Proteins from the all-cone retina of the lizard Anolis carolinensis were phosphorylated using $[\gamma^{32}\text{P}]$ ATP, separated by SDS-PAGE and detected by autoradiography. Several proteins incorporated $^{32}\text{P}$. Exposure of the retinal homogenates to light brought about a dramatic increase in phosphorylation of the protein(s) with a molecular weight nearly identical to that of rat rhodopsin. It is likely that these proteins are the cone visual pigments, and that they incorporate phosphate when bleached by light. Increasing the time of the phosphorylation reaction from 1 to 30 min led to an increase in the amount of incorporation of labeled phosphate by the putative cone visual pigments, but changing the temperature from 4°C to 20°C decreased it. The amount of phosphate incorporation was substantially increased by NaF, a phosphatase inhibitor. This latter finding, along with the changes in incorporation of $^{32}\text{P}$ with increased temperature, suggest that a phosphoprotein phosphatase is active in the lizard retina. The cation requirements, as well as the effects of cyclic nucleotides on light-induced phosphorylation of retinal lizard proteins, were also investigated. Invest Ophthalmol Vis Sci 27:1609-1614, 1986

A number of studies of protein phosphorylation in retinal tissues indicate that, upon bleaching, the protein moiety of rhodopsin undergoes phosphorylation. The kinase involved appears to be specific for rhodopsin, and up to nine phosphates may be incorporated by the serine and threonine residues of the visual pigments. In general, the phosphorylation of proteins leads to a change in their conformation and, in the case of enzymes, it modulates their function. While the visual pigment has been likened to an enzyme, the exact role of the phosphorylation of visual pigment has yet to be determined. Current suggestions include a role for rhodopsin phosphorylation in adaptation and the modulation of transduction.

Although rods and cones differ in many respects, the two cell types do, simply on the basis of similarity of function, share some of the same basic characteristics. Perhaps, then, the visual pigment of cones is also phosphorylated in response to light. Obtaining a cone preparation free from rod photoreceptor contamination is a major obstacle that must be overcome before any question concerning cone biochemistry can be answered. The problem is partially solved by using an all-cone retina such as that of the lizard, Anolis carolinensis. Morphological, biochemical, and electrophysiological evidence indicates that this retina contains only cones. Using the retina of Anolis, we undertook an investigation of the protein phosphorylation that occurs in response to light.

**Materials and Methods**

**Phosphorylation Studies**

Experimental animals were treated in conformity with the ARVO Resolution on the Use of Animals in Research. Retinas, along with pigment epithelium, were removed under dim red illumination and homogenized in 20.0 mM HEPES buffer, pH 7.2, containing 5.0 mM MgCl$_2$ and 0.5 mM DTT. The retinas were dissected together with the pigment epithelium to avoid the loss of cone outer segments. Samples were stored at $-70^\circ\text{C}$ in the dark. Using a FJW infrared sensitive headset, the rest of the procedures were conducted under infrared light. Obtaining a cone preparation free from rod photoreceptor contamination is a major obstacle that must be overcome before any question concerning cone biochemistry can be answered. The problem is partially solved by using an all-cone retina such as that of the lizard, Anolis carolinensis.
Fig. 1. Autoradiograph demonstrating the effect of light on phosphate incorporation by lizard retinal proteins. The arrow (O) indicates the opsin band. All reactions were conducted for 10 min at 20°C. Lanes 1 and 2 correspond to reactions performed in the dark and light, respectively. Lane 3 represents a reaction conducted in the light in which purified freeze/thawed rod outer segments from rat were added to the lizard homogenate before the reaction was initiated.

1) A portion of the transblot was cut and stained with Amido Black to assess the quality of the transfer of the lizard proteins from the gel to the nitrocellulose paper and to visualize the molecular weight markers.

2) The remaining portion of the transblot was blocked for 4 hr with 3% BSA in Tris-buffered saline (20.0 mM Tris-HCl, 0.5 M NaCl, pH 7.5). After rinsing with Tris-buffered saline, the transblot was incubated overnight with rabbit antiserum containing polyclonal antibodies raised against rat opsin. The antiserum was diluted 1:40 in Tris-buffered saline containing 1% BSA.

3) The transblot was washed with Tris-buffered saline and then incubated with peroxidase conjugated goat anti-rabbit IgG for 2 hr in Tris-buffered saline containing 1% BSA.

4) After washing with Tris-buffered saline, the antibody complex was detected by the addition of hydrogen peroxide and the color development reagent 4-chloro-1-naphthol.

Results

Figure 1 demonstrates the ability of light to substantially increase phosphate incorporation into proteins with a molecular weight of approximately 35,000. To compare the apparent molecular weight of phosphorylated rhodopsin with that of light-stimulated phosphorylated lizard proteins, a few micrograms of freeze/thawed rod outer segments from rat were added to the lizard homogenate prior to phosphorylation. The light-induced phosphorylation of rhodopsin in lane 3 is superimposed on that of the lizard proteins that undergo phosphorylation in response to light. Thus, it is probable that the proteins of the lizard retina that undergo light-induced phosphorylation are visual pigments.

In rods, the extent of phosphorylation of rhodopsin is determined by opposing kinase and phosphatase reactions. Lowering the phosphorylation reaction temperature to 4°C can, under certain conditions, increase the amount of phosphate incorporation by rhodopsin in retinal homogenates, suggesting the presence of phosphatase(s). If a similar system exists in cones, then lowering the temperature might lead to an increase in phosphorylation of cone visual pigments. The autoradiograph in Figure 2 demonstrates the effect of lowering temperature on phosphate incorporation in homogenates of the all-cone retina of the lizard. The phosphorylation pattern was not quantitatively the same at 4°C and 20°C. At 4°C, the overall trend was an increase in light-induced phosphorylation as the reaction time increased. In contrast, at 20°C, the amount of phosphate incorporation in response to light was reduced as reaction time was increased to 10 and 30 min.
Fig. 2. Autoradiograph depicting the effect of temperature on light-induced phosphorylation of lizard retinal proteins. The arrow (C) indicates the band corresponding to opsin. Lanes 1 and 6–9 correspond to reactions conducted at 20°C. Lane 1 represents a 10 min reaction conducted in the dark. Lanes 6, 7, 8, and 9 correspond to reactions performed in the light for 1, 3, 10, and 30 min, respectively. Lanes 2, 3, 4, and 5 correspond to reactions run in the light at 4°C for 1, 3, 10, and 30 min, respectively.

To quantitate the amount of phosphate incorporated at the different temperatures, each lane of the autoradiograph from Figure 2 was scanned with a densitometer. Peak P in Figure 3A corresponds to the 35,000 MW protein. Each peak P from lanes 1–9 of the autoradiograph is displayed in Figure 3B. Since protein band width can vary slightly within the adjacent lanes of a gel, the area under the peak, rather than the height of peak, is the better index of the amount of phosphate incorporation that occurred. These areas are graphically presented in Figure 4. From Figure 4, it is apparent that, after 1 min, the amount of phosphorylation is nearly identical at both 4°C and 20°C; however, the amount of 32P incorporation at 20°C is less than that of 4°C for reactions conducted for 3, 10, and 30 min. Moreover, at 20°C, the amount of 32P incorporation for the reactions conducted for 10 and 30 min is less than that for reactions conducted for 3 and even 1 min. In contrast, for the reactions run at 4°C, the amount of 32P incorporation increased as reaction time increased. These results are consistent with the notion that a phosphoprotein phosphatase is present in the lizard retina.

If a phosphoprotein phosphatase is present, then NaF, a known inhibitor of some phosphatases, might enhance phosphorylation. The results of a typical set of experiments to test this possibility are illustrated in...
Figure 4. Quantitative representation of the effect of temperature on light-induced phosphorylation of lizard proteins. The bars represent the area under the densitometric scans of light-induced $^{32}$P incorporation into proteins. Each area unit is .125 $\mu V \cdot$ sec. The time of each phosphorylation reaction is given on the x-axis. Reactions carried out at 4°C and 20°C are represented by the filled bars and the open bars, respectively.

Figure 5. Under control conditions in the dark, no phosphorylation was detected. When NaF (final concentration in homogenate 4.0 mM) was added at the time of addition of labeled ATP, an increase in the amount of phosphate incorporation occurred in both light and dark samples. That phosphate incorporation appears in the dark could indicate that some of the visual pigment is undergoing phosphorylation and, therefore, is bleached, although we take precautions to prevent bleaching. Alternatively, incorporation of phosphate may also occur in another protein very close in molecular weight to the visual pigment, which is phosphorylated equally in light or darkness. In the experiments in which NaF was added at the onset of the preincubation with ATP, the amount of phosphate incorporation was greatly enhanced, both in the dark and light samples, over that seen when NaF was added with [$\gamma^{32}$P] ATP. Apparently, then, the inhibition of phosphatase is more complete when NaF is added earlier and allowed to preincubate with the phosphatases.

The ionic requirements for the phosphorylation of lizard visual pigments were investigated. It was found that, when a combination of EGTA and EDTA (final concentration of each in homogenate 2.0 mM), pH 6.9, was added to the homogenate (which contained 5.0 mM MgCl$_2$) just prior to [$\gamma^{32}$P] ATP, the amount of light-induced phosphorylation nearly doubled. Since the combination of EGTA and EDTA increased the amount of light-induced phosphorylation, it would appear that some divalent cation(s) present in the homogenate either stimulated phosphatase activity or inhibited kinase activity. Adding another 5.0 mM MgCl$_2$ to the homogenate containing EGTA and EDTA increased another threefold the amount of phosphate incorporation that occurred in the presence of homogenate containing only EGTA and EDTA. That the latter was a non-specific divalent cation effect is ruled out by the fact that adding 5.0 mM CaCl$_2$ instead of 5.0 mM MgCl$_2$ to the homogenate containing EGTA and EDTA resulted in a level of phosphate incorporation less than that seen with the homogenate containing EGTA and EDTA alone, and much less than that seen in the presence of added MgCl$_2$.

In rat rod outer segments and retinal homogenates from mice, cGMP has been shown to inhibit the extent of phosphate incorporation by rhodopsin. However, in the retinal homogenates from the lizard, the addition of either 50.0 $\mu$M cGMP or cAMP did not affect the light-induced phosphorylation of proteins, even though 50.0 $\mu$M cAMP led to a substantial increase in the phosphorylation of several other proteins.

It is possible that proteins with a molecular weight of 35,000 other than opsin incorporate $^{32}$P in response to light. In an effort to alter the migration of proteins with molecular weights near that of opsin, a low cross-
linked gel (15% acrylamide; 0.08% bisacrylamide) described by Baehr et al.\textsuperscript{15} was used. To enhance phosphorylation, the homogenates contained 2.0 mM of EGTA and EDTA, as well as 5.0 mM MgCl\textsubscript{2}. Reactions were terminated with sample buffer, as before, except in the homogenates run in lanes 4, 5, and 6 of the gel. The reactions in these homogenates were terminated by boiling for 1 min and then were allowed to sit at room temperature for 1 min before the addition of SDS sample buffer. A comparison of lanes 1 and 2 of the autoradiograph displayed in Figure 6 again reveals the dramatic increase in \textsuperscript{32}P incorporation in response to light by lizard proteins with a molecular weight near 35,000. Other proteins with a molecular weight near 35,000 that may undergo light-induced \textsuperscript{32}P incorporation were not detected. For comparison, a light-exposed homogenate from mouse retina was run in lane 3, yielding the same result. Since boiling aggregates some intrinsic membrane proteins, including opsin, and the aggregated proteins do not enter the resolving gel, we boiled the homogenate prior to treatment with SDS sample buffer in order to separate opsin from the other proteins that possibly undergo \textsuperscript{32}P incorporation in response to light. Comparing lanes 3 and 6 reveals that boiling significantly reduced the amount of \textsuperscript{32}P labeled mouse opsin that entered the resolving gel. After boiling the lizard homogenates, most of the \textsuperscript{32}P labeled proteins with a molecular weight near 35,000 aggregated and remained in the stacking gel. This suggests that the proteins that become phosphorylated in response to light are likely to be intrinsic membrane proteins, and, most probably, cone opsins.

Although a visual pigment with a $\lambda_{\text{max}}$ of 500 nm appears to be present in the retina of \textit{Anolis carolinensis}, this visual pigment does not behave chemically as a typical rhodopsin, in that it is sensitive to hydroxylamine.\textsuperscript{9} We investigated whether the opsins of this lizard might also be immunologically different from rhodopsin. Figure 7 shows the results of an experiment in which we compared the cross-reactivity of \textit{Anolis}, \textit{Xenopus}, and mouse retinal proteins with an antibody raised to rat rhodopsin. Proteins with molecular weights of about 35,000 from \textit{Xenopus} (lane 2) and mouse (lane 3) homogenates cross reacted with the opsin antisera, but lizard proteins (lane 1) did not. Since the \textit{Xenopus} proteins did bind the rhodopsin antibody, the lack of antibody binding to lizard proteins probably cannot be completely attributed to evolutionary distance. Our results suggest that the visual pigment of \textit{Anolis carolinensis}, a lizard with an all-cone retina as indicated by the work of Fowlkes et al.\textsuperscript{8} and Yu and Fager,\textsuperscript{9} is different immunologically from rhodopsin. An alternative explanation is that, if a rhodopsin similar to that in rods is in cones, it must be present in very low concentrations, below the limits of detection by the antibody.
Discussion

The data presented in this paper clearly indicate that, in the all-cone retina of the Anolis carolinensis, proteins with a molecular weight of approximately 35,000 are phosphorylated in response to light. Also, our data show that, like the opsin of rods, the 32P labeled proteins of the lizard retina with an apparent molecular weight of 35,000 aggregate when boiled. Since cone visual pigments have molecular weights in this range, the light-induced 32P incorporation probably represents visual pigment phosphorylation. It has been shown that some of the photoreceptors of Anolis contain a visual pigment with a λmax at 500 nm, which is typical for rhodopsin. However, on microscopic examination of the Anolis retina, only cones are evident and, more importantly, the two visual pigments that have been extracted from this retina behave chemically like cone visual pigments, in that they are both readily destroyed by hydroxylamine. Also consistent with these observations is our immunochemical data, which suggests that a typical rhodopsin is not present in the Anolis retina. Thus, it is likely that the proteins which undergo light-induced phosphorylation are cone visual pigments.

Lowering the temperature of the reaction should slow enzymatic activity overall. Interestingly, the amount of phosphorylation detected in the lizard retina is enhanced, not depressed, by lowering temperature from 20°C to 4°C. It is doubtful that this effect is due to a breakdown of visual pigment, since all visual pigments examined to date, including the labile gecko visual pigment, are stable at the temperatures used in the present study. Perhaps a reduction in temperature leads to a reduction in dephosphorylation sufficient to bring about an increase in the final amount of phosphate incorporation into the visual pigment. Since NaF greatly enhanced the level of phosphorylation in phosphate incorporation into the visual pigment. Since NaF greatly enhanced the level of phosphorylation in the lizard retina, our contention that a kinase also requires this divalent cation. On the other hand, it is intriguing that the combination of EGTA and EDTA led to an increase in the extent of phosphorylation. It has been shown that some divalent cation(s) present in the homogenate either stimulate phosphatase activity or reduce kinase activity. To determine which of these is actually occurring will require the isolation and purification of the kinase(s) and phosphatase(s) involved.

Key words: cones, light-induced phosphorylation, lizard, visual pigment

References