Two classes of high affinity, cGMP-specific binding sites have been found in association with a peripheral membrane protein in rod outer segments. [3H]cGMP and a photoaffinity label, 8-N3-[32P]cIMP, have been used to study these cGMP binding sites. The cGMP binding sites co-migrated with rod outer segment phosphodiesterase (EC 3.1.4.17) upon Bio-Gel A-0.5m column chromatography, sucrose density gradient centrifugation, and isoelectric focusing (pl 5.55). Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the 8-N3-[32P]cIMP-labeled protein also migrated in a position identical with that of purified phosphodiesterase. Scatchard analysis, using purified phosphodiesterase, revealed the presence of two classes of cGMP binding sites with apparent Kd values of 0.16 and 0.83 \( \mu \text{M} \).

A number of observations indicated that these high affinity, cGMP-specific binding sites are distinct from the phosphodiesterase catalytic site. cAMP, which is a substrate for phosphodiesterase, did not bind to the high affinity cGMP specific sites. Limited tryptic proteolysis of phosphodiesterase resulted in a striking activation of the catalytic activity and a 96% loss of cGMP binding. 1-Methyl-3-isobutylxanthine inhibited phosphodiesterase activity and enhanced the specific binding of cGMP. Mg\(^{2+}\) was necessary for phosphodiesterase activity, but not for high affinity cGMP binding. Finally, phosphodiesterase activity and the cGMP-specific high affinity sites showed different stabilities on storage in phosphate buffer. These specific high affinity cGMP binding sites may be involved in the regulation of phosphodiesterase activity.

It is now known that the interactions of a group of disc membrane proteins support a light and GTP-dependent activation of rod outer segment (ROS) cGMP phosphodiesterase (1-7). Action spectra for ROS phosphodiesterase reveal that rhodopsin is the photopigment which participates in this light- and GTP-dependent cascade. The activation of phosphodiesterase appears to require a GTP-binding protein in addition to GTP (6-10). These observations have recently been verified in reconstitution experiments (9, 10).

These enzyme reactions mediate a rapid and striking light-induced decline in ROS cGMP levels. It has been suggested that this light-mediated decline in cGMP might play a role in photoreceptor transduction or sensitivity control (11, 12). It is not yet possible to conclude that all of the components of the light-activated reactions have been identified, nor is the mechanism of phosphodiesterase activation by light and GTP fully understood. In view of the apparent importance of cGMP for the function of vertebrate rods, we have examined extracts of ROS for the presence of specific high affinity, cGMP receptor sites. While this work was in progress a report appeared by Hamet and Coquil (13) demonstrating specific, high affinity cGMP-binding activity in an extract of rat platelets; this cGMP-binding activity co-chromatographed with cGMP phosphodiesterase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]cAMP and [3H]cGMP were purchased from New England Nuclear. 8-N3-[32P]cIMP was prepared as previously described (14, 15). Bio-Gel A-0.5m was obtained from Bio-Rad. Other reagents were of the highest purity available.

**Preparation of EDTA-washed Disc Membranes and Crude Phosphodiesterase—**EDTA-washed disc membranes and crude phosphodiesterase (EDTA-solubilized disc membrane proteins) were prepared from Rana catesbiana as previously described (8-10). ROS prepared by sucrose flotation were suspended in 1 mM EDTA, 1 mM dithiothreitol (pH 8.1), and passed successively through a No. 21 needle (three times) and a No. 25 needle (three times). This crude disc suspension was kept on ice for 60 min (to permit EDTA solubilization of peripheral membrane proteins including phosphodiesterase) and centrifuged for 60 min in a Beckman SW 27.1 rotor (65,000 x g, 4°C). The supernatant, referred to as "crude phosphodiesterase," was lyophilized and stored at ~80°C. The disc membrane pellet was washed in 30 volumes of 20 mM Tris-HCl (pH 10) containing EDTA (1 mM) and dithiothreitol (1 mM). Finally, the disc membrane pellet was washed (three times) in 30 volumes of 200 mM Tris-HCl (pH 7.5) containing MgSO\(_4\) (20 mM) and dithiothreitol (1 mM). The pellet ("EDTA-washed membranes") was resuspended in the same buffer (2 to 3 mg of protein/ml), quick-frozen with acetone and dry ice, and stored at ~80°C.

**Assay of Phosphodiesterase Activity—**Phosphodiesterase activity was measured as previously described (4, 16, 18) with 1.25 mM cGMP as substrate. The incubation medium (in a final volume of 40 \( \mu \text{l} \)) contained 50 mM Tris-HCl (pH 7.5), 6.5 mM MgSO\(_4\), 0.1 \( \mu \)M [3H]cGMP (6.22 \( \times \) 10\(^6\) cpm), 1.25 \( \mu \)M unlabeled cGMP, 40 \( \mu \)g of protamine sulfate and enzyme protein. For assay of purified phosphodiesterase activity, about 0.05 \( \mu \)g of enzyme was added/tube. After incubation at 30°C for 5 min, the reaction was stopped by boiling for 2 min. Determinations were carried out on quadruplicate samples. Results agreed within 5% and average values are given.

**Phosphodiesterase Purification—**The lyophilized crude phosphodiesterase prepared from five large R. catesbiana was suspended in...
a solution containing 10 mm Tris-HCl (pH 7.5), 1 mm dithiorethiol, and 5 mm MgSO₄ (Buffer A). The magnesium concentration was then supplemented to a final concentration of 20 mm prior to loading this material onto a Bio-Gel A-0.5m column (6.35 x 1.1 cm, bed volume 5 ml) which had been equilibrated with the same buffer. The purpose of adding magnesium was to ensure that the binding of phosphodiesterase to the rhodopsin-containing vesicles which are present in the crude phosphodiesterase preparation was not prevented by the EDTA in this preparation.

Most of the disc membranes and disc membrane fragments did not enter the Bio-Gel A-0.5m column matrix but could be visualized as a narrow zone of bleached photopigment at the very top of the column. The column was washed with Buffer A, and membrane-bound phosphodiesterase was then eluted with a solution containing 10 mm Tris-HCl (pH 7.5), 1 mm dithiorethiol, and 2 mm EDTA (Buffer B). Fractions (0.7 ml) were collected. Column fractions were concentrated by loading into washed viscose dialysis tubing which was sealed, covered with unimbibed Sephadex G-200, wrapped in aluminum foil, and kept on ice. When the crude phosphodiesterase was enriched with additional amounts of EDTA-washed disc membranes (not done routinely) and this sample loaded onto a Bio-Gel A-0.5m column, such material yielded a larger amount of phosphodiesterase during the elution step with Buffer B. This was so because a larger amount of phosphodiesterase was initially retained on the column in the form of a membrane complex which was stable during the wash step with Buffer A.

**Determination of cGMP Binding**—Binding studies were performed by a modification of the Millipore filter method (17). The incubation mixture contained 14 pg of crude enzyme protein in a total volume of 100 ul. Bound and free [³H]cGMP were separated by Millipore filtration. Each point represents the average of four determinations which agreed within 5%. The upper panel shows the specific binding of [³H]cGMP as a function of ligand concentration. The lower panel shows the effect of various concentrations of unlabeled cGMP (●) or cAMP (○) on the binding of 1 µM [³H]cGMP.

**Fig. 2. Autoradiogram of an SDS-polyacrylamide gel showing the photoactivated incorporation of 8-N3-[³²P]cIMP into specific protein components of the crude phosphodiesterase preparation.** Photoaffinity labeling with 8-N3-[³²P]cIMP was carried out in the absence or presence of 1 µM cGMP or cAMP, as indicated. Eighty micrograms of crude enzyme preparation were added to each channel. Band 1 corresponds to the high affinity cGMP binding site. Bands 2 and 3 could correspond to type II and type I cAMP-dependent protein kinases, respectively.

The incubation medium (final volume, 100 µl) contained 25 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM 1-methyl-3-isobutylxanthine, 1 µM [³H]cGMP (3.11 x 10⁶ cpm), and crude or purified phosphodiesterase. Mixtures were incubated routinely for 30 min on ice, and 80-µl aliquots were then applied to a Millipore filter (HA, pore size 0.45 µm) and washed with 20 ml of 20 mM potassium phosphate buffer (pH 6.5). Control experiments indicated that the pH optimum for cGMP binding was 7.5, that maximum binding (about 1.35% of the added counts) was reached within 20 min, that binding was stable for at least 2 h, and that the counts were virtually unaffected (less than 4% displaced) by extensive washing. (The filter itself exhibited less than 0.1% of the cGMP-binding capacity of 5 µg of purified phosphodiesterase.) The filter was dissolved (1 h) in Formula 963 (New England Nuclear) and the amount of cGMP bound was determined by liquid scintillation spectrometry in a Beckman LS-200. Determinations were carried out in duplicate and were accompanied by an additional sample to which a 100-fold excess of unlabeled cGMP was added. Addition of unlabeled cGMP inhibited binding of the labeled material by greater than 96%. All data have been corrected for nonspecific binding.

**Photoaffinity Labeling**—Photoaffinity labeling experiments with 8-N3-[³²P]cIMP were performed as described previously (15) with slight modifications. The standard reaction mixture contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH 7.4), 0.25 mM EGTA (pH 7.5), 1 µM 8-N3-[³²P]cIMP, 2.5 mM β-mercaptoethanol, cGMP or cAMP as indicated, and about 80 µg of crude phosphodiesterase in a final volume of 100 µl. β-Mercaptoethanol was used because it reduced nonspecific or background labeling without significantly reducing the incorporation of [³²P] into phosphodiesterase. 1-Methyl-3-isobutylxanthine (where indicated) was used at a final concentration of 1 mM. Preincubations were carried out for 60 min (0°C) and the samples irradiated at 0°C for 10 min with a Mineralite UVS-11 lamp at a distance of 4 cm. The sample was then mixed with 20 µl of 6% sodium dodecyl sulfate (SDS) and boiled for
and applied to a Bio-Gel A-0.5m column (6.3 cm × 1 cm), that had been equilibrated with Buffer A. The column was washed with 14 ml of Buffer A, and then eluted with Buffer B. Fractions (0.7 ml) were collected and phosphodiesterase activity and cGMP-binding activity from a Bio-Gel A-0.5m column. A 0.9% solution of phosphoric acid in 52% sucrose (w/v) was layered over the anode followed by a 47% to 0% sucrose gradient. A 2% solution of ethanolamine was layered on top of the gradient. The sample was focused at V for 60 h at 0°C. After focusing, phosphodiesterase activity, cGMP-binding activity, and the pH of each fraction (1 ml) were determined.

Protein Determinations—Protein determinations were carried out according to the method of Lowry (20) using bovine serum albumin as standard. In some cases (e.g. Bio-Gel column fractions) proteins were estimated by the procedure of Bradford (21).

RESULTS AND DISCUSSION

Demonstration of cGMP Binding—The crude disc suspension was found to contain specific, high affinity, cGMP binding sites. In the presence of Mg²⁺, this cGMP-binding activity sedimented with the disc membrane pellet upon centrifugation (data not shown). Furthermore, this cGMP-binding activity was extracted from the disc membranes by EDTA, a procedure which has been found to extract phosphodiesterase and other peripheral disc membrane proteins. The amount of [³²P]cGMP bound to this EDTA-solubilized material (crude phosphodiesterase) is shown as a function of [³²P]cGMP concentrations in Fig. 1. Binding appeared to reach half-maximal saturation at about 0.09 μM cGMP. The specificity of this binding for cGMP was demonstrated by the inability of 100 μM unlabeled cAMP to prevent the binding of 1 μM labeled cGMP. We have also observed that photoactivated incorporation of 8-NS-[³²P]cIMP (1 μM) into a band of M₁ = 120,000 could be prevented by 1 μM unlabeled cGMP but not by 1 μM cAMP (Fig. 2). Incorporation of 8-NS-[³²P]cIMP into two additional bands of about M₁ = 54,000 (Band 2) and 47,000 (Band 3) can also be observed in Fig. 2. These bands, whose labeling was prevented by cAMP but not cGMP, correspond to the regulatory subunits of type I (Band 3) and type II (Band 2) cAMP-dependent protein kinases. Bands 2 and 3 might be attributable partially to contamination by rod inner segments, or non-photoreceptor retinal cells, since their staining intensity was quite variable in different preparations and since there is little cAMP in purified rod outer segments (22).

Evidence that Phosphodiesterase is Responsible for this Specific cGMP Binding—The cGMP-binding activity behaved similarly to ROS phosphodiesterase in that it co-sedimented with the disc membranes in the presence of Mg²⁺, could be eluted from the disc membranes with EDTA, and failed to emerge from a DEAE-Sephadex G-50 column (data not shown). These results led us to suspect that the cGMP binding sites might actually be located on ROS phosphodiesterase. In order to characterize further the protein responsible for this binding, the crude disc suspension was layered at the midpoint of a continuous sucrose gradient, with either 5 mM MgSO₄ or 2 mM EDTA. Fractions (0.7 ml) were collected and phosphodiesterase activity and cGMP-binding activity were assayed.

![Fig. 3. Comparison of elution patterns of phosphodiesterase activity and cGMP-binding activity from a Bio-Gel A-0.5m column.](image)

![Fig. 4. Comparison of protein-staining patterns (A, B) and 8-NS-[³²P]cIMP labeling (C, D) of crude (A and C) and purified (B and D, Fractions 19 to 21 of Fig. 3) phosphodiesterase. Either 80 μg of crude or 8 μg of purified phosphodiesterase were analyzed upon SDS-polyacrylamide gel electrophoresis. The arrow indicates the 120,000-dalton phosphodiesterase region.](image)
for cGMP binding, we undertook its purification. It was found that the high affinity, cGMP binding sites co-purified with, and appeared to be located on, ROS phosphodiesterase. The evidence for this conclusion includes the following: The cGMP-specific binding activity co-eluted with the peak of phosphodiesterase activity upon Bio-Gel A-0.5m column chromatography (Fig. 3). Moreover, when the phosphodiesterase peak fractions (19-21) were pooled and an aliquot examined on an SDS (7.5%) polyacrylamide gel for incorporation of [3-32P]cIMP into protein, a single band of radioactivity was observed, which corresponded to a single band of Coomassie blue staining (Fig. 4).

The protein content (5 μg/100 μl) of the purified phosphodiesterase activity peak (Fractions 19 to 21 in Fig. 3) corresponds to 20 pmol of phosphodiesterase/100 μl. The observed phosphodiesterase activity (225 pmol of cGMP hydrolyzed/min/mg of protein) gives a catalytic constant of 54,000 mol of cGMP hydrolyzed/min/mol of phosphodiesterase, in good agreement with that (48,000) calculated previously (4). The observed [3H]cGMP-binding activity was 16 pmol/100 μl, indicating that about 80% of the phosphodiesterase molecules bind [3H]cGMP.

A comparison of the isoelectric points for phosphodiesterase activity and cGMP-binding activity is shown in Fig. 5. It was found necessary to include the nonionic detergent Brij 35 in the ampholyte system to prevent aggregation of ROS proteins, which otherwise occurred in the region of pH 4.6. Incorporation of 0.5% Brij 35 prevented the formation of such aggregates without inhibiting phosphodiesterase or cGMP-binding activ-
ity. Under these conditions, both phosphodiesterase and cGMP-binding activities focused at pH 5.35.

When crude phosphodiesterase was layered onto a continuous sucrose gradient, catalytic activity sedimented as a peak with a molecular weight of 240,000 (4). When a similar preparation was mixed with EDTA-washed disc membranes, in the presence of 2 mM EDTA, the same pattern for phosphodiesterase sedimentation was observed (Fig. 6). If, in the place of EDTA, 5 mM Mg²⁺ was added together with the disc membrane suspension, the phosphodiesterase activity sedimented to the bottom of the gradient with the disc membrane pellet (Fig. 6). Under either condition, the cGMP-binding activity sedimented in the same position as the phosphodiesterase activity. The small amount of phosphodiesterase activity and cGMP-binding activity, found at the top of the sucrose gradient when the sedimentation was carried out in the presence of MgSO₄, is attributable to phosphodiesterase binding to minute fragments of the disc membrane which did not sediment into the sucrose gradient (data not shown). A Scatchard analysis of cGMP binding to purified phosphodiesterase indicated the presence of two classes of binding sites with Kₐ values of 0.16 μM and 0.83 μM, respectively (Fig. 7).

Evidence that the cGMP Binding Sites on Phosphodiesterase are Distinct from the Phosphodiesterase Catalytic Site—1. The addition of 1 mM unlabeled cAMP to 10 μM [³H]cGMP inhibited the hydrolysis of cGMP by about 90%. In contrast, 100 μM cAMP did not inhibit the binding of 1 μM [³H]cGMP to its specific high affinity site. Thus, while cAMP competed with cGMP for hydrolysis at the catalytic site, it did not appear to compete with cGMP for the high affinity binding sites.

2. When crude phosphodiesterase was exposed to 20 μg/ml of bovine pancreatic trypsin, the activity of phosphodiesterase was enhanced about 67%. Maximal activation was observed at about 4 min. Trypsin had an opposite effect on cGMP binding. Thus, there was a 96% loss of binding at a time when enhancement of phosphodiesterase activity was maximal (Fig. 8). In addition, the photoactivated incorporation of 8-Nβ-P⁴⁺IMP into the 120,000-dalton band was abolished by prior exposure of the crude phosphodiesterase preparation to trypsin (data not shown).

3. When 1 μM cGMP was added to the crude phosphodiesterase preparation, it substantially decreased the photoactivated incorporation of 8-Nβ-P⁴⁺IMP into the 120,000-dalton protein, under conditions designed to prevent cGMP hydrolysis by phosphodiesterase (Fig. 9, Channels A and B) but not under conditions designed to permit cGMP hydrolysis by phosphodiesterase (Fig. 9, Channels C and D). These results are consistent with the conclusion (23, 24) that 8-substituted cyclic nucleotides are poor substrates for certain phosphodiesterases, whereas the unsubstituted cyclic nucleotides are readily hydrolyzed in the absence of a phosphodiesterase inhibitor. The results also demonstrated that 8-Nβ-P⁴⁺IMP binding, and presumably cGMP binding, to the high affinity site was not inhibited by 1-methyl-3-isobutylxanthine per se and did not require Mg²⁺ (Fig. 9, compare Lanes B and D), in apparent contrast to binding of cGMP at the catalytic site. In fact, we have found that 1-methyl-3-isobutylxanthine enhanced cGMP binding in the presence of 1 μM, but not of 10 μM, added cGMP under conditions in which 1-methyl-3-isobutylxanthine markedly inhibited phosphodiesterase catalytic activity (data not shown). Hammet and Coquil also found, using 0.1 μM [³H]cGMP in their assay mixture, that 1-methyl-3-isobutylxanthine enhanced cGMP binding.
believe this difference reflects a factor(s) in the crude material which enhances the binding of cGMP to phosphodiesterase. This factor may also contribute to the modest differences in binding/activity ratios found between more highly purified phosphodiesterase preparations (Figs. 3 and 5) and crude phosphodiesterase preparations (Figs. 6 and 10). These differences in cGMP binding, which are associated with the degree of purification of the phosphodiesterase, may also reflect the fact that the cGMP binding sites are much more sensitive to proteolysis than is the catalytic activity (Fig. 8).

After the completion of this manuscript, S. H. Francis, T. M. Lincoln, and J. D. Corbin kindly informed us that they have also characterized a specific cGMP binding protein from rat lung which co-purifies with a cGMP phosphodiesterase (25). Their results with the lung phosphodiesterase differed in certain respects from the results obtained with the light-sensitive enzyme. Nevertheless, the specific cGMP binding site of the lung enzyme was distinct from the phosphodiesterase catalytic site (25), in agreement with the results obtained with rat platelets by Hamet and Coquil (13) and with rod outer segments in the present study.

Acknowledgment—We thank Dr. Jon Morrow for his critical reading of the manuscript.

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4. When the crude phosphodiesterase preparation was stored in phosphate buffer at ice temperature (pH 7.9), and was lost after storage at ice temperature for 9 days. Under these same conditions, cGMP-binding activity deteriorated (Fig. 10). This difference in stability between phosphodiesterase activity and cGMP-binding activity was not seen in Tris.

5. activity and cGMP binding were stable at ice temperature for 7.9, HC1 buffer (pH 7 to 8.5), and were lost after storage at ice temperature for 9 days. In acetate buffer (pH 4.0 to 6.0), both phosphodiesterase activity and cGMP binding deteriorated rapidly, and were lost after storage at ice temperature for 48 h (data not shown).

Concluding Remarks—The data presented here indicate the existence of two classes of specific, high affinity, saturable cGMP binding sites on photoreceptor phosphodiesterase. A variety of experimental procedures described here suggest that these sites are distinct from the catalytic site. Clearly, further studies are needed to elucidate the physiological role of these sites. One attractive possibility would be that the high affinity cGMP binding sites are involved in regulating the catalytic activity of the phosphodiesterase molecule. Preliminary evidence2 indicates that two physiological regulators of phosphodiesterase activity (light and GTP) are capable of decreasing the binding of cGMP to the high affinity sites.

We note that binding of cGMP to crude phosphodiesterase (Fig. 1) half-saturates at somewhat lower concentrations than binding to highly purified phosphodiesterase (Fig. 7). We

Fig. 10. Effect of storage at various pH values on cGMP-binding activity. Crude phosphodiesterase (280 μg of protein) was suspended in a solution containing 50 mM potassium phosphate, 1 mM dithiothreitol, and 5 mM MgSO4. The solution was stored at ice temperature at the indicated pH values and phosphodiesterase activity and [3H]cGMP binding were measured at the indicated time points.

A. Yamazaki and M. Bitensky, unpublished observations.