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Phosphorylation of Rhodopsin in Bovine Photoreceptor Membranes. A Dark Reaction after Illumination†

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ABSTRACT: We have found protein kinase activity in purified bovine rod outer segments (ROS). This activity requires Mg^{2+} and, as assayed with histones or protamine, is light independent. Rhodopsin, presumably the normal substrate, must be bleached before it can be phosphorylated. The kinase can be extracted from ROS fragments, demonstrated in the extract, and added back to restore the original light-dependent phosphorylation of rhodopsin. This reaction has not been demonstrated *in vivo*. A hypothetical mechanism is discussed in which phosphorylation blocks ionic channels which have been opened in response to light absorption and reduces the light sensitivity of the rod cell.

The retinal rod cell is a specialized neuron containing a receptor system triggered by light. The receptor molecule, rhodopsin, can be isolated relatively easily in amounts that are useful for biochemical and protein chemical studies. Thus, the rod is a useful model system for the study of membrane receptors.

The outer segment (a modified cilium) is the photodetector unit of the rod cell. The rat ROS,[1] for example, contains *ca.* 4×10^7 rhodopsin molecules densely packed in about 1000 parallel "disks" (Penn and Hagins, 1972). The disks are flattened, closed sacs containing primarily rhodopsin and phospholipids in their membranes. Their internal volume is reported to be high in Na^+ concentration (Govardovskiĭ, 1971).

The light sensitivity of the visual system can adapt so as to permit it to function over a wide range of light intensities, perhaps as much as nine orders of magnitude (Rushton, 1969). Two sorts of "light adaptation" can be observed. The one involves changes in the ability to detect, for example, intensity differences between a test spot and a background; this "increment threshold," as it is called, depends on the intensity of the background. Relatively low light intensities are used and the changes occur rapidly. This type of light adaptation presumably involves retinal processing (Rushton, 1969). The other sort of light adaptation involves slow, long-term changes in the sensitivity of the visual system and presumably occurs at the level of the receptor (Dowling and Ripps, 1972; Hodgkin, 1972; Hagins, 1972; Penn and Hagins, 1972). A fully dark adapted rod can, upon absorption of a single photon, respond and trigger the bipolar cell with which it synapses (Hecht *et al.*, 1942; Hagins *et al.*, 1970; Dowling and Ripps, 1972). On the other hand, the threshold and response–stimulus intensity function of skate rods can be shifted by several orders of magnitude in response to a steady background (Dowling and Ripps, 1972). Excessive triggering has also been observed to result in the desensitization of other receptor systems such as those involving the acetylcholine receptor (Thesleff, 1970). The molecular basis of these phenomena is not clear at this time.

It is generally accepted that the primary effect of light absorption by rhodopsin is the isomerization of the 11-*cis*-retinal chromophore to the all-trans form (Wald, 1968). Other changes then occur with, at least *in vitro*, the eventual formation of free *all-trans*-retinal and opsin (Wald, 1968). This process is called bleaching. The original light sensitivity of rhodopsin is restored when, through a cycle of reactions, 11-*cis*-retinal is again linked to the protein. Little is known about other chemical reactions in this cycle.

We have found, as described in a preliminary report (Kühn

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[1] Abbreviation used is: ROS, rod outer segment.

and Dreyer, 1972), evidence for an ATP-dependent reaction in which rhodopsin in bovine ROS is phosphorylated after exposure to light. Bownds *et al.* (1972) have reported similar findings with frog ROS. The present paper points out in more detail the role of light in this reaction. A water-soluble kinase has been found which can phosphorylate rhodopsin and also histones and protamine. The activity of this enzyme is not affected by light. Its substrate rhodopsin, however, must be bleached before it can be phosphorylated. We believe that this reaction, if it occurs *in vivo*, may play a role in the regulation of the rod's light sensitivity.

Materials and Methods

Rod Outer Segments from Bovine Eyes. ROS fragments from bovine eyes were prepared according to the procedure of McConnell (1965) with the following modifications (Dreyer *et al.*, 1972): the homogenizing solution (40% sucrose + 1 mM Tris-acetate) also contained NaCl (65 mM) and $MgCl_2$ (1.0 mM) and the discontinuous sucrose gradient solutions contained $MgCl_2$ (0.1 mM) in order to reduce nuclear rupture and contamination by endoplasmic reticulum membranes. The preparation was done under dim red light (General Electric 25-W BAS) at 0–4°.

The final ROS membrane suspensions (protein concentrations 3–6 mg/ml) were stored in small portions in the dark, either frozen at −20° in 8% sucrose or frozen at −185° in 20% glycerol after sonication at 0° (five times 1 sec at level 3, Branson sonicator).

Freezing and thawing ruptured the cell membrane surrounding the parallel disks and the disks formed vesicles (Papermaster, D. S., unpublished results). Sonication was employed to make the vesicles more uniform in size.

Protein concentrations were determined by amino acid analysis of acid hydrolyzed samples using a Beckman Model 121 analyzer.

Phosphorylation Conditions. Concentrations were, unless stated otherwise in the text or figure legends, 3 mM [^{32}P]ATP, 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.3), 20 mM KCl, 6 mM Na^+ (from ATP disodium salt), and 2% sucrose (or instead, 4% glycerol). The volume was 50 or 100 μl and the concentration of ROS membrane was 0.6–1.2 mg of protein/ml. The ATP stock solution was neutralized with Tris to pH 7.3 and its concentration determined by absorption at 259 nm. The specific activity of [^{32}P]ATP was usually between 10^3 and 10^4 cpm/nmol and was determined in each experiment.

The reaction was started by adding ROS suspension to the prewarmed (2–3 min at 37°) buffer–[^{32}P]ATP mixture under dim red light. The tube was shaken briefly using a Vortex mixer and incubated at 37° for the time and illumination conditions indicated in each case. The reaction was stopped by adding 0.3 ml of ice-cold 12% trichloroacetic acid (Cl_3CCOOH) containing 20 mM ATP and 5 mM H_3PO_4 when the precipitated membrane was to be filtered and the radioactivity counted on the filters (see section on filtering). When the reaction mixture was to be separated by sodium dodecyl sulfate gel electrophoresis, the reaction was stopped by adding sodium dodecyl sulfate denaturing cocktail (see next section).

Sodium Dodecyl Sulfate Gel Electrophoresis. Gels containing 5.8% polyacrylamide (3.6% cross-linked), 1% sodium dodecyl sulfate, 40 mM Tris-acetate (pH 7.4), and 2 mM EDTA were used according to the procedure of Fairbanks *et al.* (1971). Samples were prepared for electrophoresis either by solubilization of the acid precipitated and centrifuged material with a denaturing cocktail containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 10 mM Tris-HCl (pH 8.0), 10% sucrose, and Pyronin Y (tracking dye) for 30 min at 37° or by adding 0.1 vol of a 10 times concentrated denaturing buffer (containing 10% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 10 mM Tris, and Pyronin Y) to the reaction mixture after phosphorylation and denaturing for 30 min at 37°. In the latter case, a few sucrose crystals were added to each sample before application to the gels.

After electrophoresis, [^{32}P]ATP and $^{32}PO_4^-$, which migrated together with the tracking dye, were removed either by cutting off the lower end of the gel or by fixing the whole gel with 25% Cl_3CCOOH for about 1 day (three changes of Cl_3CCOOH).

Radioactively labeled protein bands were detected and analyzed by one of three methods.

(i) The gels were cut into two strips of equal thickness with a razor blade. One strip was wrapped with thin plastic film to prevent it from drying and shrinking and was exposed to X-ray film. Overnight exposure was sufficient when the radioactivity was about 5000 cpm or more per band. Smaller activities necessitated longer exposure times. The other half of the gel was stained for proteins with Coomassie Brilliant Blue.

(ii) The gels were cut into 1-mm slices (100–110 per gel) using a Brinkmann "Mickle" gel slicer. Each slice was put into a scintillation vial and extracted for 1–2 days by shaking in the dark with 5 ml of a mixture containing 0.5 ml of 0.6 M NCS (tissue solubilizer, Amersham-Searle) and 4.5 ml of scintillation fluid containing 42 ml of Liquifluor (New England Nuclear) per 960 ml of toluene. ^{32}P was counted in a Beckman liquid scintillation counter.

(iii) Instead of cutting the gel into 1-mm slices, whole ^{32}P-labeled protein bands of 0.5–2.0 cm length were cut out. Their position on the gel was determined either by previous radioautography or by scanning at 280 nm using a Gilford spectrophotometer. This was possible because essentially only rhodopsin, and in some experiments histones and protamine, were expected to be radioactively labeled. Rhodopsin gave an absorption peak at 280 nm because of its aromatic amino acids, and the histones and protamine because of light scattering by the acid-precipitated proteins. The slices were extracted by shaking for 1–2 days with 1.5–3 ml of 0.6 M NCS solubilizer. Then 5–10 ml of scintillation fluid (42 ml of Liquifluor/960 ml of toluene) were added and ^{32}P was counted as above.

Filtering. The acid-precipitated reaction mixture was filtered through Millipore HAWP 0.45-μ filters using slight pressure. For small amounts of membrane containing as much as 40 μg of protein, small filters of 13-mm diameter were used as described (Kuhn and Dreyer, 1972). With larger amounts of material, however, the small filters tended to plug up and 25-mm diameter filters were used with the following modifications to minimize the background of low molecular weight radioactivity [^{32}P]ATP and $^{32}PO_4^{3-}$) adsorbed to the filters.

The filters were presoaked with ice-cold 5% Cl_3CCOOH–20 mM ATP–50 mM H_3PO_4 for a few minutes and 5 ml of the same solution was placed in the syringe connected to the filter. The precipitated membrane was rinsed into the syringe with 5–7 ml of the above Cl_3CCOOH solution and filtered with pressure. The filter was washed three times with 6–7 ml of Cl_3CCOOH—first with 5% Cl_3CCOOH–20 mM ATP–50 mM H_3PO_4 as above and then with 5% Cl_3CCOOH–2 mM ATP–5 mM H_3PO_4. If histones or protamine were present, 25% Cl_3CCOOH was used instead of 5%. Blank values were obtained by filtering [^{32}P]ATP with Cl_3CCOOH-precipitated, unlabeled

membrane under the same conditions as above. The blank values were subtracted from the sample values; they amounted to about 3% of the sample values at maximum incorporation.

The filters were dried in scintillation vials and 10 ml of scintillation fluid (42 ml of Liquifluor/960 ml of toluene) was added to each. They were counted in a Beckman liquid scintillation counter.

Exposure to Light during or before Incubation with [^{32}P]ATP. Two different light sources were used, as indicated later in the text for the particular experiments: white light and orange light.

WHITE LIGHT. A General Electric reflector flood lamp, 120 V/150 W, was installed vertically above the water bath to shine directly into the tubes. The light intensity at 100 cm from this lamp was about 100 footcandles, as measured with a General Electric light meter. Half of the rhodopsin was bleached in 30 sec under these conditions.

ORANGE LIGHT. In some experiments orange light was used in order to avoid secondary isomerization of the *all-trans*-retinal produced by bleaching of the rhodopsin (Wald and Brown, 1956). The light source was a 100-W film projector lamp equipped with an orange filter (OG 570), which absorbed light of wavelengths shorter than 570 nm, and an infrared filter. Bleaching and phosphorylation were carried out in 0.5-ml quartz cuvets of 1-cm path length. The distance from the lamp to the cuvet was 7 cm. Half of the rhodopsin was bleached in 35 sec at ROS concentrations of 0.3–0.6 mg of protein/ml. Each sample was divided after phosphorylation into smaller portions for filtration and/or gel electrophoresis.

Spectroscopy. Adsorption spectra of ROS suspensions (0.3–0.6 mg of protein/ml) from 700 to 300 nm were measured using a Cary 14 spectrophotometer. Quartz cuvets of 0.5-ml volume and 1-cm path length were used. Bleaching difference spectra were obtained by subtracting the spectrum of a bleached sample from its spectrum before bleaching. The absorption due to light scattering, assumed to be invariant upon bleaching, was thus eliminated. Indeed, the changes in E_{300} and E_{700} upon complete bleaching were both negative and rarely greater than 0.03. "Bleaching" of rhodopsin was defined as the relative decrease in E_{500} after exposure to light. No detectable bleaching occurred during the measurement of spectra.

Regeneration of Rhodopsin after Bleaching. All operations were done in dim red light. 11-*cis*-Retinal was dissolved in methanol to 15 mg/ml and suspended in water at about 1 mg/ml. After sonication at 0° and evaporation of the methanol with N_2, the concentration was adjusted to 0.5 mg/ml with H_2O. The purity of the 11-*cis*-retinal was checked by thin layer chromatography (silica gel, 3% acetone in 30–60° petroleum ether) and its concentration was determined by spectroscopy. Aliquots of this suspension were stored at −185° until used. An 11-*cis*-retinal concentration of 8–20 μg/ml was used for the regeneration of ROS suspensions at 0.3–0.6 mg of protein/ml. Regeneration, determined by rebleaching, was maximal after 2–3 hr at 25°. Between 60 and 120% of the original E_{500} could be regenerated depending on the ROS preparation used.

Tris–EDTA Extraction of ROS. The ROS suspension (6 mg of protein) was diluted to 3.0 ml in 10 mM Tris-HCl (pH 8.0)–5 mM EDTA and sonicated lightly at 0°. One-third was taken aside and the rest (2.0 ml) was centrifuged for 30 min at 98,000*g*. The pellet was sonicated in 3 ml of 10 mM Tris-HCl (pH 8.0)–5 mM EDTA–2.7 M urea, diluted to 9 ml with 10 mM Tris-HCl (pH 8.0)–5 mM EDTA, and centrifuged for 30 min at 98,000*g*. This pellet was resuspended by sonication in 6 ml of 10 mM Tris-HCl (pH 8.4)–5 mM EDTA and centrifuged for 30 min at 98,000*g*. Half of the final pellet was resuspended in 1.0 ml of the first Tris–EDTA extract and the rest in 1.0 ml of 10 mM Tris-HCl (pH 8.0)–5 mM EDTA. All operations were done under dim red light at 0–4°. The original ROS suspension, the pellet after the Tris–EDTA–urea and Tris–EDTA washings, and the pellet resuspended in the first Tris–EDTA extract were tested for light-dependent rhodopsin phosphorylation.

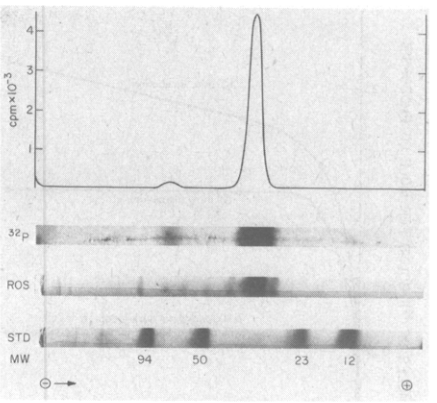

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of ^{32}P phosphorylated ROS membrane: (a) molecular weight standard gel containing phosphorylase *a* (94,000), bovine γ-globulins (50,000 and 23,000), and cytochrome *c* (12,000); (b) ^{32}P phosphorylated ROS membrane, half-gel, stained with Coomassie Brilliant Blue; the mol wt of rhodopsin is about 35,000; (c) radioautograph of the nonstained half of gel b; (d) distribution pattern of ^{32}P in a gel run under the same conditions as b, and cut into 100 slices of 1-mm thickness. ROS (30 μg) was phosphorylated in a 50-μl suspension containing 3 mM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 20 mM KCl, 20 mM Tris-HCl (pH 7.3), 6 mM Na^+, and 2% sucrose for 1 hr at 37° under continuous illumination with white light.

Materials. Histones I and II from pea seedlings were kind gifts of Dr. Jerry Johnson. Protamine sulfate from salmon was purchased from Sigma. [γ-^{32}P]- and [α-^{32}P]ATP were purchased from ICN. Chemicals used were analytical grade. 11-*cis*-retinal was prepared by photoisomerization of *all-trans*-retinal (Eastman Kodak Co.) in methanol and separation of the isomers by tlc (Akhtar *et al.*, 1968).

Results

Radioactivity is incorporated in to the ROS membrane when membrane suspensions are incubated with [γ-^{32}P]ATP and $MgCl_2$ at neutral pH in the presence of light (Kühn and Dreyer, 1972). Sodium dodecyl sulfate gel electrophoresis (of the acid-precipitable material) shows that the radioactivity migrates together with rhodopsin at an apparent mol wt of *ca.* 35,000 (Figure 1).

A faint radioactively labeled band sometimes appears at an apparent mol wt of *ca.* 70,000. Its intensity relative to that of rhodopsin depends on the denaturing conditions; we assume that it is a dimer of rhodopsin due to incomplete dissociation. In some instances of insufficient denaturation, we found a series of higher molecular weight radioactive proteins including trimers, tetramers, and higher oligomers of rhodopsin. However, none of the minor bands that normally appear on the Coomassie Brilliant Blue stained gels (Figure 1) were ever found to be radioactively labeled. Quantitative scintillation counting of the acid-precipitated material could therefore be carried out without further purification of [^{32}P]rhodopsin.

When [α-^{32}P]ATP was used instead of [γ-^{32}P]ATP, only

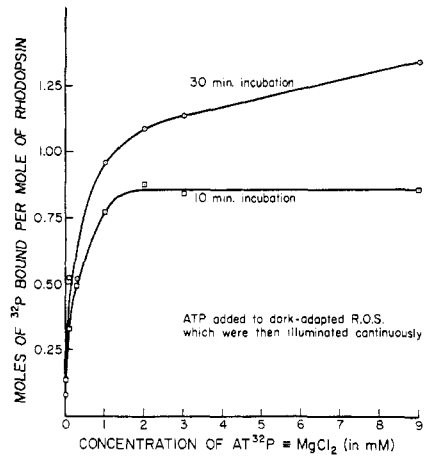

FIGURE 2: Phosphorylation of rhodopsin, dependence on ATP concentration: upper curve, phosphorylation extent after 30 min at 37° at various ATP concentrations; lower curve, phosphorylation extent after 10 min at 37°. Each sample contained 30 μg of ROS proteins in a 50-μl suspension containing 20 mM Tris-HCl (pH 7.3), 20 mM KCl, 2% sucrose, and various concentrations of [γ-^{32}P]ATP and $MgCl_2$. The concentration of $MgCl_2$ was similar to the [^{32}P]ATP concentration. Samples were mixed in dim red light and then illuminated during the full incubation period with white light of 100 footcandles intensity. Radioactivity of the Cl_3CCOOH-precipitated material was counted on filters.

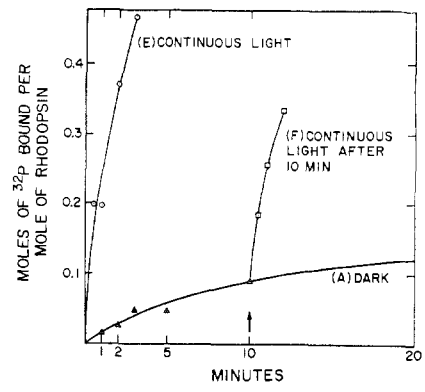

FIGURE 3: Phosphorylation rate of rhodopsin, light dependence: curve A, samples were mixed under dim red light and incubated at 37° in the dark for the times indicated; curve E, samples were mixed under dim red light; they were exposed to white light (400 footcandles) for the whole incubation time; curve F, samples were mixed under dim red light, incubated in the dark for 10 min, and then (arrow) exposed to white light (400 footcandles) for the rest of the incubation period. ATP concentration in all samples was 3 mM.

negligible amounts of radioactivity were bound to rhodopsin (about or less than 1%, compared to [γ-^{32}P]ATP as checked by gel electrophoresis or collection of the acid-precipitated material on Millipore filters). This indicates that rhodopsin is phosphorylated by transfer of the γ-phosphate group from ATP to the protein, and that the whole nucleotide is not bound tightly to the protein.

Reaction Conditions. The ROS membrane must be bleached during or before the incubation with [^{32}P]ATP. Details about the influence of light are discussed later in the paper.

The reaction requires Mg^{2+} ions. Ca^{2+} cannot substitute for Mg^{2+}. EDTA at higher concentrations than Mg^{2+} inhibits completely. Neither Na^+ nor K^+ is required although our reaction mixture usually contained 20 mM KCl and low concentrations of Na^+ ions (from disodium ATP). These ions seem to have little effect, but no systematic investigation on the influence of Na^+ and K^+ ions has yet been made.

Phosphorylation of rhodopsin in the membrane suspension is prevented by heat denaturation (5 min at 100°), precipitation with Cl_3CCOOH, or solubilization with 1% sodium dodecyl sulfate prior to incubation with [^{32}P]ATP. Some detergents that are reported to preserve the optical properties of rhodopsin, such as 1% Emulphogene (Shichi *et al.*, 1969) or 0.04 M cetyltrimethylammonium bromide (Heller, 1968), also prevent phosphorylation. Phosphorylation takes place, on the other hand, in the presence of the nonionic detergent Triton X-100 at concentrations of 0.1% or lower, although to a lower extent compared to detergent free suspension (unpublished observations).

ATP Concentration. The influence of the [^{32}P]ATP concentration on the phosphorylation extent at two different incubation times at 37° is shown in Figure 2. Above 2 mM ATP, the initial phosphorylation rate (in the first 10 min) is independent of the ATP concentration. At longer incubation times, however, higher ATP concentrations permit a higher final phosphorylation extent. This may in part be due to competition from other processes which consume ATP, such as hydrolysis by a Na^+–K^+-dependent ATPase (Sekoguti, 1960) or formation of cyclic AMP by an adenylate cyclase (Miller *et al.*, 1971).

The maximum phosphorylation extent shown in Figure 2 is about 1.3 mol of phosphate bound/mol of rhodopsin. However, with most preparations we obtained lower phosphorylation extents, usually between 0.6 and 0.9 mol/mol.[2] Prolonged reaction at high ATP concentration apparently leads to the slow incorporation of one or more additional phosphate groups.

Effect of Freezing and Thawing. All phosphorylation data given in this paper were obtained with ROS membrane that was stored frozen and was thawed shortly before incubation with [^{32}P]ATP. This treatment (as well as sonication) destroys the original structure of the outer segment. Instead of parallel disks surrounded by the cell membrane, random vesicles are formed (Papermaster, D. S., unpublished results). Nonfrozen outer segment fragments were phosphorylated to only about one-third the extent of the vesicles obtained after freezing and thawing when incubated with [^{32}P]ATP under the same conditions.

Although subsequent freezing and thawing had no significant effect compared to the first freezing and thawing, repeated freezing and thawing of the same sample of membrane decreased slightly the extent of phosphorylation.

Phosphoprotein Bond. Hydroxylamine did not displace ^{32}P from phosphorylated rhodopsin; this excludes the possibility of an acyl phosphate bond to aspartic or glutamic acid, as reported for phosphorylated intermediates of ATPases (Kahlenberg *et al.*, 1968). Partial acid hydrolysis (3–6 hr at 102° with 2 N HCl) yielded phosphoserine and some (approximately 10%) phosphothreonine together with a series of acidic, phosphorylated peptides (Kühn and Dreyer, 1972). [^{32}P]Serine and [^{32}P]threonine were purified by preparative paper

[2] Molar concentrations of rhodopsin were calculated on the basis of two assumptions: (1) its molecular weight is *ca.* 35,000 (Cavanagh and Wald, 1969) although published values range between 27,000 (Heller, 1968, 1969; Heller and Lawrence, 1970; Shields *et al.*, 1967) and 40,000 (Daemen *et al.*, 1972; Heitzmann, 1972; Hubbard, 1954; Robinson *et al.*, 1972) and (2) rhodopsin makes up 85% of the protein in our membrane preparations (Dreyer *et al.*, 1972; Heitzmann, 1972; Daemen *et al.*, 1972). These values, and hence the molar phosphorylation extents calculated on their basis, may be subject to change.

electrophoresis at pH 1.7 and identified by comparison with commercial phosphoserine and phosphothreonine standards by paper electrophoresis at pH 1.7 (6.7% formic acid) and at pH 3.5 (1% pyridine–10% acetic acid–water). After complete hydrolysis with 6 N HCl, the amino acids serine and threonine were identified by paper electrophoresis as well as with a Beckman amino acid analyzer.

Preliminary analyses of ^{32}P-labeled peptides obtained by cyanogen bromide or tryptic cleavage of rhodopsin indicate that the phosphorylated site or sites are localized in a restricted portion of the rhodopsin polypeptide chain (Hargrave, P. A., *et al.*, unpublished results). Sequence studies of rhodopsin and the peptides containing the phosphorylated site(s) are in progress.

Light Dependence. Figure 3 shows that phosphorylation of rhodopsin is stimulated by light. Some phosphorylation at a very low rate and extent took place in the dark; we assume that this is due to the incomplete dark adaptation of our ROS preparations. Illumination significantly increased both the phosphorylation rate and extent (curves E and F).

It is not necessary to illuminate the membrane during the full incubation period. Under our illumination conditions, complete bleaching was achieved in about 3 min. Complete phosphorylation, on the other hand, took much longer—at least 1 hr. There was virtually no difference in the final phosphorylation extent when ROS were illuminated during the full incubation period or when the light was turned off after bleaching was complete and the rest of the phosphorylation was effected in the dark.

This indicates that phosphorylation is a dark reaction *after* bleaching of rhodopsin, rather than an immediate response to light itself. Accordingly, addition of [^{32}P]ATP in the dark to prebleached ROS membrane and incubation in the dark also led to high phosphorylation extents, although not as high as when [^{32}P]ATP was present during bleaching.

To measure the correlation between bleaching extent and phosphorylation extent more quantitatively, ROS were illuminated in the presence of 3 mM [^{32}P]ATP and 3 mM $MgCl_2$ with orange light for various times (5 sec to 10 min); incubation at 37° was then continued in the dark. The bleaching extent was measured spectrophotometrically and the phosphorylation extent was measured after 1 hr of total incubation time. The correlation between bleaching and phosphorylation was roughly linear; the more rhodopsin bleached, the more phosphorylated. We are in the process of studying this relationship further in order to determine whether the correlation is exactly linear when completely dark adapted ROS are used.

Phosphorylation of Histones and Protamine. Histones I and II from peas and protamine from salmon were incubated with ROS suspension and [^{32}P]ATP for 1 hr at 37°. The phosphorylated proteins were separated from each other by sodium dodecyl sulfate gel electrophoresis and radioactivity was counted in 1-mm slices of the gels. Figure 4 shows the result of a typical experiment with ROS and histone II. Both rhodopsin and histone II were phosphorylated when the mixture was illuminated for the first 5 min of the incubation period (Figure 4a). When, however, the incubation was carried out completely in the dark, almost no radioactivity was bound to rhodopsin while histone II was phosphorylated to the same extent as in light (Figure 4b). Both samples contained the same amounts of ROS membrane and were run under the same conditions except for illumination.

The same result was obtained when histone I or protamine was added to ROS. Quantitative phosphorylation data for these different substrates are given in Table I. Neither bovine

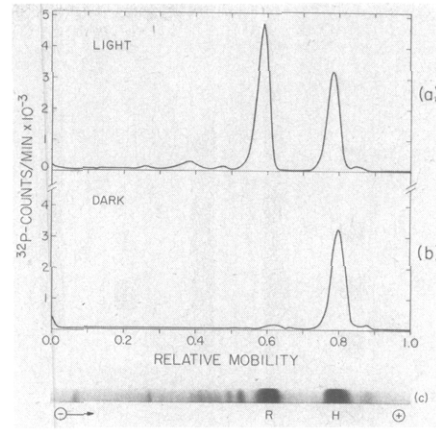

FIGURE 4: Phosphorylation of ROS and histone II, followed by sodium dodecyl sulfate gel electrophoresis. The gels a, b, and c contained 70 μg of ROS proteins and 70 μg of histone II. The samples were incubated in a volume of 40 μl with 1.3 mM [^{32}P]ATP, 3 mM $MgCl_2$, 20 mM KCl, 20 mM Tris-HCl (pH 7.3), and 5% glycerol for 30 min at 37°. Sample a was illuminated with white light (100 footcandles) for the first 5 min of its incubation period and incubated in the dark for the remaining 25 min. Samples b and c were both incubated completely in the dark. Gels a and b were sliced into 1-mm thick slices each of which was counted for ^{32}P; gel c was stained with Coomassie Brilliant Blue, after fixing with 25% Cl_3CCOOH in order to precipitate the histone. See Materials and Methods for details.

serum albumin, γ-globulins, nor cytochrome *c* was phosphorylated when incubated with ROS membrane and [^{32}P]ATP under the above conditions.

The kinase activity was soluble in Tris–EDTA (Table II). The pellet after extraction and urea treatment incorporated no phosphate unless resuspended in the Tris–EDTA extract; this restored the original light-dependent phosphorylation of rhodopsin.

Sodium dodecyl sulfate electrophoresis of the final pellet and the Tris–EDTA extract demonstrated that several of the minor proteins present in ROS are indeed removed by the Tris–EDTA (Figure 5). The pellet contained mostly rhodopsin, while some of the minor ROS proteins were considerably enriched in the Tris–EDTA extract.

Reversibility of Phosphorylation; Regeneration. Phosphorylation was essentially irreversible in our system; no dephosphorylation occurred with a rate comparable to that of phosphorylation. An excess of cold ATP added after phosphorylation with [^{32}P]ATP did not displace ^{32}P from rhodopsin. Neither did addition of 11-*cis*-retinal to bleached and phosphorylated ROS suspensions. Phosphorylated, as well as nonphosphorylated, opsin was regenerated to rhodopsin by added

TABLE I: Phosphorylation of Different Proteins by the ROS Kinase (nmol of ^{32}P Bound per nmol of Protein).[a]

Protein	Light	Dark
Rhodopsin	0.50	0.05
Histone I	0.16	0.17
Histone II	0.14	0.13
Protamine	0.24	0.27

[a] Experimental conditions as in Figure 4. The radioactive bands were cut out of sodium dodecyl sulfate gels and counted after solubilization. Molecular weights were assumed to be 35,000 for rhodopsin, 25,000 for histone I, 14,000 for histone II, and 4200 for protamine.

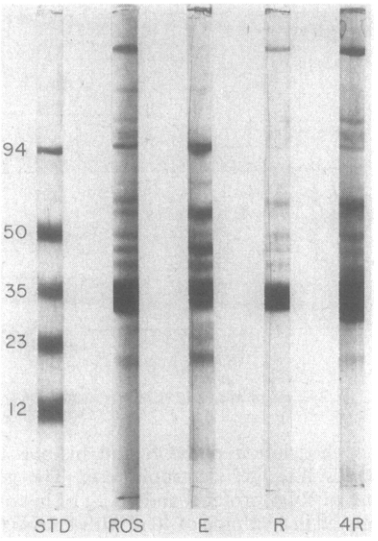

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis of ROS extracted with 10 mM Tris-HCl–5 mM EDTA (pH 8.0); STD, mol wt standard containing phosphorylase *a* (94,000), γ-globulins (50,000 and 23,000), pepsin (35,000), and cytochrome *c* (12,000); ROS = complete, nonextracted ROS, 40 μg of protein; E, first extract of 180 μg of ROS proteins; R, insoluble residue of 45 μg of ROS proteins after three extractions; 4R, the same as R, but with a four times higher loading on the gel (corresponding to 180 μg of extracted ROS).

11-*cis*-retinal, and no radioactivity was removed from the protein in the process (Table III). Furthermore, no phosphate was lost upon rebleaching of the regenerated phosphorylated rhodopsin. Thus, it appears that phosphorylation and regeneration after bleaching are two processes quite independent from each other.

Discussion

It has been shown that rhodopsin binds roughly equimolar amounts of phosphate (as phosphoserine and to a much lower extent as phosphothreonine) when suspensions of disrupted ROS containing ATP and $MgCl_2$ are exposed to light. Light does not stimulate phosphorylation directly; illumination of the ROS prior to or at the beginning of the incubation with ATP leads to phosphorylation in the dark as well as in the light.

One can think of several ways in which the initial light exposure might influence the reaction. Does light activate a kinase? Or does it "activate" the substrate rhodopsin? That is, does light activate rhodopsin so as to permit it to act as its own kinase or do molecular alterations resulting from the bleaching process expose one or more phosphorylation sites on rhodopsin to a kinase? Our results strongly favor the last possibility. Removal of the kinase activity with Tris–EDTA prevents the light-dependent phosphorylation of rhodopsin and the reaction can be restored by readdition of the enzyme extract. The kinase, either in the ROS or in the extract, is able to phosphorylate certain other proteins, such as histones or protamine, *independently* of light (see Figure 4 and Table I). The only substrate found to require illumination is rhodopsin; it could be phosphorylated only after it had been bleached. Plots of the extent of phosphorylation as a function of the extent of bleaching were roughly linear. The latter result is, however, somewhat puzzling since it differs radically from results recently reported by a group working with frog retinas. Thus, Bownds *et al.* (1972) report light-induced phosphorylation of

TABLE II: Kinase Activity in the Various Fractions of the Tris–EDTA Extraction of ROS.[a]

Fraction	Rhodopsin (nmol)	^{32}P (light) (nmol)	^{32}P (dark) (nmol)
Unextracted ROS	*Ca.* 1.2	0.90	0.08
Extracted ROS	*Ca.* 1.0	0.03	0.01
Extracted ROS + kinase extract	*Ca.* 1.0	0.95	0.01

[a] Each sample tested corresponded to 50 μl of ROS proteins subjected to extraction. Phosphorylation was done either in room light or in the dark with the following concentrations: 3 mM Na_2 [^{32}P]ATP, 5 mM $MgCl_2$, 2.5 mM EDTA, 20 mM KCl, and 20 mM Tris-HCl (pH 7.3). The samples were incubated 1 hr at 37°, denatured with 1% sodium dodecyl sulfate–1% β-mercaptoethanol for 1 hr at 37°, and run on sodium dodecyl sulfate–polyacrylamide gels. The gels were frozen on Dry Ice after electrophoresis and cut into 1-mm slices. The slices were solubilized overnight in 0.5 ml of NCS and, after the addition of 4.5 ml of Liquifluor in toluene, were counted for ^{32}P.

frog rhodopsin under similar conditions. They find, however, that rhodopsin need not be bleached in order to be phosphorylated; bleaching of 1% of the rhodopsin led to the incorporation of 0.5 mol of phosphate/mol of rhodopsin, whereas we require 40–60% bleaching to achieve the same extent of phosphorylation in our bovine system. It is hard, at the present, to quantitatively compare our results with those of Bownds' laboratory. Where Bownds *et al.* used intact frog ROS in isotonic solution, we used freeze-thawed or sonicated bovine ROS which were not completely dark adapted.

The question naturally arises whether the soluble kinase is intrinsic to ROS or is a contamination. We believe that it is intrinsic. There are three steps during McConnell's (1965) preparation procedure which should remove soluble enzymes: two pelletings and one sucrose gradient. Complete nuclei should also be separated easily, and Mg^{2+} and Na^+ ions were added to prevent nuclear rupture. Electron micrographs of our preparations showed very clean ROS fragments without other structures present (Papermaster, D., unpublished results). Hence, it seems unlikely that nuclear or other kinases may contaminate our ROS preparations and cause artifactual phosphorylation of rhodopsin.

Phosphorylation was irreversible in our system. This should obviously not be the case *in vivo*, if phosphorylation plays any role there, since very little of the rhodopsin in dark adapted ROS is phosphorylated (Hall and Bacharach, 1970). It is possible that, *in vivo*, a phosphatase dephosphorylates phosphorylated rhodopsin. We might easily have removed or inactivated this phosphatase in the course of our ROS isolation and purification procedure.

Although *in vivo* phosphorylation of rhodopsin has not been demonstrated, we feel that the reaction is not an *in vitro* artifact. We have found kinase activity in rod outer segments which is specific for bleached rhodopsin and such proteins as histones and protamine which have been shown to be kinase substrates; unbleached rhodopsin and other proteins such as bovine serum albumin, γ-globulins, and cytochrome *c* are not phosphorylated. These data imply that changes in rhodopsin upon bleaching make it a suitable substrate for the kinase. It is known from other biological systems that the phosphorylation of proteins by kinases is used to control quite diverse pro-

TABLE III: Regeneration of Rhodopsin: Comparison of Nonphosphorylated with Phosphorylated Opsin.[a]

		Before Bleaching	After Bleaching	Change	After Regeneration	After Rebleaching	Change
Not phosphorylated	E_{500}	1.96 ± 0.05	1.63 ± 0.04	0.34 ± 0.03	1.89 ±0.09	1.65 ± 0.07	0.24 ± 0.04
Phosphorylated	E_{500} mol of ^{32}P/mol of rhodopsin	0.07	0.90	0.83	1.80 0.86	1.58 0.77	0.22 −0.08

[a] The values for the phosphorylated case are the result of a single experiment run in parallel with nonphosphorylated samples; all were done with the same preparation. Those for the nonphosphorylated case are the average of five experiments. The phosphorylated sample contained 1.2 mg of protein/ml, 3 mM [^{32}P]ATP, 3 mM $MgCl_2$, 25 mM Tris-HCl (pH 7a–3), 6 mM Na^+, and 4% glycerol. It was bleached for 5 min with orange light ($\lambda > 570$ nm) and incubated in the dark for 70 min to complete phosphorylation. 11-*cis*-Retinal was then added as a water suspension to a final concentration of 15 μg/ml and regeneration was allowed to take place for 3 hr at 25° in the dark. The sample was diluted to 0.6 mg of protein/ml with 3 mM $MgCl_2$–25 mM Tris-HCl (pH 7.3) for spectrophotometry. Phosphorylation extents were measured with aliquots taken before the addition of 11-*cis*-retinal, after 3 hr of regeneration, and after rebleaching with white light (~100 footcandles) for 5 min. In two cases, the nonphosphorylated samples were treated the same as the phosphorylated one, except that no ATP was present. In the other three, however, no incubation at 37° was done. There was no significant difference and the results have been averaged together. The E_{500} values in all cases correspond to a protein concentration of 0.6 mg/ml.

cesses (*e.g.*, Krebs *et al.*, 1966; De Lange *et al.*, 1968; Balhorn *et al.*, 1972). It can be argued that, since a unique phosphorylated site has not yet been demonstrated and, furthermore, since some phosphothreonine as well as phosphoserine are found after partial acid hydrolysis, the reaction is not likely to be part of the normal visual cycle. That may be true, but such site specificity does not appear to be a requirement for biological function (*e.g.*, Riley *et al.*, 1968). In any case, certain properties of the reaction such as the specificity of the kinase for bleached rhodopsin, and the localization of the phosphorylated sites to a restricted portion of the polypeptide chain suggest that it may be involved in the functioning of the rod cell.

The phosphorylation of rhodopsin is, in our system, much too slow to be part of the primary response to light. However, as significant phosphorylation does occur within a few minutes after bleaching (Figure 3), the reaction is fast enough to be involved in the regulation of the rod's light sensitivity (Dowling and Ripps, 1972). Our first proposal was that phosphorylation of opsin blocked its regeneration to rhodopsin. This would keep the bleached and phosphorylated rhodopsin molecules nonfunctional. This is not the case, since phosphorylated rhodopsin can be regenerated as well as nonphosphorylated (Table III).

It has been proposed (Yoshikami and Hagins, 1972; Hagins, 1972; Wu and Stryer, 1972) that the message of photon absorption is transferred from disk to cell membrane by the diffusion of Ca^{2+} ions normally contained within the disks. Bleaching of a rhodopsin molecule might open an associated "channel" and release ions, just as the Na^+–K^+ channels open in the acetylcholine receptor complex. It seems possible that phosphorylation of the bleached rhodopsin might lead to the closing of a transmitter channel and thus to desensitization of the receptors. This would allow the "recharging" of the disk by ATP-powered ion pumps present in the disk membrane and, at the same time, prevent further light activation of that channel. Subsequent dephosphorylation by a phosphatase, perhaps after regeneration, could restore the ability of the rhodopsin molecule to open its channel upon bleaching.

There are, of course, a number of alternative hypotheses which might explain the biological significance of the *in vitro* studies and further experiments will be needed to determine what, if any, *in vivo* role is played by the kinase-mediated phosphorylation reaction reported here.

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Oxidation of 3,3′-Diaminobenzidine by Rat Liver Mitochondria†

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ABSTRACT: The dye 3,3′-diaminobenzidine (DAB) has been used previously (Seligman, A. M., Karnovsky, M. J., Wasserkrug, H. L., and Hanker, J. S., *J. Cell Biol. 38*, 1 (1968)) for ultrastructural location of cytochrome oxidase in liver and kidney mitochondria. An *in vitro* study of the biochemical site of DAB oxidation by liver mitochondria is reported here. Studies of mitochondrial oxygen utilization, using the optimal DAB concentration of 4 mM in a highly buffered medium, showed that DAB oxidation was not inhibited by antimycin A but was inhibited by cyanide. Mitochondria depleted of cytochrome *c* required addition of exogenous cytochrome *c* in order to oxidize DAB, and polylysine inhibited mitochondrial oxidation of DAB. These studies show that the site of interaction of DAB with the respiratory chain is at cytochrome *c* and therefore support the conclusion that the ultrastructural location of cytochrome *c* is at the outer surface of the inner mitochondrial membrane. Reduction of cytochrome *c* by DAB was demonstrated spectrophotometrically and indicated a reduction of three to four cytochrome *c* molecules by each DAB molecule. Mitochondrial oxidation of DAB gave a stoichiometry of one DAB molecule oxidized by each oxygen molecule. Production of free radicals during oxidation of DAB was demonstrated using the sulfite chain reaction. Based on these data, a mechanism is proposed where DAB loses four electrons to yield a free radical intermediate, which subsequently polymerizes to give the final product.

Seligman *et al.* (1968) have reported cytological evidence that the product of mitochondrial oxidation of the dye DAB[1] accumulates on the outer surface of the mitochondrial inner membrane. They concluded that the reaction of electron donors with cytochrome *c* was located at that intramitochondrial site. Because such information is important in understanding enzyme compartmentation in mitochondria, a thorough *in vitro* study of the biochemical steps involved in DAB oxidation by rat liver mitochondria is being reported here.

Materials and Methods

DAB was obtained as the tetrachloride from the Sigma Chemical Co. and was dissolved in 10 mM Tris-chloride (pH 7.4). TMPD was obtained as the dichloride from Eastman Organic Chemicals. Sodium sulfite was obtained from Merck and Co. and sodium hydrosulfite was obtained from Baker Chemical Co. Other reagents and substrates were obtained from the Sigma Chemical Co. Stock solutions of glutamic, malic, and succinic acids were titrated to pH 7.4 using Trizma base, and NaADP was kept at pH 6.9. Polylysine hydrobromide (Sigma Types VI-B and VII-B) was dissolved in the respiration medium described below.

Rat liver mitochondria were prepared by the usual method (Johnson and Lardy, 1967) of homogenization and differential

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[1] Abbreviations used are: DAB, 3,3′-diaminobenzidine; TMPD, tetramethylphenylenediamine; ADP, adenosine 5′-diphosphate.