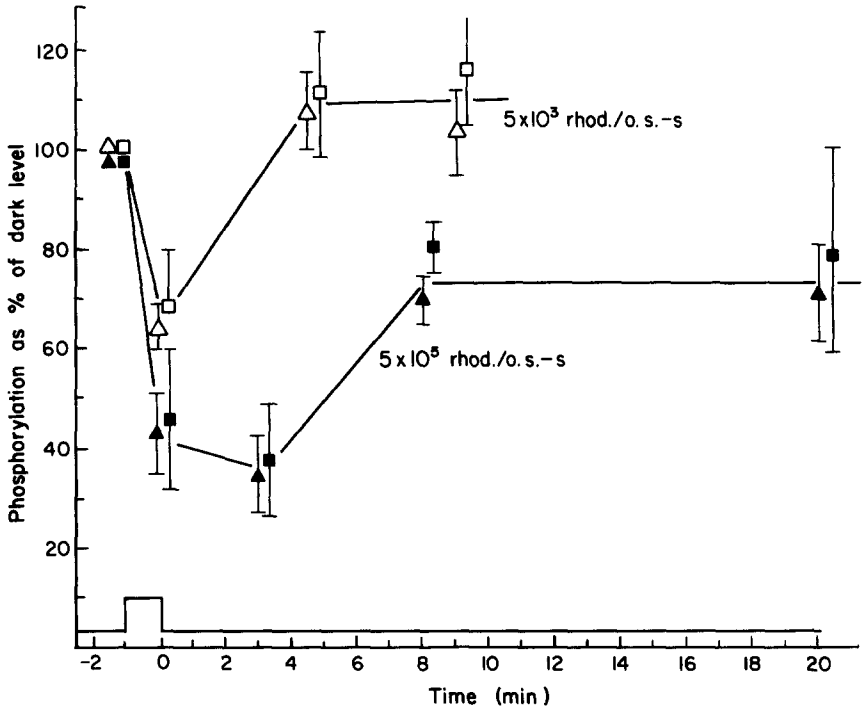

FIGURE 4. Phosphorylation level of components I (△,▲) and II (□,■) as a function of time after the end of illumination. Retinas are incubated in the dark for 8 min each in 200 μl Ringer's solution containing ~ 1 mCi carrier-free $^{32}P_i$. After 8 min, retinas are transferred into 300 μl Ringer's solution without the isotope. Control retinas are kept dark while points other than those at time = 0 are exposed to 1 min of light bleaching either 5.0×10^5 rhodopsin molecules/outer segment per second (■,▲) or 5.0×10^3 rhodopsin molecules/outer segment per second (□,△). 2 min before quenching, retinas are shaken gently to detach rod outer segments. Samples of the rod outer segment suspension are quenched with TCA and processed for electrophoresis and autoradiography as described in Materials and Methods. Points taken at time = 0 are illuminated for 1 min at the two light intensities and then shaken gently and quenched in the light. The data represent at least triplicate experiments for each value (mean ± SEM).

with the light intensity as shown in Fig. 4. Both components dephosphorylate ~ 65% from their initial dark levels when retinas are exposed to 1 min of light which bleaches 5.0×10^5 rhodopsin molecules/outer segment per second (i.e., 1% of the total rhodopsin is bleached). Higher intensities which bleach 100% of the rhodopsin do not cause further dephosphorylation (data not shown). When retinas are exposed for 1 min to light which bleaches 5.0×10^3

rhodopsin molecules/outer segment per second (0.01% bleach), components I and II dephosphorylate ~ 35% from their dark values (Fig. 4). This same level of illumination causes half-maximal decreases in the cyclic GMP content and permeability of isolated outer segments (Brodie and Bownds, 1976; Woodruff et al., 1977). The remaining 35% of the ^{32}P which cannot be displaced by saturating illumination might represent light-insensitive phosphate binding sites. It is known that light does not eliminate entirely the levels of either cyclic GMP (Woodruff et al., 1977) or GTP[1] in frog rod outer segments.

The two components are rephosphorylated towards their original dark levels when retinas are maintained in the dark following illumination. Rephosphorylation is observed whether 0.1 or 100% of the rhodopsin molecules have been bleached. If retinas are illuminated and the outer segments are then detached, rephosphorylation is observed in the isolated outer segments kept in the dark and is more rapid after smaller bleaches. Only component I and II demonstrate the reversible phosphorylation sequence; rhodopsin and the 12,500 dalton protein which are phosphorylated in the light do not show significant dephosphorylation when retinas are returned to the dark. (This point will be discussed in more detail below.)

Fig. 5 demonstrates that addition of 5 mM cyclic GMP to dark-adapted retinas during incubation with $^{32}P_i$ enhances the phosphorylation level of components I and II. No other cyclic GMP-stimulated protein phosphorylation was detected. Addition of cyclic AMP to the retina also enhances the phosphorylation of only components I and II, and to the same extent as cyclic GMP. (A 30,000 dalton soluble protein, found in extracts from bovine rod outer segments and reported by Farber et al. [1979] to undergo cyclic nucleotide-dependent phosphorylation, is not observed in these experiments on frog material.) It is unlikely that components I and II could be subunits of such a protein, for altering reducing conditions, which might be expected to influence the association of protein subunits, does not alter the migration of components I and II or of proteins in the 30,000 mol wt range. Further, phosphorylation of the 30,000 dalton protein is not reported to be light sensitive.)

Drugs that influence the concentration of cyclic GMP when added to retinas during incubation with $^{32}P_i$ also alter the phosphorylation of components I and II (Table I). In taking values for the dark phosphorylation of components I and II in Ringer's solution as the control (100%), addition of cyclic GMP to an incubating retina enhances the level of phosphorylation about twofold. EGTA [ethylene glycol-bis-(β-aminoethyl ether) *N,N'* tetraacetic acid], a calcium chelator, increases the phosphorylation of components I and II threefold. IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase inhibitor, increases the phosphorylation of components I and II about sixfold. Both EGTA and IBMX have been shown to increase cyclic GMP concentration in isolated outer segments (Woodruff et al., 1977; Woodruff and Bownds, 1979) and in the outer segment layer in retina (Cohen et al., 1978). Addition of both IBMX and dibutyryl cyclic GMP enhances the level of phosphorylation of components I and II about eightfold. Calcium, which decreases the concentration of cyclic GMP in dark-adapted rod outer segments

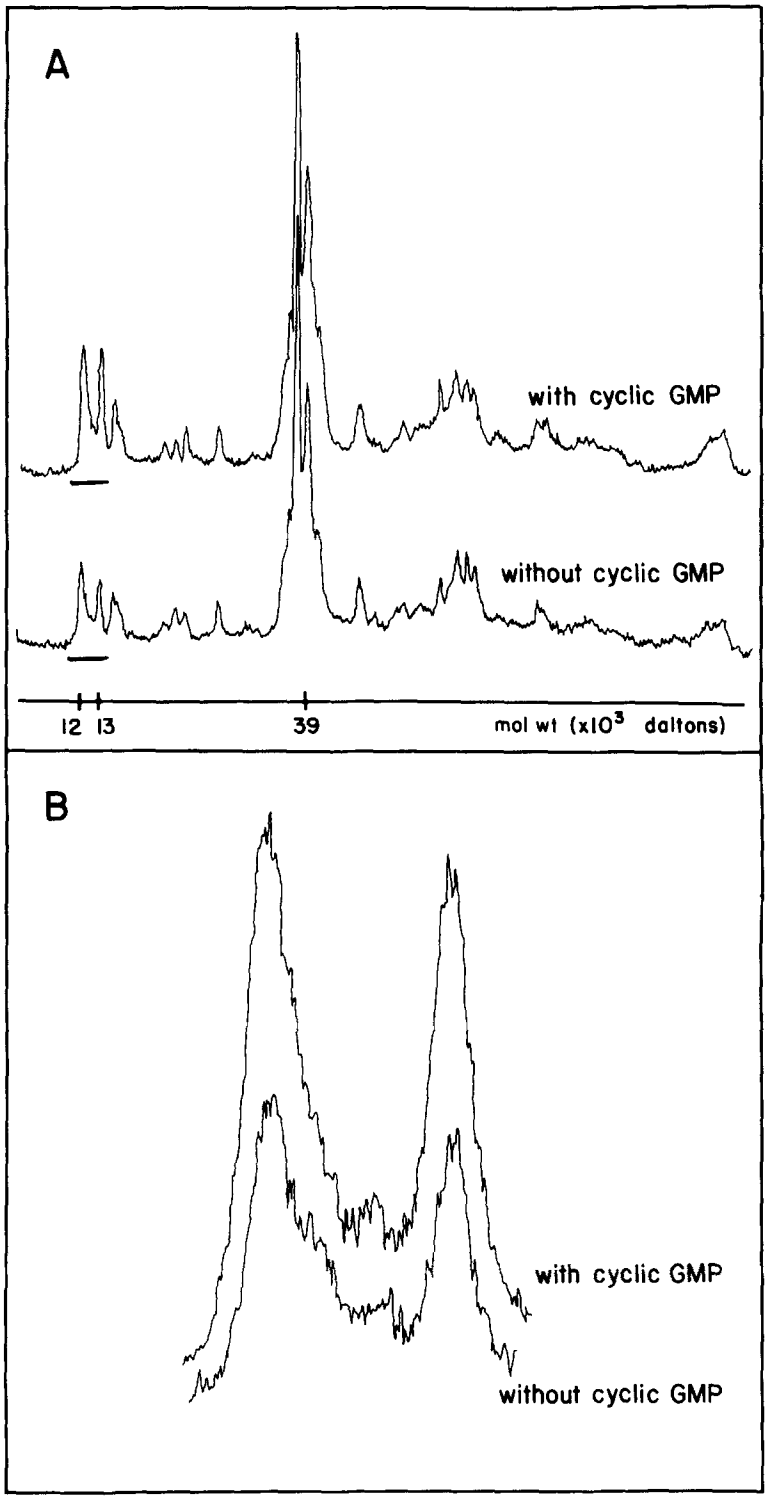
A
with cyclic GMP
without cyclic GMP
12 13
39
mol wt (x10³ daltons)
B
with cyclic GMP
without cyclic GMP

FIGURE 5

(Woodruff and Bownds, 1979), also decreases the phosphorylation level of components I and II by half when added to incubating retinas. Calcium addition to retinas after incubation still elicits a 50% decrease in the phosphorylation level of components I and II. In some experiments (data not shown) entire retinal samples were subjected to electrophoresis after incubation with

TABLE I
PHOSPHORYLATION LEVEL OF COMPONENTS I AND II

	I	II
Control	100%	100%
cGMP	238±48	176±22
EGTA	309±31	339±48
IBMX	622±59	587±81
IBMX/$(BU)_2$cGMP	979±90	743±103
Ca^{+2}	43.4±5.3	59.5±8.5

Influence of pharmacological agents on the phosphorylation level of components I and II. Retinas are incubated with ~ 1 mCi carrier-free $^{32}P_i$ for 8 min in the dark, each in 200 μl Ringer's solution containing either no addition, 5 mM $CaCl_2$, 5 mM cyclic GMP, 3 mM EGTA, 3 mM IBMX, or 3 mM IBMX and 5 mM dibutyryl cyclic GMP. After incubation, retinas are gently agitated for 2 min in 300 μl Ringer's solution without the isotope or additives. Samples are processed for electrophoresis and autoradiography as described in Materials and Methods. Phosphorylation level for the control retina is set at 100% and all other values are expressed as a percent of this value. The data represent at least three experiments (mean ± SEM).

$^{32}P_i$ and IBMX or cyclic GMP. Only the phosphorylation of components I and II was enhanced.

Corresponding effects of these perturbations on the permeability of isolated outer segments (Bownds and Brodie, 1975; Brodie and Bownds, 1976) and intact receptor cells (Lipton et al., 1977 *a*) have been noted, with calcium addition apparently decreasing and EGTA and IBMX addition possibly increasing plasma membrane permeability.

FIGURE 5. *(Opposite)* Cyclic GMP-stimulated phosphorylation of components I and II. Dark-adapted retinas are incubated for 8 min in the dark in 200 μl Ringer's solution containing ~ 1 mCi carrier-free $^{32}P_i$ with or without 5 mM cyclic GMP. At the end of the incubation period, the retinas are each transferred to 300 μl Ringer's solution without the isotope or cyclic GMP and gently shaken for 2 min. Samples from each outer segment suspension are processed for electrophoresis and autoradiography as described in Materials and Methods. (A) Densitometric scans from an autoradiograph of rod outer segment suspensions obtained from retinas incubated with or without cyclic GMP. The solid bar indicates the location of components I and II. (B) Amplified densitometric scan of components I and II shown in part A. This data are from one of four experiments with similar results. The phosphorylation increase due to addition of cyclic GMP is calculated in Table I. The densitometric scans of samples with cyclic GMP are displaced upwards in this figure for clarity.

Phosphorylation in Isolated Rod Outer Segments

When crude suspensions of isolated rod outer segments are incubated with $^{32}P_i$, components I and II become phosphorylated. The labelled inorganic phosphate is incorporated into nucleoside triphosphates,[1] which presumably serve as substrate for the phosphorylation. (The nucleotide pools remain labelled for several minutes after outer segments are removed from $^{32}P_i$.) The level of phosphorylation is never as large as can be obtained by incubating the retina with isotope, possibly because less $^{32}P_i$ is incorporated into nucleoside triphosphates in isolated outer segments. Alternatively, the drop in cyclic GMP concentration that occurs rapidly after detachment of outer segments from the retina (Woodruff et al., 1977) may result in lower phosphorylation of components I and II.

If dark-adapted rod outer segments are suspended in a Ringer's solution containing 5 mM cyclic GMP the phosphorylation level of only components I and II is enhanced and by approximately twofold. Addition of cyclic AMP has a similar effect. IBMX addition increases the phosphorylation of components I and II approximately threefold. While these findings are comparable to those with intact retinas, neither calcium nor EGTA affect the phosphorylation of the components.

No light-induced dephosphorylation of components I and II is observed in isolated rod outer segments (18 separate experiments). In an attempt to observe a light-activated dephosphorylation, the following parameters were considered:

(*a*) Higher light intensities that bleach up to 100% of the rhodopsin molecules present failed to elicit a dephosphorylation.

(*b*) Supplements of 5 mM ADP, GDP, ATP, and GTP in varying combinations did not influence dephosphorylation. These were added to replenish nucleotide pools normally supplied by the inner segment.

(*c*) Apyrase, which hydolyzes ATP and causes partial dephosphorylation of rhodopsin (Miller and Paulsen, 1975) had no effect on the dephosphorylation of components I and II.

(*d*) No effect was observed upon variation of calcium and magnesium concentrations between 10^{-9} and 10^{-2}M, which might induce any phosphatase activity with a divalent cation requirement (Colowick and Kaplan, 1955).

(*e*) Since the initial phosphorylation level was lower in isolated outer segments incubated with $^{32}P_i$ a further light-induced decrease in phosphorylation levels might be difficult to detect. The initial level of phosphorylation therefore was increased either by the addition of 5 mM IBMX or 5 mM cyclic GMP to isolated outer segments or by using outer segments detached from retinas incubated with $^{32}P_i$. No light-induced dephosphorylation was observed in these preparations.

(*f*) A modified Ringer's solution, which is optimal for observing both cyclic GMP and permeability changes in vitro (Woodruff, et al., 1977), does not influence dephosphorylation in isolated outer segments.

Thus, the dephosphorylation of components I and II is severely limited upon

detachment of the outer segments from the retina and in this way is similar to rhodopsin dephosphorylation (Miller and Paulsen, 1975). This will be discussed below.

Solubility Characteristics of Components I and II

When outer segment suspensions that have been phosphorylated in the dark are centrifuged at 1,500 *g* for 1 min, components I and II are found associated with the pellet. This result is not dependent upon the intactness of the outer segments, assayed by the didansyl cysteine fluorescent-staining procedure of Yoshikami et al (1974). Components I and II remain with outer segments or outer segment fragments through the washing procedure whether gentle treatment (Woodruff et al., 1977) has kept more than 90% of the outer segments intact, or whether forcing the outer segments through a syringe needle has reduced them to fragments. Washing the outer segment suspension in Ringer's solution containing either 3 mM EGTA, 3 mM EDTA, 5 mM $CaCl_2$, 1 mM dithiothreitol, or 1 mM GTP does not influence the elution of the components. The elution of the components is not influenced by illumination of the outer segment suspensions (cf. Kühn, 1978).

Components I and II dissociate from outer segment membranes under hypoosmotic conditions. If outer segments are sedimented at 1,500 *g* in Ringer's solution for 1 min, and the pellet is then resuspended in Ringer's solution diluted 20-fold with water and recentrifuged, the phosphorylated components are found in the supernate. This supernate contains almost all of the components originally present and only 2% of the total outer segment membrane (i.e., 2% of the total rhodopsin originally present). Further evidence that the components in the supernate are soluble proteins, rather than possibly being associated with small membrane vesicles, comes from the observation that they remain in the supernate after centrifugation at 100,000 *g* for 15 min.

DISCUSSION

The experiments demonstrate a light-activated dephosphorylation of two low molecular weight proteins of frog rod outer segments. The two proteins (designated components I and II) are shown to undergo rephosphorylation when illumination ceases. While the two protein components have similar molecular weights, it is not known whether they represent different primary sequences, or whether one forms from the other by some post-translational modification. The phosphorylation-dephosphorylation sequence and its sensitivity to drugs appear identical in both. The dark levels of phosphorylation of the two proteins are enhanced by the addition of cyclic GMP or cyclic AMP to retinas or isolated rod outer segments. Several observations, however, suggest that cyclic GMP might normally regulate their level of phosphorylation. In isolated outer segments (Fletcher and Chader, 1976; Woodruff et al., 1977) and in outer segments in the receptor layer of retina (Orr et al., 1976; Goridis et al., 1977; Cohen et al., 1978), the level of cyclic GMP is approximately 10-fold higher than that of cyclic AMP, and in both these preparations only cyclic GMP is light sensitive. After decreasing on illumination, cyclic GMP levels return towards their dark values during a subsequent period in

the dark (Cohen et al., 1978; Woodruff and Bownds, 1979), and this parallels the behavior of the phosphorylation reaction. Further, Cohen et al. (1978) have shown that although EGTA addition to retina increases cyclic GMP concentration, it does not significantly alter levels of cyclic AMP. EGTA similarly enhances the level of phosphorylation of components I and II. To resolve this issue more completely, it would be necessary to isolate the kinase activity responsible for the phosphorylation of components I and II and determine the effectiveness of both cyclic GMP and cyclic AMP in stimulating the phosphorylation reaction. Farber et al. (1979) recently have isolated a protein kinase from cattle outer segments that is activated maximally by 10^{-4} M cyclic GMP. This is close to the concentration found for cyclic GMP in intact frog rod outer segments (Woodruff and Bownds, 1979). Thus, the observed decreases in cyclic GMP would appear to be appropriate for influencing the phosphorylation of components I and II.

Isolated rod outer segments are capable of phosphorylating components I and II and responding to exogenous cyclic GMP, indicating that the kinase activity and its control by cyclic nucleotides are maintained, at least partially, during detachment of the rod outer segment from the retina. However, the finding that components I and II do not dephosphorylate in isolated outer segments might indicate a decrease or absence of a phosphatase activity. The deficiency of a presumed phosphatase activity is not unique to components I and II, for dephosphorylation of rhodopsin is less evident in isolated outer segments (Miller and Paulsen, 1975) than in the living frog (Kühn, 1974). It is possible that the outer segment preparation is missing the phosphatase, a factor necessary for its activity, or that the isolation procedures interfere with a light-activation of the enzyme.

In this regard, it is relevant to note that a lesion of the permeability of outer segments, like the dephosphorylation of components I and II, occurs rapidly after they are detached from the retina, so that the light-suppressible permeability is only 0.1–1% as large as the same permeability in living receptor cells (Bownds and Brodie, 1975; Wormington and Cone, 1978). If the ability of isolated outer segments to perform dephosphorylation undergoes a similar decay, the light-induced dephosphorylation in isolated outer segments might be only 0.1–1% of the amount observed in intact retinas. Such a small change could not be detected with the present techniques.

Although both the light-induced permeability decrease and the dephosphorylation of components I and II appear to be reduced in isolated outer segments, a light-induced decrease in cyclic GMP levels measured by Woodruff and Bownds (1979) has stoichiometry and time-course which agree fairly closely with the permeability changes measured in living receptor cells. This suggests that the changes in components I and II and permeability may occur subsequent to the cyclic GMP changes in the normal sequence of events. If this were the case, one possible sequence for steps in transduction would be: light absorption by rhodopsin → activation of cyclic GMP phosphodiesterase → lowering of cyclic GMP levels → dephosphorylation of components I and II → decrease in plasma membrane permeability. The step involving dephos-

phorylation of components I and II would be limited in isolated outer segments.

The present data are consistent with such a relationship between cyclic GMP levels, the phosphorylation of components I and II, and outer segment permeability. The light-induced dephosphorylation of components I and II upon illumination of the retina is observed over the same range of illumination that suppresses both cyclic GMP levels and permeability in isolated outer segments. Changes in calcium concentration, and the other pharmacological perturbations listed in Table I, influence cyclic GMP, the phosphorylation of components I and II, and the permeability of isolated outer segments similarly. These same agents, at similar concentrations, were used by Lipton et al. (1977 *b*) on living retinas to demonstrate that conditions which increase the concentration of cyclic GMP depolarized the rod membrane potential, most likely by increasing its light-sensitive permeability.

It should be emphasized, however, that a scheme linking components I and II directly to membrane permeability control represents only one of many possibilities. These components might play a different role in visual transduction, perhaps regulating slower events which occur during light or dark adaptation. It has been suggested that cyclic GMP also might participate in these slower mechanisms (Woodruff et al., 1977). Alternatively, components I and II might function in regulating the general metabolism of the outer segment and be very indirectly linked to the permeability mechanism. Experiments to further resolve their function would include determining the kinetics of the dephosphorylation-rephosphorylation reaction and noting correlations with changes in cyclic GMP and permeability during transduction. Direct comparisons of the behavior of components I and II with data on cyclic GMP and permeability are difficult to make at this time, because the phosphorylation of the components has been studied in retinas, whereas cyclic GMP and permeability studies have been performed on isolated outer segments (Brodie and Bownds, 1976; Woodruff and Bownds, 1979). Measurements of cyclic GMP in the outer segment layer of intact retinas (see Orr et al., 1976; Cohen et al., 1978) have not been made at comparable levels of illumination. It would be extremely useful to measure all the reactions in the same preparation. Experiments to localize these proteins within the outer segment may also aid in determining their function.

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