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A Link between Rhodopsin and Disc Membrane Cyclic Nucleotide Phosphodiesterase. Action Spectrum and Sensitivity to Illumination†

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ABSTRACT: Frog (*Rana pipiens*) rod outer segment disc membranes contain guanosine 3′,5′-cyclic monophosphate phosphodiesterase (EC 3.1.4.1.c) which, in the presence of ATP, is stimulated 5- to 20-fold by illumination. The effectiveness of monochromatic light of different wavelengths in activating phosphodiesterase was examined. The action spectrum has a maximum of 500 nm, and the entire spectrum from 350 to 800 nm closely matches the absorption spectrum of rhodopsin, which is apparently the pigment which mediates the effects of light on phosphodiesterase activity. *trans*-Retinal alone does not mimic light. Half-maximal activation of the phosphodiesterase occurs with a light exposure which bleaches $1/2000$ of the rhodopsins. Half-maximal activation can also be achieved by mixing 1 part of illuminated disc membranes in which the rhodopsin is bleached with 99 parts of unilluminated membranes. Regeneration of bleached rhodopsin by addition of 11-*cis*-retinal to illuminated disc membranes reverses the ability of these membranes to activate phosphodiesterase in unilluminated membranes. If the rhodopsin regenerated by 11-*cis*-retinal is illuminated again, it regains the ability to activate phosphodiesterase. These studies show that the levels of cyclic nucleotides in vertebrate rod outer segments are regulated by minute amounts of light and clearly indicate that rhodopsin is the photopigment whose state of illumination is closely linked to the enzymatic activity of disc membrane phosphodiesterase.

In 1971 Bitensky et al. reported an effect of illumination on the concentrations of adenosine 3′,5′-cyclic monophosphate (cAMP)[1] in suspensions of frog rod outer segment disc membranes. At first this effect was attributed to inhibition of adenylate cyclase by illumination, since in the absence of ATP illumination had no effect on phosphodiesterase activity. Later, however, Miki et al. (1973, 1974) found that the light-sensitive enzyme is phosphodiesterase. In the presence of ATP, or other nucleoside triphosphates, the activity of phosphodiesterase is stimulated 5- to 20-fold by visible light. The disc phosphodiesterase has a lower K_m for guanosine 3′,5′-cyclic monophosphate (cGMP) than for cAMP, so the enzyme is better designated as a cGMP phosphodiesterase (at 10^{-6} *M* or below, cGMP is hydrolyzed 23 times more rapidly than cAMP). The percentage activation of phosphodiesterase by light in the presence of ATP is comparable for the two substrates (Miki et al., 1973).

At the time when the locus of light regulation was thought to be adenylate cyclase, Bitensky et al. (1972) examined the apparent change in cyclase activity as a function of the fraction of rhodopsins bleached, and also examined the action spectrum for the apparent change in cyclase activity. These early experiments suggested that the change in cyclase activity was proportional to the fraction of rhodopsins bleached, and that the action spectrum had a maximum around 500 nm.

With the knowledge that the light-sensitive enzyme is phosphodiesterase rather than cyclase and more information about the technical factors which affect the stability of the light-regulated enzyme, we decided to reexamine the questions of action spectrum and sensitivity to illumination with the following changes in procedure: the more active phosphodiesterase was monitored, permitting the use of more dilute suspensions of disc membranes which allowed more uniform illumination of the sample; we used fresh, fully dark adapted frog retinae; and, finally, we took precautions (rapid assay and incorporation of dithiothreitol) to prevent the gradual decline in the activity of phosphodiesterase which follows activation. These changes in technique have permitted us to generate a very precise action spectrum for the activation of phosphodiesterase, and to precisely determine the relationship between the fraction of rhodopsin bleached and the activity of phosphodiesterase.

We had found (Miki et al., 1973) that complete activa-

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[1] Abbreviations used are: cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate.

tion of phosphodiesterase in suspensions of frog discs could be achieved by mixing three parts of illuminated discs with 97 parts of unilluminated discs, so we wished to compare the efficiency of this mode of activation with the activation produced by low intensity illumination which bleaches an equal fraction of the rhodopsins in a suspension of discs. Finally, we wished to see whether reversal of rhodopsin bleaching by addition of 11-*cis*-retinal in the dark (Futterman and Rollins, 1973) also reverses the ability of membranes with illuminated rhodopsin to activate phosphodiesterase.

Experimental Procedure

Materials. Frozen bovine retinae were from Hormel (Austin, Minn.). *all-trans*-Retinal, cGMP, ATP, hexadecyltrimethylammonium bromide, and ethylene glycol bis(β-ethanolamine)-*N,N,N',N'*-tetraacetic acid were purchased from Sigma Chemical Co. 11-*cis*-Retinal was a gift from Hoffman-LaRoche, Inc.

Exclusion of Visible Light. All manipulations (dissection and assay) except for deliberate bleaching were performed in the complete absence of visible light using infrared lamps (a 12-V tungsten automobile headlight lamp fitted with a Corning CS 7-56 filter) and infrared image converters (Metascope 9902E, Varo Inc., Garland, Texas).

Preparation of Photoreceptor Outer Segments. Frogs (*Rana pipiens*) were dark adapted overnight, then sacrificed; retinae (and some attached portions of pigment epithelium) were removed and placed in a half-full centrifuge tube of 46% (w/w) sucrose (six retinae/5-ml tube). The tube was shaken manually 15 times, filled with the sucrose solution, and centrifuged at 50,000*g* for 1 hr. Outer segments form a thick paste at the air–sucrose interface. This paste was harvested with a spatula and suspended in 1 ml/retina of 10 m*M* Tris-HCl buffer (pH 7.4) containing 1 m*M* dithiothreitol. In some of the experiments, the method of Papermaster and Dreyer (1974) was used to prepare the disc membranes. Experiments with these more highly purified membranes give results indistinguishable from the results obtained with membranes prepared by the simpler procedure, so the latter was used routinely.

Assay of Phosphodiesterase. Phosphodiesterase activity was assayed in a 20-μl volume with 2 m*M* [^{3}H]cGMP (12.5 Ci/mol, New England Nuclear), 1 m*M* ATP, 3 m*M* $MgSO_4$, 50 m*M* Tris-HCl buffer (pH 7.4), and 4 μg of photoreceptor disc membrane protein. Samples were incubated for 1 min at 30° and stopped by boiling for 1 min. cGMP was isolated on polyethylenimine-cellulose thin layers as previously described (Keirns et al., 1974), and quantitated by counting in a Beckman LS-200 liquid scintillation counter. A unit of enzyme activity is 1 μmol of cGMP hydrolyzed/min. In a few experiments the method of Thompson and Appleman (1971) was used to assay phosphodiesterase and similar results were obtained.

Other Analytical Methods. Absorption spectra were measured following dilution of the photoreceptor membranes with 4 parts of 1% hexadecyltrimethylammonium bromide, either with or without 0.1 *M* hydroxylamine. The incorporation of hydroxylamine did not alter the measured absorbance at 500 nm. Protein concentrations were determined by the method of Lowry et al. (1951).

Results

Use of the ATP Requirement to Estimate the Percent of Rhodopsin Bleached. The cGMP phosphodiesterase activity of dark adapted frog disc membranes is 0.2 μmol of cGMP hydrolyzed per min per mg of protein (at 2 m*M* cGMP). In the presence of ATP phosphodiesterase activity of membranes with bleached (white light) rhodopsin is 1.6 μmol of cGMP hydrolyzed per min per mg of protein. The photoreceptor phosphodiesterase is activated by very small exposures to visible light (see below), so the question arises whether even the use of infrared light and image converters permits the phosphodiesterase activity of truly dark membranes to be estimated. The answer to this question is provided by the ATP requirement for activation of phosphodiesterase by light. In the absence of ATP, both illuminated and unilluminated preparations have the same phosphodiesterase activity as the unilluminated preparation has in the presence of ATP (Miki et al., 1973). In the experiments with frog disc membranes the addition of ATP did not change the phosphodiesterase activity of the unilluminated membranes. This indicates that the amount of unintentional bleaching of rhodopsin which occurs during routine preparation and assay is too small to produce measurable effects on the phosphodiesterase, that is, inadvertent bleaching must be less than 0.01% of the rhodopsin present. The phosphodiesterase activity which is present in unilluminated disc membranes does not represent inadvertent bleaching followed by activation by endogenous ATP, for the following reasons. If we take the concentration of ATP in the intact rod outer segment as 4 m*M* (Robinson et al., 1975), dilution from the intact outer segment to the suspension of disc membranes in the assay tube would reduce it to 10 μ*M*. Also, activation is rapidly reversed when ATP is removed. On the other hand, ATP produces a substantial activation of phosphodiesterase (from 0.072 to 0.20 μmol of cGMP hydrolyzed per min per mg of protein) in unilluminated bovine disc membranes, indicating that significant prior bleaching had occurred. The activity in illuminated bovine membranes in the presence of ATP is 0.38 μmol per min per mg of protein.

Gradual Decline in Phosphodiesterase Activation and Its Prevention by Dithiothreitol. If one part of illuminated disc membranes is mixed with 99 parts of unilluminated membranes, half-maximal activation of phosphodiesterase in the mixture is achieved. If the mixture is allowed to sit at room temperature for 1 hr, approximately one-half of this activation is lost. This decline in activation is prevented by 1 m*M* dithiothreitol. The procedure of mixing small amounts of illuminated with large amounts of unilluminated disc membranes has permitted the localization of the labile sulfhydryl group in the phosphodiesterase (unilluminated) fraction, rather than in the activator (illuminated) fraction. Aged illuminated discs are fully effective in activating fresh phosphodiesterase, while phosphodiesterase in aged unilluminated discs is not activated by fresh illuminated discs unless dithiothreitol is added. For this reason 1 m*M* dithiothreitol was included in the disc suspension for the experiments described below.

Exposure of Disc Membranes to Monochromatic Light. Photic bleaching was performed with a Gilford 2000 spectrometer modified as follows. All light leaks from the instrument to the room were masked with aluminum foil. The detector and sample holder were removed. A new sample holder was placed 11 cm from the focal point of the light beam, with an intervening shutter. This arrangement provides uniform illumination of the entire cross section of the sample and permits accurate and convenient timing of the exposure. A sample of photoreceptor outer segment mem-

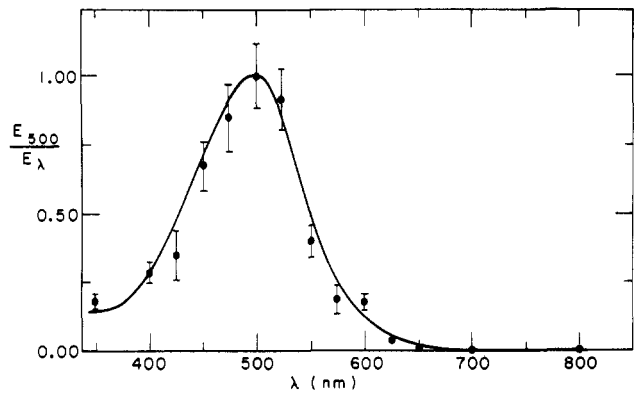

FIGURE 1: Action spectrum for activation of cGMP phosphodiesterase. Frog rod outer segment disc membranes were prepared in the complete absence of visible light and exposed to various amounts of monochromatic (1–10 nm bandwidth) visible light. The relative number of photons required to half-maximally activate phosphodiesterase (E) was determined. The points represent the mean and standard error of E_{500}/E_λ. Two to six determinations were performed for each wavelength. The curve is the absorption spectrum of rhodopsin in 1% hexadecyltrimethylammonium bromide.

branes (150 μl) was placed in a self-masking cuvet (Hellma cells) which had a 5-mm path length, and was 2-mm wide. The slit was set to provide a uniform bandwidth (1 or 10 nm) for the different wavelengths, based on the dispersion curve obtained from Gilford. There is some reduction in the intensity of light reaching the back of the cuvet compared to the front due both to absorption by rhodopsin and to Rayleigh scattering by the disc membranes. To reduce artifacts due to these factors, (1) the membrane suspension in the cuvet was thoroughly mixed before an aliquot was removed, (2) 5 mm rather than a 1-cm path length cell was used, and (3) a moderately dilute suspension of membranes (0.4 mg of protein/ml) was used. At this concentration the absorbance at 500 nm due to rhodopsin was 0.1. This results in an observed action spectrum which differs from the true spectrum in having a slightly reduced magnitude (a few percent) at 500 nm compared to 400 or 600 nm. Scattering results in a small shift (a few nanometers) to longer wavelengths. Absorption and scattering effects are reduced to experimentally insignificant levels by the measures described above. A 150-μl aliquot of rod outer segment membranes was removed from a cold (0°) dark safe (an aluminum ultracentrifuge bucket with a screw cap) and exposed to visible light of a given wavelength and bandwidth for varying lengths of time (from 10 sec to 5 min). In some experiments the membranes were exposed to different intensities of light for a fixed time. The two procedures (varying time of exposure or intensity of light) gave the same results, indicating that the fraction of rhodopsin bleached or the fraction of phosphodiesterase activated depends only on the total energy of light received by the membrane suspension. Immediately after each exposure 10-μl aliquots were assayed for phosphodiesterase activity. The total time elapsed from removal of the disc membrane sample from the dark safe to completion of the phosphodiesterase assay never exceeded 20 min.

Calculation of the Action Spectrum. Phosphodiesterase activities (A_λ) of samples exposed to light for various times (t) fit the following equation:

$$A_\lambda - D = (L - D)(t/2K_\lambda) \quad (1)$$

as long as A_λ is less than 90% of L. D and L are the phosphodiesterase activities of unilluminated membranes and membranes with all the rhodopsins bleached (white light). K_λ is an empirical parameter (time for half-maximal activation of phosphodiesterase). Values of K_λ for the given wavelength and slit settings were obtained from a linear regression.

The exposure (E_λ) (which is proportional to the number of photons) required to give half-maximal activation at a given wavelength was then obtained from the following equation:

$$E_\lambda = K_\lambda I_\lambda F_\lambda (\lambda/500) S_\lambda^{0.75} \quad (2)$$

where S_λ is the slit width, I_λ is the spectral intensity of the tungsten lamp, F_λ is the percent transmittance of the filter (a violet filter is used for wavelengths shorter than 410 nm and a red filter for wavelengths longer than 660 nm), and K_λ is derived from eq 1. The dependence of I_λ and F_λ on wavelength and the dependence of intensity on slit width were determined using the Gilford detector which contains an RCA 1P28 photomultiplier tube. The measurements of I_λ and F_λ made in this way were corrected for the dependence of the response of the detector on wavelength which was provided by RCA. The factor ($\lambda/500$) converts equal intensities at different wavelengths to equal numbers of photons at different wavelengths.

Figure 1 shows a plot of E_{500}/E_λ vs. wavelength, that is, an action spectrum for activation of cyclic nucleotide phosphodiesterase by light, and for comparison shows the absorption spectrum of rhodopsin. It is clear that the correlation between wavelength and efficiency is the same for bleaching of rhodopsin as for activation of phosphodiesterase in disc membranes.

Quantitative Effects of Uniform Whole Sample Illumination of Varying Intensity and Duration on Phosphodiesterase Activity. Following exposure of disc membranes to 500-nm visible light for various lengths of time, phosphodiesterase was assayed and the fraction of rhodopsin which had been bleached was determined from the absorbance at 500 nm. Since activation of phosphodiesterase occurs with bleaching of very small amounts of rhodopsin, it is impractical to directly determine the amounts of rhodopsin bleached when phosphodiesterase is half-maximally activated. However, since at low light intensities bleaching of rhodopsin is proportional to the duration and intensity of light exposure, bleaching was measured with long exposures and then extrapolated to short exposures. Figure 2 shows that half-maximal activation of phosphodiesterase occurs when 0.05% of the rhodopsin has been bleached.

Quantitative Effects of Mixing Illuminated and Unilluminated Membranes on Phosphodiesterase Activity. Unilluminated photoreceptor membranes and illuminated membranes with all the rhodopsins photoisomerized were mixed in varying proportions. Approximately 5 min after the membranes were mixed, phosphodiesterase activity was assayed in the absence of light (Figure 2). With this procedure half-maximal activation of frog disc membranes occurs with a 1% admixture of bleached membranes. For bovine disc phosphodiesterase, half-maximal activation occurs with a 10% admixture.

Effects of a Red Safelight on Phosphodiesterase Activity. The need for complete exclusion of visible light during preparation and manipulation of disc membrane suspensions is shown by the finding that half-maximal activation of phosphodiesterase is obtained by bleaching less than 1% of the rhodopsin. In order to further clarify this point, a red

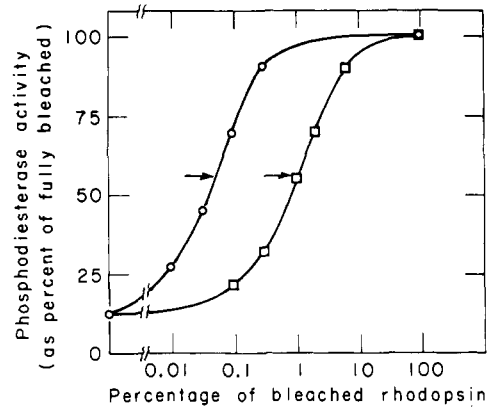

FIGURE 2: Effectiveness of bleaching with a short flash of light or admixture of a small amount of membranes with bleached rhodopsin in activating cGMP phosphodiesterase: The ordinate is phosphodiesterase activity expressed as percent of the activity of illuminated membranes. The abscissa is the percent of the rhodopsin bleached. In the first experiment (○) unilluminated membranes were exposed to 500-nm light of measured intensity for a measured time. Phosphodiesterase was assayed and the percentage of rhodopsin which had been bleached was determined from the absorbance at 500 nm. The small percent bleaches were estimated by extrapolating from the percentage bleached by longer exposures. Alternatively, dark adapted frog disc membranes from the same preparation were divided into two fractions, one held in the dark and the other exposed to fluorescent light to bleach the rhodopsin. Then various mixtures of illuminated and unilluminated membranes were prepared and assayed in the dark (□). The arrows indicate the points of half-maximal activation of the phosphodiesterase.

safelight (Kodak Model A with a 50-W bulb and 1Å filter mounted 4 ft overhead) was used during 4 min of preparation, prior to assay. During this time the disc membranes were pipetted into reaction tubes. This brief exposure resulted in a 50% activation of the phosphodiesterase although bleaching much less than 1% of the rhodopsin (Table I). If the whole experiment (including dissection of retinas) is done with dim red light, virtually all of the phosphodiesterase is activated and no effects of light are observable (Table I).

Effects of all-trans-Retinal on the Activity of Photoreceptor Phosphodiesterase. all-trans-Retinal was suspended in Tris-HCl buffer by 10-sec sonication (Branson Model 185C) and then added to unilluminated disc membranes in the dark. The phosphodiesterase was assayed in the dark and following illumination. Several concentrations of retinal were used including 10^{-7}, 10^{-6}, and 10^{-5} *M*, which correspond to the concentration of retinal achieved by bleaching 1, 10, or 100% of the rhodopsin. None of these concentrations significantly alter phosphodiesterase activity of illuminated or unilluminated disc membranes.

Loss of Activator Function upon Regeneration of Rhodopsin with 11-cis-Retinal. Illuminated rhodopsin whose retinal is in the all-trans form can be restored to the unilluminated state by adding purified 11-*cis*-retinal (Futterman and Rollins, 1973). We have utilized this procedure to see whether reversal of the photochemical state of rhodopsin is accompanied by reversal of the ability to activate phosphodiesterase. The rhodopsin concentration (about 2×10^{-5} *M*) of a disc suspension was estimated from the absorbance at 500 nm (ϵ = 40,600, Hubbard et al., 1971) following dilution with 1% hexadecyltrimethylammonium bromide. Then, using near-infrared light 11-*cis*-retinal was dissolved in ethanol (approximately 10 mg/ml), and the concentration estimated from the absorbance at 380 nm (ϵ = 24,900, Hubbard et al., 1971). The ethanol was removed by evaporation, and the retinal was suspended in 10 m*M* Tris-HCl buffer (pH 7.4) by sonication (10 sec at half-maximum power). During sonication the sample was cooled with ice-water. The suspension of 11-*cis*-retinal was mixed with a suspension of illuminated disc membranes by sonication (10 sec) to give a final concentration of 6×10^{-5} *M* 11-*cis*-retinal. Dithiothreitol and disodium ethylene glycol bis(β-ethanolamine)-*N,N,N',N'*-tetraacetic acid were added to give final concentrations of 10 and 1 m*M*, respectively. Then the mixture was incubated for 1 or 2 hr at 25° in the dark. Incubation for longer than 2 hr did not produce greater regeneration. These membranes were evaluated for their ability to activate the phosphodiesterase in unilluminated disc membranes by mixing two parts of the membranes with regenerated rhodopsin with 98 parts of unilluminated membranes (Table II). This experimental design in which only the illuminated membranes (with 2% of the phosphodiesterase in the final mixture) were subjected to the regeneration procedure, avoided exposure of the bulk of the phosphodiesterase to the traumata of sonication and a 2-hr incubation at room temperature. The membranes in which the rhodopsin was 75% regenerated on the basis of recovery of 500 nm absorbance were found to have lost more than 60% of the ability of the untreated unilluminated membranes to activate phosphodiesterase. If the regenerated membranes were bleached with white light, they regained 90% of their original ability to activate phosphodiesterase. The addition of 11-*cis*-retinal by itself has no effect on the activity of phosphodiesterase in unilluminated membranes.

Table I: Effects of Dim Red Light on Photoreceptor Phosphodiesterase.[a]

Previous Light Exposure	Phosphodiesterase Activity		Percentage of Rhodopsin Bleached
	Unilluminated	Illuminated	
None	0.22 ± 0.02	1.63 ± 0.07	None
4 min of red safelight prior to assay	0.95 ± 0.05	1.55 ± 0.07	0.07
Entire dissection and assay performed with red light	1.59 ± 0.07	1.64 ± 0.08	~1

[a] Phosphodiesterase activity was assayed with 2 m*M* [^{3}H]cGMP and 1 m*M* ATP incubating at 30° for 1 min. A unit of activity is 1 μmol of cGMP hydrolyzed per min per mg of protein. The activities shown are the mean and standard error for three determinations. Except as noted the phosphodiesterase activity of unilluminated membranes is assayed using near-infrared light and image converters. The fraction of the rhodopsin bleached was estimated from the phosphodiesterase activity using Figure 2.

Discussion

The data presented in Figure 1 indicate that the first step in the activation of phosphodiesterase by light is an interaction between light and rhodopsin. This result is not surprising since rhodopsin is the principal photopigment in the rod outer segment. We find that *all-trans*-retinal does not mimic the effect of light on phosphodiesterase activity. Thus, it is likely that opsin, the protein portion of illuminated rhodopsin, or a retinal–opsin complex, functions as an intermediate in the activation of phosphodiesterase by light. However, we cannot exclude the possibility that changes in the physical properties of the disc membrane or release of a transmitter mediates the effects of rhodopsin bleaching on

Table II: Reversal of the Ability of Illuminated Rhodopsin to Activate Phosphodiesterase in Unilluminated Disc Membranes by Regeneration of Bleached Rhodopsin with 11-*cis*-Retinal.[a]

Expt	Incubation Time (hr)	Absorbance		Phosphodiesterase Activity of 2:98 Mixture	
		$\frac{\Delta A_{500}(RD - RL)}{\Delta A_{500}(D - L)}$	Fraction of rhodopsin regenerated (%)	$\frac{RL - RD}{L - D}$	Loss of activiting capacity (%)
1	1	$\frac{0.047}{0.172} = 27$		$\frac{1.35 - 1.00}{1.44 - 0.22} = 29$	
2	2	$\frac{0.087}{0.158} = 55$		$\frac{1.04 - 0.61}{1.12 - 0.19} = 46$	
3	2	$\frac{0.120}{0.161} = 74$		$\frac{1.59 - 0.52}{1.74 - 0.23} = 71$	
4	2	$\frac{0.130}{0.172} = 75$		$\frac{1.15 - 0.58}{1.21 - 0.22} = 58$	

[a] Unilluminated frog disc membranes (D) were illuminated with white light to bleach the rhodopsin (L); then the rhodopsin was regenerated with 11-*cis*-retinal (RD) as described in the text; finally, the regenerated rhodopsin was bleached again with white light (RL). At each stage the photochemical state of rhodopsin was determined from the absorbance at 500 nm, and the capacity of the membranes to activate phosphodiesterase in unilluminated membranes was evaluated. To examine the absorbance at 500 nm, the membranes were dissolved in 1% hexadecyltrimethylammonium bromide (4 parts) and A_{500} was measured. Then the cuvet was exposed to room light and A_{500} measured again. The difference between the two values is ΔA_{500} given above for unilluminated (D − L) or regenerated (RD − RL) membranes. The values given above are the means of two values which agreed to 0.01 or better. The activating capacity of unilluminated (D), illuminated (L), illuminated and then regenerated (RD), or illuminated, regenerated, and then reilluminated membranes (RL) was tested by mixing two parts with 98 parts of unilluminated membranes. Then phosphodiesterase activity (μmol of cGMP hydrolyzed per min per mg of protein) of the 2:98 mixture was measured. The numbers in the table are the means of three values which differed by less than 5%.

phosphodiesterase activity. Our previous experiments (Miki et al., 1973) would appear to rule out calcium as the transmitter between rhodopsin and phosphodiesterase.

The data presented in Figure 2 indicate that phosphodiesterase is maximally activated by a very small amount of bleached rhodopsin. An exposure to visible light which uniformly bleaches 0.05% of the rhodopsin is as effective as addition of an admixture of fully illuminated membranes (which contain only bleached rhodopsin) to give a final concentration of 1% bleached rhodopsin in the entire membrane suspension. The difference between the two procedures presumably reflects differences in the distribution of the bleached rhodopsin in the two cases. The rhodopsin is an integral part of membrane vesicles or disc membranes. When unilluminated membranes are exposed to a small amount of light some of the rhodopsins on each membrane are bleached. In contrast, in experiments utilizing the mixing design all of the bleached rhodopsins are confined to a small percentage of the disc membranes. These differences between the two methods of activation suggest that in experiments where phosphodiesterase activation is achieved by an admixture of fully illuminated membranes, the efficiency of activation is compromised by the necessity for interaction between rhodopsin and phosphodiesterase on different disc membranes. At present it is not clear whether this activation of phosphodiesterase in unilluminated membranes by addition of illuminated membranes occurs by membrane fusion, collision mediated exchange of rhodopsin, release of a soluble mediator, or movement of phosphodiesterase from one membrane to another. Physical studies have indicated that rhodopsin undergoes rapid rotational (Cone, 1972) and translational (Poo and Cone, 1973) movement within a disc membrane, but the facility of movement of rhodopsin from one membrane to another is not known. We have been unable to demonstrate the existence of a water-soluble mediator. Phosphodiesterase is bound to the disc membrane much less tightly than rhodopsin, and can be released as a soluble enzyme in magnesium free buffers of low ionic strength (Miki et al., 1975). However, with 100 m*M* Tris-HCl buffer containing 2 m*M* $MgCl_2$, the phosphodiesterase is firmly attached to the membrane, and under these conditions illuminated membranes efficiently activate phosphodiesterase in unilluminated membranes.

In experiments reported previously (Miki et al., 1973; Bitensky et al., (1974), we took illuminated membranes, treated them in various ways to probe the nature of the activator which is generated by illumination, and then tested the activator by mixing 1 part of the illuminated membranes with 50 parts of untreated unilluminated membranes and assayed phosphodiesterase activity. These experiments showed that the activator is resistant to digestion by trypsin or phospholipase C, heating at 60° for 20 min, and dialysis. The activator is inactivated by heating at 90° for 10 min and by exposure to 0.5% Triton X-100. The activator remains associated with the disc membranes when the membranes are sedimented to their equilibrium position in a continuous sucrose density gradient. Most of these observations as well as the experiments reported in this paper are consistent with the idea that the activator function is closely associated with bleached rhodopsin.

The action spectrum for activation of phosphodiesterase in frog disc membranes agrees with the preliminary result obtained with bovine disc membranes (Bitensky et al., 1972). On the other hand, the relationship of frog disc phosphodiesterase activity to the fraction of rhodopsin bleached is different from the previously reported (Bitensky et al., 1972) relationship of bovine adenylate cyclase activity to the fraction of rhodopsin bleached. With fresh fully dark adapted frog disc membranes, bleaching of less than 1% of the rhodopsin molecules gives virtually complete activation of photoreceptor phosphodiesterase. There are several ex-

perimental differences between the earlier experiments and the ones reported here. First, in the earlier work cyclase activity was monitored rather than phosphodiesterase. It is not clear that this should have any systematic effect on the results, although it did require the use of concentrated suspensions and resulted in data of lower precision. Second, the phosphodiesterase in the bovine photoreceptors is already partially activated by light exposures which occurred during slaughter, dissection, and packaging. Thus, there is only a 1.5- to threefold activation by further illumination. Third, the bovine enzyme is less sensitive to illumination, requiring admixture of 10% of illuminated membranes to achieve half-maximal activation of the enzyme in unilluminated membranes (compared to a 1% admixture for frog discs). Fourth, the spontaneous decline in the activity of partially activated phosphodiesterase, which occurs on standing in the absence of dithiothreitol, was not appreciated when the early experiments were performed, and hence suitable precautions to prevent this decline were not taken.

There are a number of physical or chemical properties of disc membranes which change upon illumination. Among these are the visible absorption spectrum of rhodopsin (Wald, 1968), low angle X-ray scattering (Blasie, 1972), exposure of sulfhydryl groups (Wald and Brown, 1952; Zorn and Futterman, 1971), accessibility of the Schiff base linkage of retinal to reducing agents (Bownds and Wald, 1965; Fager et al., 1972), accessibility of ionizable groups (Radding and Wald, 1955; Ostroy, 1974), and the availability of hydroxyl (serine) groups of rhodopsin as substrates for a protein kinase (Kuhn et al., 1973; Bownds et al., 1972). Most or all of these changes can be considered as direct manifestations of the photoisomerization of retinal or associated changes in the conformation of opsin, since in quantitative terms the properties in question change on a molecule per molecule basis as rhodopsin is bleached. One possible exception is the phosphorylation reaction. Bownds et al. (1972) report that for the fully dark adapted frog photoreceptor bleaching of one rhodopsin produces phosphorylation of 50. A number of light-induced changes in the activity of cyclic nucleotide enzymes have been reported, including inhibition of adenylate (Bitensky et al., 1972) or guanylate cyclase (Pannbacker, 1973; Bensinger et al., 1974), decreases in the cGMP content of intact retina (Goridis and Virmaux, 1974), and the ATP-dependent stimulation of phosphodiesterase (Miki et al., 1973; Chader et al., 1974). These effects of illumination on cyclic nucleotide metabolism probably all reflect the light-induced stimulation of phosphodiesterase activity which occurs in the presence of ATP. The report of Chader et al. (1974) is similar to our observations with bovine rod outer segments in that we both find that ATP is an essential cofactor in the activation of phosphodiesterase by light. We find that ATP stimulates phosphodiesterase in unilluminated bovine disc membranes, and attribute this stimulation to the fact that the cattle were not fully dark adapted at the time of sacrifice, and that dissection (which was done at Hormel) was performed using a red safelight (see also DeGrip et al., 1973). We find that the phosphodiesterase in frog disc membranes which are fully dark adapted is not stimulated by ATP. Chader et al. (1974), who prepare bovine retinae from fresh eyes and thus may have more completely dark adapted membranes, report that ATP has no effect on phosphodiesterase in the unilluminated bovine disc. Activation of phosphodiesterase has the same action spectrum as other light-induced changes in disc membrane properties. However, the other changes which occur on illumination appear to directly reflect changes in rhodopsin itself, while the activation of phosphodiesterase represents the communication of information about illumination from rhodopsin to another disc membrane protein.

These studies demonstrate an explicit link between the photoisomerization of rhodopsin and the enzymatic activity of disc phosphodiesterase. Furthermore, the studies emphasize the remarkable sensitivity of this regulatory mechanism to light and thereby provide a new and simple technique for estimating the percent of rhodopsin bleaching in a range where spectrophotometric measurements are less than satisfactory (from 0.005 to 1%). The reversal of the capacity of illuminated membranes to activate phosphodiesterase by regeneration with 11-*cis*-retinal further strengthens our conclusion that rhodopsin is the initial locus of the interaction between light and disc membrane phosphodiesterase.

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The Conformation of Proteins in Chromatin. A Circular Dichroism Study below 250 nm†

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ABSTRACT: This paper is an investigation of the circular dichroism (CD) spectra of DNA and protein in chromatin. The circular dichroism (CD) of chromatin below 250 nm is due to DNA and protein peptide chromophores. The spectrum in this region is resolved into contributions from salt-extractable proteins (histone and non-histone proteins extractable with sodium chloride), residual non-histone proteins (not extractable with 3 *M* sodium chloride), and DNA. Below 250 nm, DNA in chromatin has the same CD spectrum as DNA free in solution, in contrast to the CD of DNA above 250 nm (Hjelm, R. and Huang, R. C., (1974), *Biochemistry 13,* 5275). Histones and salt-extractable non-histone proteins in chromatin are seen to have an average CD like those observed for globular proteins. The average CD of the residual non-histone proteins is consistent with a population of proteins with more extended conformation. The CD of each of these components is found to be the same in chromatins isolated from tissues having different nuclear synthetic activities: chick embryo brain, pig cerebellum, myeloma K41, calf thymus, and chicken erythrocyte.

Isolated interphase chromosomes of higher plants and animals, chromatin, are a complex of DNA, proteins, and some RNA (Stellwagen and Cole, 1969; Hearst and Botchan, 1970; DeLange and Smith, 1971; Elgin et al., 1971; Huang and Hjelm, 1975). Among the chromatin proteins two classes are readily distinguished: the small, basic, well-defined histones and the heterogeneous non-histone proteins. Although much study has been devoted to the problem of chromatin structure and to the conformation of DNA in chromatin, little attention has been paid to the structure of protein in chromatin. To obtain some information on the structure of proteins in chromatin, in this paper, we extend our earlier studies on the circular dichroism of chromatin above 250 nm (Hjelm and Huang, 1974, 1975) into the region of the spectra between 200 and 250 nm.

From the studies on the CD of chromatin above 250 nm—where the signal is mainly due to DNA—we learned that the interaction of the histone proteins with DNA results in secondary structure of DNA in chromatin that is different from the conformation of DNA in solution at very low ionic strength (Shih and Fasman, 1970; Permogarov et al., 1970; Henson and Walker, 1970). Observations of the CD spectrum of chromatin between 200 and 250 nm—where protein peptide and DNA chromophores contribute—have indicated that the proteins in chromatin may contain considerable secondary structure not found in the isolated material at low ionic strength (Permogarov et al., 1970; Henson and Walker, 1970; Fric and Sponar, 1971; Ramm et al., 1972). No effort, however, was made to resolve the signal in this region into contributions from different chromatin components. Their conclusion on the secondary structure of proteins in chromatin was, therefore, based on indirect evidence.

We have attempted to separate the CD contribution of chromatin DNA from that of chromatin proteins. In this paper, we report the resolution of the CD spectra of chromatin between 200 and 250 nm into contribution from three components: salt soluble proteins (histones and salt-extractable non-histones), residual non-histone proteins, and DNA. In chromatin the salt-extractable non-histone and histone proteins have very similar CD spectra and are like those observed for globular proteins. The CD of the residual non-histone proteins is unlike that of the salt-soluble proteins and is consistent with a structure that is more extended than the other chromosomal proteins. The contribution of the DNA to the CD in this region is indistinguishable from the spectrum of free DNA in solution. The spectra for the three protein classes and DNA appear to be the same in all the chromatins studied, regardless of composition of origin.

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