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Light-Induced Phosphorylation of Rhodopsin in Cattle Photoreceptor Membranes: Substrate Activation and Inactivation†

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ABSTRACT: The ability of rhodopsin in cattle rod outer segment (ROS) membranes to be phosphorylated in the presence of $AT^{32}P$ is induced by light, and thereafter slowly decreases in the dark. This light activation and dark inactivation are shown to be properties of the membrane-bound rhodopsin and to be independent of the presence of the extractable kinase. The dark inactivation is reversible under appropriate conditions; ROS membranes, which had been thoroughly bleached in the absence of ATP and allowed to lose their phosphorylation ability by a subsequent prolonged incubation in the dark, could be "reactivated" for phosphorylation if they were reilluminated for long times with white light in the presence of $AT^{32}P$. This apparent reactivation is shown to be due to "recycling" of the substrate, rhodopsin. A likely pathway of recycling is regeneration of rhodopsin from opsin and *11-cis*-retinal produced by photoisomerization of *all-trans*-retinal; subsequent bleaching then makes it available again as a substrate for phosphorylation. Conditions which minimize recycling, namely, short illumination with orange light, also suppressed "reactivation". If ROS membranes containing various mixtures of rhodopsin and opsin were illuminated in the presence of $AT^{32}P$ and kinase under conditions of minimum recycling, the phosphate incorporation corresponded exactly to the amount of freshly bleached rhodopsin and was independent of the amount of opsin present, suggesting that only the freshly bleached rhodopsin was phosphorylated. All the data presented are consistent with the model that the kinase activity is not affected by light, and that the substrate, rhodopsin, is changed by light such that it can be phosphorylated by the kinase, but this ability to be phosphorylated slowly decays. Thus, cattle opsin at long times after bleaching is not a substrate for the kinase, similar to unbleached rhodopsin.

Rhodopsin, the visual pigment located in the photoreceptor membranes of the vertebrate retina, is phosphorylated in a slow reaction consequent to photon capture (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973). On bleaching, the γ-phosphate group of ATP is enzymatically transferred to serine and threonine residues of rhodopsin in the presence of Mg^{2+} and a soluble protein kinase present in ROS. While light is necessary to initiate the reaction, the phosphorylation itself is a dark reaction. Although the reaction appears to be too slow to be involved in visual excitation (half time 1–2 min; Kühn and Bader, 1976), it is a possible candidate for a role in light/dark adaptation.

The light stimulation of the phosphorylation reaction has been well established; however, the mechanism of this stimulation is not clear. Several investigators using cattle ROS have proposed that the kinase is always active, independent of light, and that rhodopsin is converted into a substrate for this kinase only on bleaching (Kühn et al., 1973; Frank et al., 1973; Weller et al., 1975; Frank and Buzney, 1975); i.e., the *substrate is activated.* However, Bownds et al. (1972) have presented evidence obtained with frog ROS which suggests that bleaching a small number of rhodopsin molecules results in the phosphorylation of many unbleached rhodopsin molecules. Therefore, the role of light would not be simply the conversion of rhodopsin into a substrate for the kinase. Also, Frank and Buzney (1975) showed that illumination of cattle "opsin" preparations containing only small amounts of residual rhodopsin results in the same phosphate incorporation as illumination of previously unbleached rhodopsin preparations. These data might be interpreted as evidence for *activation of the kinase* mediated by bleaching of the rhodopsin. Nevertheless, Frank and Buzney (1975) concluded from other experiments that the kinase was not light activated, though they could not explain how the opsin could then be phosphorylated as a consequence of illumination if the kinase were not light activated. The present work presents specific experiments aimed at determining whether the light stimulation of the phosphorylation reaction is due to light activation of the kinase, or to light activation of the substrate, rhodopsin.

Methods

Preparation of Cattle and Frog Rod Outer Segments. Retinas were dissected from cattle eyes which had been enucleated shortly after slaughter and placed in the dark on ice for a few hours before use. The preparation of ROS[1] followed a

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[1] Abbreviations used: ROS, rod outer segments; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; cGMP, guanosine 3′,5′-monophosphate; sem, standard error of the mean; DTT, dithiothreitol.

slight modification of the method described by McConnell (1965) and Papermaster and Dreyer (1974). ROS were sometimes prepared immediately after dissection, but more often the retinas were frozen by dropping into liquid N_2 and stored at −60 °C until use. The highest phosphorylation yields were obtained with preparations in which all solutions contained 70 mM sodium phosphate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1–5 mM DTT. DTT was found to prevent loss of phosphorylation activity of ROS preparations on prolonged standing at room temperature in atmospheric oxygen. The retinas were vigorously shaken in 45% (w/v) sucrose. The ROS were first floated on 45% sucrose, then sedimented in 15% sucrose, and finally subjected to discontinuous sucrose density gradient centrifugation. Purified ROS were collected from the interface between layers of 0.77 and 0.92 M sucrose. After diluting with buffer and pelleting, the ROS were suspended in buffer and stored frozen in small aliquots at −60 °C. The yield was normally 10–20 nmol of rhodopsin per retina with an OD_{280}/OD_{500} ratio of 2.3 to 2.6.

Frog ROS were prepared as above from the retinas of *Rana esculenta* dark adapted overnight.

Assuming a molar extinction coefficient of 40 600 (Wald and Brown, 1953), the rhodopsin content of the preparations was determined by the light-sensitive OD_{500} of an aliquot solubilized in emulphogene BC 720 (1–5%) containing hydroxylamine hydrochloride (25–50 mM).

Preparation of Extracts Containing Kinase Activity. Kinase activity was extracted from ROS membranes into aqueous buffers at 0–5 °C under dim red light as described earlier (Kühn et al., 1973) with some modifications. Homogenization of the ROS suspended in the extracting buffer, 10 mM Tris-HCl–5 mM EDTA–0–5 mM DTT (pH 8.0), was preferred to sonication because even mild sonication caused difficulties in the separation of the membrane fragments from the supernatant. The most active extracts were obtained when DTT was present and when the combined extracts from two to three subsequent extractions were concentrated using Centriflo membrane cones (CF-50, Amicon Corp.) with a molecular weight cut-off of 50 000. The kinase activity was retained by the cones and no activity was found in the small molecular weight fraction. The extracted kinase was stored in small aliquots at −60 °C. Incubation of the most active extracts with $AT^{32}P$, but without addition of ROS membranes, generally resulted in a small, light-independent incorporation of phosphate (0.2 nmol per 20 μL of kinase extract). This small phosphate incorporation was subtracted from the total phosphate incorporation in experiments in which kinase extract was added to ROS membranes. No attempt was made to determine the source of this incorporation.

Preparation of Kinase-Depleted ROS Membranes. The membrane fragments obtained after extraction of kinase were treated with alum to destroy any residual kinase activity. The membranes were washed once with water, suspended in 4% alum solution, and allowed to sit in the dark for 30 min at 22 °C. The membranes were then pelleted, washed once with water, and finally suspended in 100 mM phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$ and 1–5 mM dithiothreitol. The intrinsic phosphate incorporation of such preparations in the absence of the extracted kinase was generally between 0.03 and 0.09 mol of phosphate per mol of rhodopsin in light as well as in the dark.

Preparation of "Opsin". "Opsin" was prepared by bleaching ROS suspensions for 30 min at 30 °C with white light in the absence of ATP. The bleached ROS suspension was then incubated in the dark at 30 °C for 4 h to allow for the inactivation of the phosphorylation ability. The residual rhodopsin content of such preparation was about 13%.

Preparation of Retinal-Depleted "Opsin". ROS suspensions were bleached in the presence of 0.5–1 mM NADPH in order to reduce free *all-trans*-retinal to its alcohol (see deGrip et al., 1972). The sample was continuously stirred and illuminated at room temperature for 4 h by a 150-W flood lamp about 35 cm from the sample. A heat filter and a Schott RG 610 cut-off filter (transmission <0.1% at 595 nm) were placed between the lamp and the sample. If first-order bleaching kinetics were followed througout the illumination period, less than 0.1% rhodopsin should be present after 4 h. (In fact, the final rhodopsin content after 4 h was still 2%.) The sample was then placed in the dark for 4 h at 30 °C to allow the light-induced phosphorylation ability to decay. The membranes were collected by centrifugation, washed twice with 100 mM phosphate buffer–1 mM $MgCl_2$–0.1 mM EDTA (pH 7.0), and then suspended in the same buffer. The OD_{280} of the final suspension was measured in order to correct for any losses in protein occurring during the washing procedure. In the cases where ethanolic solutions of *all-trans*-retinal were added to these suspensions, care was taken that the final concentration of ethanol did not exceed 1%. The *all-trans*-retinal (Fluka) was checked by high-pressure liquid chromatography to contain no detectable amounts (less than 1%) of the 11-cis and 9-cis isomers (P. Carl, personal communication).

Phosphorylating Procedure. The final concentration of $AT^{32}P$ was 3 mM in all experiments presented. Phosphorylating buffer was prepared by adding small amounts of [γ-^{32}P]ATP in 50% ethanol (Amersham Buchler) to a buffer containing 4.5 mM ATP–12 mM $MgCl_2$–20 mM Tris-HCl, pH 7.3. The ethanol concentration never exceeded 1%. The specific activity of the phosphorylating buffer was determined from the concentration of the ATP measured spectrophotometrically, and the measured radioactivity of aliquots; it was usually between 1000 and 10 000 cpm per nmol of ATP. The following operations were carried out on ice under dim red light. ROS (20–25 μL) suspension containing 1–5 nmol of rhodopsin was pipetted into small tubes and the volume was brought to 50 μL by adding appropriate amounts of water and/or extracted kinase. The samples were then mildly sonicated for 2 min in a Buehler Ultramet III sonic cleaner. One hundred microliters of the above phosphorylating buffer was added to the samples which were then placed in a thermostated water bath, usually at 30 °C, and kept in the dark for a few minutes to allow temperature equilibration. The addition of phosphorylating buffer is referred to in the text as addition of $AT^{32}P$. The samples were then incubated in the dark or in light for 1 h to allow completion of the phosphorylation reaction (Kühn et al., 1973). The samples were then precipitated and filtered as described below. Dark incubated samples were run simultaneously with identical light samples to facilitate comparison. If phosphorylating buffer was added to ROS suspensions after bleaching, both the buffer and the ROS sample were prewarmed before bleaching.

After incubation, the samples were precipitated by addition of 200 μL of an ice-cold solution containing 25% (w/v) Cl_3CCOOH–20 mM ATP–5 mM H_3PO_4 and placed on ice for a few minutes before filtering. The precipitated samples were poured into a suction filtering apparatus containing 10 mL of ice-cold 10% Cl_3CCOOH–2 mM ATP–5 mM H_3PO_4 and were then filtered. The reaction tubes were rinsed into the filtering apparatus with five 1-mL portions of the above 10% Cl_3CCOOH solution. The filters were washed once with 10 mL of the above solution and then three times with 10% Cl_3CCOOH–70 mM H_3PO_4. The filters were placed in scintillation vials and dried. A standard scintillation cocktail in

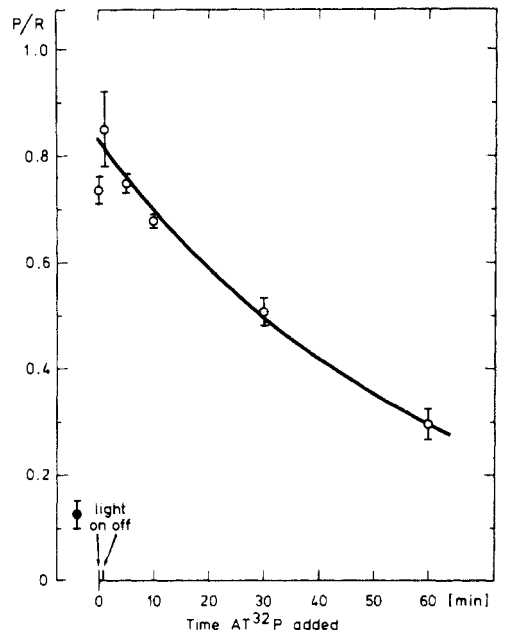

FIGURE 1: Dependence of the phosphorylation extent on the time elapsed between illumination and addition of $AT^{32}P$. Whole (= unextracted) ROS suspensions were illuminated for 1 min with white light which bleached about 90% of their rhodopsin. $AT^{32}P$ was then added in the dark at the times indicated on the abscissa. The extent of phosphorylation was determined after a further dark incubation of 1 h after addition of $AT^{32}P$ for each sample.[2] The ordinate represents phosphate incorporation in mol of phosphate per mol of total rhodopsin (as determined before bleaching). Each point is the average of three to five determinations with the sem represented by the error bars. The filled circle to the left of time zero represents the dark phosphorylation level of the ROS preparation used. The solid line is the least-squares fit of the data, excluding time zero, to a first-order process. T = 30 °C.

toluene was added, and the radioactivity was measured in a Packard scintillation spectrometer. Teflon filters (LSWP 02500, Millipore) were used because they did not interfere with the counting or filtering efficiency and had lower backgrounds of adsorbed ^{32}P than the HAWP filters normally used. The ^{32}P background, as measured by filtering samples of ROS which were first Cl_3CCOOH-precipitated followed by addition of phosphorylating buffer, was usually 0.01–0.02 nmol of phosphate and was subtracted from the rhodopsin phosphorylation values presented.

Illumination Conditions. The samples were illuminated from above with white light from a 150-W flood lamp at a distance of ca. 32 cm. The intensity at the samples was about 400 footcandles. Orange illumination was obtained using the above geometry, but with orange plexiglass (orange 478, Röhm GmbH) covering the sample compartment. The spectral characteristics of the orange plexiglass are shown in the insert in Figure 3. The intensity of the light could be varied by changing the distance between the lamp and the samples. But normally, if not stated otherwise, "bleaching with white light" means illumination at 400 footcandles which bleached half of the rhodopsin present within the first 13 s, and "bleaching with orange light" occurred under a light intensity by which half of the rhodopsin was bleached within the first 91 s (see Figure 3). If bleaching was not intended, the ROS samples were handled in dim red light, which is sometimes denoted as "dark" condition.

Results

Phosphate Incorporation in the Light and in the Dark. A small amount of phosphate incorporation is observed on incubating cattle ROS with $AT^{32}P$ in the dark. However, if the incubation is carried out in light, the phosphate incorporation is greatly enhanced. The light-induced phosphate incorporation can therefore be defined as the incorporation observed in light minus that observed in the dark. Preparations with the highest light-induced phosphate incorporation were generally obtained using isotonic phosphate buffer in the presence of DTT, and starting from freshly dissected retinas. The ratio of light-induced to dark phosphorylation was about 10:1 or even higher in these preparations, and the phosphorylation yield was between 1 and 2 (maximally 2.4) mol of phosphate incorporated per mol of rhodopsin at full bleaching. Preparations in which hypotonic Tris buffer was used (McConnell, 1965; Papermaster and Dreyer, 1974) showed significantly lower light-induced phosphate incorporation (0.34 mol of phosphate per mol of rhodopsin; ratio light:dark = 3.4). For most of the following experiments, such preparations with high light-induced phosphorylation activity were used. It should be stated that all these ROS preparations had completely lost their capability to dephosphorylate phosphorylated rhodopsin (opsin) in the dark. Phosphate once bound to the protein remained so irreversibly, in contrast to the living organism (Kühn, 1974) and to crude frog ROS preparations (Miller and Paulsen, 1975) where dephosphorylation takes place in the dark. Therefore, dephosphorylation does not interfere with the activation/inactivation experiments described in this report and does not complicate their interpretations.

Decay of Phosphorylation Ability in the Dark. It is known that phosphorylation of rhodopsin is a dark reaction (e.g., Kühn et al., 1973), but on closer examination it is seen that the phosphate incorporation obtained in the dark after bleaching is in fact somewhat lower than that obtained in continuous illumination. If, for instance, ROS were bleached for 1 min in the presence of $AT^{32}P$, subsequent incubation for 1 h in the dark resulted in a phosphate incorporation that was only 75% of the value observed for an identical sample incubated in continuous white light for 1 h. This suggests that the ability of the system to phosphorylate bleached rhodopsin in the presence of $AT^{32}P$ (the *"phosphorylation ability"*) may decay in the dark. To check this in more detail, ROS suspensions were bleached for 1 min with white light, and then $AT^{32}P$ was added at various times after bleaching. The samples were further incubated in the dark for 1 h after addition of $AT^{32}P$ to allow completion of the phosphorylation reaction, and the phosphate incorporation was then determined.

The amount of phosphate incorporated decreased as the time interval between bleaching and addition of $AT^{32}P$ was increased (Figure 1). The solid line in Figure 1 is a least-squares fit of the data to a first-order process (omitting time zero), assuming that, at infinite time between bleaching and $AT^{32}P$ addition, no phosphate would be incorporated. The data fit the line reasonably well, but this is not adequate proof that the decay of phosphorylation ability is a first-order process. The rate constant for the line drawn is 0.017 min^{-1} which gives a half-time of 41 min.

It should be mentioned that the rate of inactivation varied considerably from one preparation to another. In most preparations this rate was much faster than in the one shown in Figure 1. In one ROS preparation, for instance, the phosphorylation activity was below the original dark value after only

[2] Using the Student t test, the higher $^{32}PO_4$ incorporated on adding $AT^{32}P$ after the 1-min illumination as compared with addition before the illumination was found to be significant for cattle ROS (n = 24, t = 2.19, 2% < P < 5%). In contrast, for dark-adapted frog ROS, more $^{32}PO_4$ was incorporated on adding $AT^{32}P$ before the illumination than after (n = 6, t = 2.28, 5% < P < 10%).

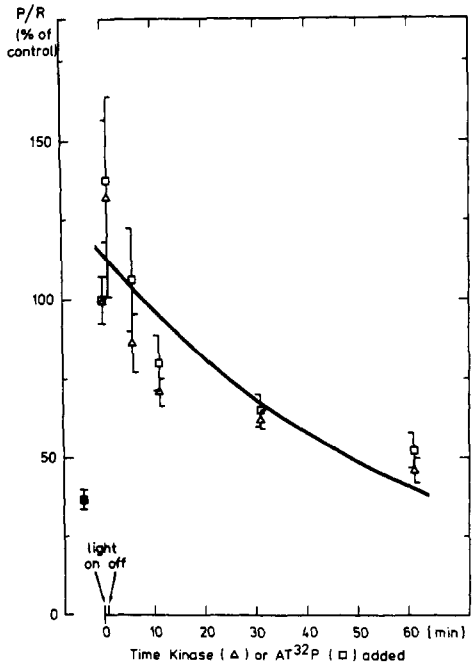

FIGURE 2: Dependence of the phosphorylation extent on the time elapsed between illumination and addition of either kinase or $AT^{32}P$. (Δ) $AT^{32}P$ was added to samples of kinase-depleted ROS. The samples were then illuminated for 1 min with white light, and kinase extract was added in the dark at the times indicated. The sem is indicated by error bars to the right of each point. (□) Kinase extract was added to kinase-depleted ROS. The samples were then illuminated for 1 min with white light, and $AT^{32}P$ was added in the dark at the times indicated. The sem is indicated by error bars to the left of each point. Phosphate incorporation was determined in both sets of samples 1 h after the addition of kinase extract (Δ) or $AT^{32}P$ (□), respectively. Each data point is the average of four to five determinations. The phosphate incorporation is given on the ordinate as percent of the control. The control was prepared by addition of both $AT^{32}P$ and kinase extract to kinase-depleted ROS before illumination. The filled square represents the dark phosphate incorporation of the kinase-depleted ROS in the presence of both $AT^{32}P$ and kinase extract. The solid line was taken from Figure 1 where data obtained with whole ROS (a different preparation) are shown. $T = 30$ °C.

30 min in the dark at 22 °C, while another preparation treated in the same way still retained about 50% of its phosphorylation ability. Four hours in the dark at 30 °C after bleaching was found to be sufficient for most preparations to reach phosphorylation extents in the dark near those of original, unbleached dark samples from the same preparation. The decay of phosphorylation ability in the dark is fully reversible, as will be shown later (see reactivation section).

Activation and Decay of Activity in Kinase-Depleted Rod Outer Segments. The next experiment was done to determine if it is the kinase, or the substrate, rhodopsin, or both together which is (are) *transiently* activated by light. In Figure 2, $AT^{32}P$ was added to one set of kinase-depleted ROS samples before illumination and kinase extract was added at various times after illumination. For another set of samples, kinase extract was added before illumination and $AT^{32}P$ was added at various times after illumination. The data show that the activation and inactivation of the phosphorylation ability in both cases are similar and roughly follow that observed for whole, unextracted ROS. These experiments show that: (1) the activation of the phosphorylation ability occurs in the absence of kinase in agreement with other workers (Frank and Buzney, 1975; Miller and Paulsen, 1975); and (2) the inactivation of the phosphorylation ability also occurs in the absence of the kinase.

"Reactivation" of the Phosphorylation Ability. The phosphorylation ability has been shown to decay in the dark after bleaching. However, virtually complete reactivation by a second bleach could be obtained under appropriate conditions. If the data in the light columns of rows A and B of Table I are used to compute the mol of phosphate incorporated per mol of opsin, one sees that illumination of opsin preparations for 1 h with white light yields almost the same phosphate incorporation (0.61 mol of phosphate per mol of opsin) as does similar illumination of a previously unbleached preparation (0.79 mol of phosphate per mol of rhodopsin). Similar results have been reported by Frank and Buzney (1975). However, as will be shown in the following, special conditions are necessary to produce this high reactivation, namely, prolonged illumination with white light. If "opsin" was illuminated for only 1 min with white light and then incubated for an additional hour in the dark, only 22% of the reactivation achieved with a 1-h white light illumination was observed. Thus 1-min illumination is not sufficient to produce high reactivation, although the light intensity was sufficient to bleach most of the

TABLE I: Light-Stimulated Phosphorylation of "Opsin" and of a 10:1 Mixture of "Opsin" and Rhodopsin.[a]

Sample, amounts	Absolute amount of phosphate (nmol) incorp on incubation with $AT^{32}P$	
	Dark	Light
(A) "Opsin", 9.6 nmol	1.18 ± 0.08 (6)	5.90 ± 0.31 (3)
(B) "Fresh-bleached" rhodopsin, 0.96 nmol	0.50 ± 0.08 (6)	0.76 ± 0.13 (4)
Pred for mixture, if no stimulation occurs (=A + B)	1.68	6.66
(C) Measured for mixture of 9.6 nmol of "opsin" and 0.96 nmol of "fresh-bleached" rhodopsin	1.44 ± 0.06 (6)	6.28 ± 0.35 (4)

[a] "Opsin" was prepared as described in Methods and contained ca. 13% rhodopsin. "Fresh-bleached" rhodopsin was bleached in the absence of ATP for 1 min with white light and then immediately added in the dark either to $AT^{32}P$ buffer alone (row B), or to "opsin" plus $AT^{32}P$ buffer (row C). The samples were then mildly sonicated and incubated for 1 h at 30 °C either in the dark ("dark" column), or in white light ("light" column), before the phosphate incorporation was determined. The phosphorylation of "opsin" alone was measured under the same conditions. The values are given ± sem with number of determinations in parentheses.

TABLE II: Phosphate Incorporation by Retinal-Depleted "Opsin" in the Dark, in Orange Light, and in White Light; Effect of the Addition of *all-trans*-Retinal.[a]

Sample	Mol of phosphate incorp per mol of total opsin + rhodopsin		
	Dark	Orange light	White light
"Opsin"	0.332 ± 0.004	0.420 ± 0.012	1.14 ± 0.01
"Opsin" + retinal	0.394 ± 0.001	0.572 ± 0.015	1.92 ± 0.04

[a] Samples were mixed and $AT^{32}P$ was added in the dark before they were incubated for 1 h at 30 °C in continuous illumination. They were run in duplicates so that the errors shown represent the range of the individual measurements. "Opsin" means retinal-depleted "opsin" prepared as described in Methods, resulting in >70% reduction of the retinal content. Each sample contained 2.0% rhodopsin (=0.0167 nmol) and 98% opsin (=0.807 nmol) in a final incubation volume of 150 μL. A 1.66-fold molar excess of *all-trans*-retinal over the amount of opsin was added to the samples in the lower row.

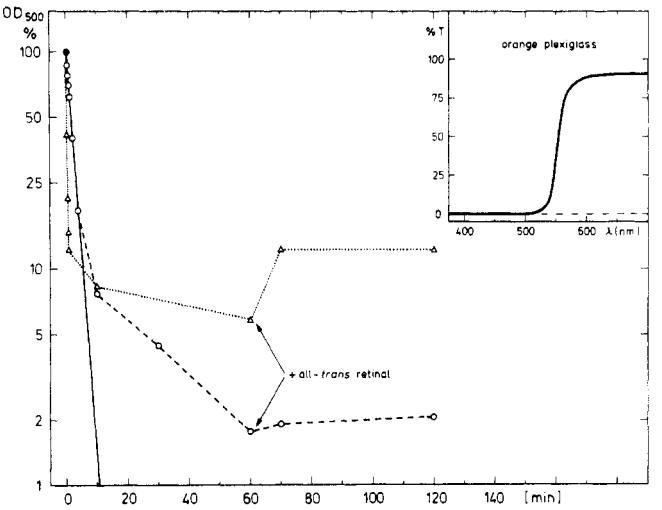

FIGURE 3: Dependence of OD_{500} on the total illumination time in orange or white light; effect of adding *all-trans*-retinal. ROS suspensions were illuminated with orange (○) or white (△) light (see Methods) under phosphorylating conditions. After illumination for the times indicated on the abscissa, the samples were solubilized by addition of a concentrated solution of emulphogene and hydroxylamine. The photosensitive OD_{500} was determined (see Methods) as a measure of the apparent rhodopsin concentration and is shown logarithmically on the ordinate as the percentage of an unbleached sample (●). After 60 min of illumination, *all-trans*-retinal (2.45 mol per mol of rhodopsin originally present) was added, and the illumination was continued. The solid line is a least-squares fit of the data obtained for the first 2 min of orange illumination to a first-order process (half-time 1.5 min). The insert shows the absorption characteristics of the orange filter used.

rhodopsin present. As will be shown later, prolonged illumination with orange light will also not lead to high reactivation (Table II).

Activation Is Not Observed on Mixing "Active" Membranes with "Inactive" Membranes. The experiment shown in Table I was done to test whether it is possible to reactivate the phosphorylation of dark-kept opsin, whose phosphorylation ability had decayed, by adding a "fresh-bleached" ROS preparation, whose phosphorylation ability was still potent. If the soluble kinase in the fresh-bleached ROS was activated, mixing it with opsin in the dark should result in the phosphorylation of both opsin and fresh-bleached ROS. This experiment is analogous to those reported by Bitensky et al. (1973) and Goridis and Virmaux (1974) in which adding a small portion of bleached rhodopsin led to full light activation of the cGMP phosphodiesterase in the dark. If phosphorylation of the opsin in the mixture of row C, Table I, occurs due to addition of the fresh-bleached ROS, the amount of phosphate incorporated on incubation of the mixture in the dark should be higher than the sum of the phosphates incorporated by each component of the mixture. The data show clearly that this was not observed. Therefore activation of the phosphorylation of an opsin preparation cannot be achieved by addition of fresh-bleached ROS. Similar results were also obtained with a 1:1 mixture of opsin and fresh-bleached ROS.

Effect of all-trans-Retinal on Reactivation in Orange and White Light. The following experiments were performed to test if the reactivation phenomenon described above may be due to the continuous production of regenerated rhodopsin simply by photoisomerization of *all-trans*-retinal in continuous white light. An opsin preparation was depleted of free retinal as much as possible by bleaching in the presence of NADPH and subsequent washing (see Methods). The residual rhodopsin content of this opsin preparation was 2.0%. AT^{32}P was added to all samples in the dark, and a 1.66-fold molar excess of *all-trans*-retinal over opsin was added to some of the samples in the dark. The phosphate incorporations after 60-min incubation at 30 °C in the dark, in orange light, and in white light, are shown in Table II. The light values shown include the dark incorporation.

White light causes a much greater phosphate incorporation than orange light, and this effect is enhanced by addition of *all-trans*-retinal. The differences in the extent of reactivation observed under the different incubation conditions of Table II become more obvious if light-induced (= light minus dark) phosphate incorporations are calculated per mol of residual rhodopsin present in the opsin preparation before illumination. In the sample incubated in white light with added *all-trans*-retinal, this value is as high as (1.92 − 0.394) mol of phosphate/mol of (opsin + rhodopsin) ÷ 0.02 mol of residual rhodopsin/mol of (opsin + rhodopsin) = 76.3 mol of phosphate per mol of residual rhodopsin; in the sample incubated in orange light in the absence of added *all-trans*-retinal, this value is only 4.4 phosphates per residual rhodopsin. Since it is highly unlikely that a single rhodopsin molecule binds 76 phosphate groups, we have to assume that in white light many opsin molecules become phosphorylated in addition to the few residual rhodopsin molecules present. The data in Table II suggest that photoisomerization of *all-trans*-retinal and the subsequent regeneration and bleaching of rhodopsin, i.e., recycling, is indeed a major cause of the reactivation observed. Similar results were obtained with other retinal-depleted opsin preparations. If less *all-trans*-retinal was added than in the samples shown in Table II, less increase in phosphorylation was observed for both orange and white light illumination.

Bleaching Kinetics of Rhodopsin in White and Orange Light. Bleaching experiments with ROS suspensions were carried out with white and orange light under the same conditions as above, with the exception that nonradioactive ATP was used. A series of identical samples was prepared, illuminated for various times, and then immediately solubilized by addition of emulphogene and hydroxylamine. The light-sensitive OD_{500} of these solutions was determined as a measure of the residual rhodopsin (and isorhodopsin) content. As can be seen in Figure 3, bleaching initially follows first-order kinetics in both orange and white light, but, in both, deviation from first-order kinetics is obvious at long illumination times indicating that recycling was occurring.

The initial bleaching rate in white light (half-time 13 s) was faster than in orange light (half-time 91 s). However, the rhodopsin content after long illumination times was higher in white light than in orange light. This apparent discrepancy can be explained by the assumption that recycling of rhodopsin is much greater in white light than in orange light. Free *all-trans*-retinal should absorb negligible amounts of the orange light, but it absorbs white light, and this is known to lead to some formation of 11-*cis*- (and 9-*cis*-) retinal (Dieterle and Robeson, 1954) which can then combine with opsin to form rhodopsin (isorhodopsin). In fact, addition of *all-trans*-retinal to samples illuminated for 60 min and then further illuminated caused an increase in the rhodopsin content (Figure 3). This strongly supports the recycling hypothesis.

Some recycling also occurs in orange light. The spectral characteristics of the orange light make it unlikely that isomerization occurs due to absorption of light by free retinal. However, retinal bound to a protein or to phosphatidylethanolamine by a protonated Schiff base linkage absorbs in the visible region and could absorb under these conditions leading to isomerization (cf. Erickson and Blatz, 1968; Shichi and Somers, 1974) and therefore recycling. This was the major difficulty encountered in preparing highly bleached opsin samples for the phosphorylation experiments described above.

TABLE III: Effect of the Presence of Opsin on Phosphate Incorporation under Conditions of Minimal Recycling.[a]

Sample	Experimental conditions	Phosphate incorporation 1 % bleach in presence of $At^{32}P$	2 Total (nmol of ^{32}P)	3 Light induced (nmol of ^{32}P)	4 Light-induced P/R bleached in presence of $AT^{32}P$
(A) Light	Light 15 s, dark 3 h, + $AT^{32}P$, light 15 s, dark 1 h	9.3	0.538 ± 0.007 (2)	0.192	0.812 ± 0.059
Dark	Light 15 s, dark 3 h, + $AT^{32}P$, dark 1 h		0.346 ± 0.013 (2)		
(B) Light	Dark 3 h, + $AT^{32}P$, light 15 s, dark 1 h	11.8	0.576 (1)	0.241	0.803
Dark	Dark 3 h, + $AT^{32}P$, dark 1 h		0.335 ± 0.030 (2)		
(C) Light	+ $AT^{32}P$, light 15 s, dark 3 h, dark 1 h	11.8	0.694 ± 0.001 (2)	0.238	0.793 ± 0.010
Dark	+ $AT^{32}P$, dark 3 h, dark 1 h		0.456 ± 0.002 (2)		
(D) Light	Dark 3 h, + $AT^{32}P$, light 30 s, dark 1 h	21.1	0.696 ± 0.047 (2)	0.361	0.673 ± 0.106
Dark	As dark in part B		0.335 ± 0.030 (2)		
(E) Light	+ $AT^{32}P$, light 15 s, dark 3 h, light 15 s, dark 1 h	21.1	0.819 ± 0.015 (2)	0.362	0.676 ± 0.028
Dark	As dark in part C		0.456 ± 0.002 (2)		

[a] Each sample contained 2.54 nmol of rhodopsin from the same ROS preparation. The samples were illuminated for 15 or 30 s with orange light. The orange light intensity was such that each 15-s illumination bleached ca. 11% of the rhodopsin present; thus, in the first illumination, 11.8%, and in the second illumination, 9.3% of the rhodopsin originally present were bleached. The first column shows the amount of rhodopsin bleached in the presence of $AT^{32}P$; this number equals the total amount bleached for most samples, except for sample A where $AT^{32}P$ was present only in the second bleach. The second column shows the absolute amounts of phosphate incorporated per sample ± difference to the individual measurements. The light-induced phosphate incorporation shown in the third column was obtained by subtracting the dark from the light values in the second column. In the fourth column, the light-induced phosphate incorporation is given in mol of phosphate per mol of *rhodopsin bleached in the presence of* $AT^{32}P$. The temperature was 30 °C.

NADPH reduces the free retinal concentration in outer segment suspensions by converting retinal to retinol (deGrip et al., 1972). With less retinal present to be photoisomerized, less rhodopsin will be produced by recycling. Therefore, higher bleaches can be obtained by prolonged bleaching in the presence of NADPH.

Conditions Which Suppress Recycling Also Suppress Reactivation of Phosphorylation. The following experiments were performed under conditions which should minimize the recycling of rhodopsin, namely, orange light and relatively short illumination times (15 s). The aim was to determine whether the phosphorylation of opsin can be activated by bleaching neighboring rhodopsin molecules *in the same membrane* under conditions suppressing the recycling mechanism of opsin reactivation. If activation occurs, one should find higher phosphate incorporation per rhodopsin bleached in the presence of $AT^{32}P$ in membranes containing both opsin and rhodopsin than in membranes containing only rhodopsin.[3]

For sample A of Table III, the opsin was produced by a 15-s illumination in the absence of $AT^{32}P$. The samples were then kept dark for 3 h at 30 °C to allow complete decay of the light-induced phosphorylation ability. $AT^{32}P$ was then added and the sample was illuminated an additional 15 s. For this second bleach performed in the presence of $AT^{32}P$, the total amount of light-induced phosphorylation was slightly less than in other samples (B and C) which had been subjected to only one 15-s bleach which was in the presence of $AT^{32}P$. Thus, although a total of nearly twice as much rhodopsin had been bleached in A as compared with B and C, the phosphate incorporation was not higher. In fact, the phosphate incorporation per rhodopsin bleached in sample A is virtually identical with that in B and C when it is calculated on the basis of the amount of rhodopsin bleached in the presence of $AT^{32}P$ (last column of Table III). Therefore, phosphorylation of sample A can be quantitatively accounted for by assuming that only the freshly bleached rhodopsin and none of the opsin was phosphorylated. Similar results were obtained in three other experiments using two different ROS preparations and different light intensities adjusted to bleach from 10.7 to 18.3% of the rhodopsin present during a 15-s illumination period.

In control experiments done simultaneously with those of Table III, the 3-h dark incubation was shown to have no effect on the light-induced phosphate incorporations. Samples (D and E, Table III) which received a total of two 15-s bleaches in the presence of $AT^{32}P$ both had the same light-induced phosphate incorporation regardless of whether the two bleaches were separated by a 3-h dark period (E) or delivered consecutively (D). Similarly, the 15-s bleach in the absence of $AT^{32}P$ was shown to cause no irreversible loss in phosphate incorporation if the samples were subsequently bleached for 1 h with white light (see also the reactivation section).

Discussion

The light-induced phosphorylation is known to be a dark reaction, initiated by bleaching of rhodopsin. However, as is shown in this report, the ability to phosphorylate rhodopsin lasts only for a limited time after activation; it decays in the dark as the time between illumination and addition of $AT^{32}P$ increases. The half-time of this decay has been measured to be on the order of 30–40 min at 30 °C (and faster) with considerable variation among preparations. Similar results have been obtained in two other laboratories (R. Paulsen, personal communication; R. N. Frank, personal communication). This decay of phosphorylation ability is not due to denaturation of the phosphorylating system since almost full reactivation can be obtained under appropriate conditions. Therefore, any mechanism proposed for the phosphorylation reaction must include inactivation as well as activation.

Our results clearly indicate that not only the light-induced

[3] Since dark-kept cattle ROS normally contain some bleached opsin, to be exact, the above sentence should read: "If activation by neighboring molecules occurs, one should find a higher phosphate incorporation per rhodopsin bleached in the presence of $AT^{32}P$ in membranes containing a higher opsin/rhodopsin ratio than in membranes with a lower opsin/rhodopsin ratio."

activation, but also the decay of this activity in the dark are properties of the membrane-bound rhodopsin and not of the extractable kinase (Figure 2). This is consistent with the action spectrum observed for the phosphorylation reaction (Bownds et al., 1972; Frank and Buzney, 1975), suggesting that the light stimulation of the phosphorylation is a consequence of light absorption by rhodopsin. However, this does not completely rule out indirect activation of the kinase via interaction with some photoproduct(s), for instance, in a manner similar to the light activation of the cGMP phosphodiesterase of ROS (Bitensky et al., 1973; Goridis and Virmaux, 1974).

Several attempts were made to demonstrate (indirect) activation of the kinase. Some of them initially seemed to indicate enzyme activation, but closer examination proved that this was not the case. One such observation was that extensively bleached opsin preparations which had lost their ability to phosphorylate in the dark and which contained little residual rhodopsin could be almost fully phosphorylated if they were reilluminated with white light for an hour in the presence of $AT^{32}P$ (see also Frank and Buzney, 1975). However, this reactivation can also be explained without the assumption of kinase activation, i.e., by the recycling mechanism. In this mechanism, freshly bleached rhodopsin is continuously produced and is the only substrate for the (always active) kinase. Since regeneration of rhodopsin from opsin and 11-*cis*-retinal is virtually complete within a few minutes (Paulsen et al., 1975), 1-h of illumination in white light would be sufficient to allow for several cycles of regeneration and rebleaching of each rhodopsin and "old" opsin molecule present. Thus all the "old" opsin molecules could be activated and consequently phosphorylated.

In fact, high reactivation for opsin preparations was observed only under conditions favoring recycling, namely, long illumination times with white light and high amounts of retinal present. The spectrophotometric data in Figure 3 clearly show that recycling occurs under these conditions and comparison of these spectral data with the phosphorylation data shown in Tables I, II, and III demonstrates that the more the conditions favor recycling, the higher is the apparent reactivation of the phosphorylation. In the highest reactivation experiment, up to 76 phosphates were incorporated per rhodopsin present at the beginning of the illumination. However, if the same experiment was carried out in orange instead of in white light and without the addition of *all-trans*-retinal, only 4 phosphates were incorporated per rhodopsin present at the beginning of the illumination. This is still somewhat higher than expected, but, even in orange light, some recycling occurs at long illumination times. We conclude that in all cases where reactivation occurred, it was due to the recycling mechanism and not to light activation of the kinase.

To show this more directly, recycling was virtually eliminated by using orange light and illumination times too short to allow much regeneration and rebleaching to occur. Under these conditions, the light-induced phosphate incorporation into ROS preparations which contained some "old" opsin could be quantitatively accounted for by assuming that only the freshly bleached rhodopsin was phosphorylated. If any kind of light activation of the kinase occurs, one would certainly expect that the old opsin molecules located on the same membrane fragments as the freshly bleached rhodopsin would be phosphorylated. Therefore this experiment also failed to give any evidence for kinase activation.

Similarly, mixing fresh-bleached rhodopsin and old opsin located on different membrane fragments (Table I) did not lead to activation of opsin phosphorylation. However, neither this mixing experiment nor that of Frank and Buzney (1975) excludes the possibility that the kinase is activated by interaction with freshly bleached rhodopsin to form a *membrane-bound* active complex such that the active complex remains bound to the membrane in which the activating rhodopsin molecule is located. Only the experiment shown in Table III where both opsin and freshly bleached rhodopsin were located on the same membrane fragments and yet no phosphorylation of the old opsin was observed could rule out the above mechanism of an activated membrane-bound kinase.

In this study, no evidence was found to support the possibility that the kinase may be activated by light. We therefore conclude that the light activation of the phosphorylation reaction is due solely to activation of the substrate. This activation decays with time so that opsin, at long times after bleaching, is also not a substrate for the kinase, like unbleached rhodopsin.

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