GUANOSINE 3',5'-MONOPHOSPHATE RECEPTOR PROTEIN: SEPARATION FROM ADENOSINE 3',5'-MONOPHOSPHATE RECEPTOR PROTEIN

Gordon N. Gill and Cynthia B. Kanstein

Department of Medicine, Division of Endocrinology
University of California, San Diego, School of Medicine
La Jolla, California 92037

Received February 12,1975

Summary. A specific cGMP receptor protein has been identified and separated from the cAMP receptor protein by chromatography on 8-(6-aminohexyl)-amino-cAMP-Sepharose. Scatchard analysis of cGMP binding indicates a single affinity class of receptor sites with KD = 1.4×10^{-8} M. The specificity of the cGMP receptor site has been defined by using a number of nucleotides as competitors for cGMP binding. The cGMP receptor protein sediments at 7S in glycerol density gradients.

In a number of biological systems the accumulation of guanosine 3',5'-monophosphate (cGMP) produces cellular responses different from those produced when the concentration of adenosine 3',5'-monophosphate (cAMP) is increased in the same tissue or cells (1-10). Cellular mechanisms are required not only to differentiate between cAMP and cGMP but to provide for these differing biological responses. In prokaryotes cAMP and cGMP interact with the same receptor protein. The binding of cAMP results in a configuration which binds to DNA and stimulates transcription of certain inducible operons; cGMP, which is a competitive inhibitor of cAMP binding, confers a configuration to the receptor which is inactive for DNA binding and for stimulation of transcription (11-14). Kuo and Greengard have proposed that, in eukaryotes, cGMP regulates cellular function via activation of a distinct cGMP-dependent protein kinase analogous to regulation of cellular function by cAMP-dependent protein kinase (15). cGMP-dependent protein kinase has recently been described in several mammalian tissues (16-18) and there is some evidence for biological specificity occurring through substrates for phosphorylation (19). cGMP binding activity has been identified in rat pancreas and rat cerebellum but lability has hampered characterization and separation from cAMP receptor protein (17,20).

In the present studies 8-(6-aminohexyl)-amino-cAMP-Sepharose (C8-NC_6NH_2-cAMP-Sepharose) has been utilized to effectively separate specific cGMP receptor protein from cAMP receptor protein. Separate and specific receptor proteins provide recognition and discrimination of cGMP and cAMP; the differing biological responses to the two cyclic nucleotides occur through mechanisms distal to the initial nucleotide binding step.

MATERIALS AND METHODS. [^{3}H]cGMP (16 Ci/mmole) was obtained from Schwarz/Mann; [^{3}H]cAMP (38.4 Ci/mmole) was obtained from New England Nuclear. Unlabeled nucleotides were from P-L Biochemicals Inc. and Sigma Chemical Company; concentration was determined from OD measurements in 5 mM sodium phosphate buffer, pH 7.0.

Initial Preparation of Binding Protein. Solid ammonium sulfate was added to 50% saturation to bovine adrenal cortical cytosol prepared by differential centrifugation. The resulting precipitate containing > 90% of total cAMP and cGMP receptor activity was resuspended in .05 M Tris-HCl (pH 7.4), .006 M 2-mercaptoethanol, and 10% glycerol (TMG) and dialyzed overnight against this same buffer. Fresh guinea pig tissues were prepared in a similar manner.

Chromatography Using C8-NC_6NH_2-cAMP-Sepharose. C8-NC_6NH_2-cAMP-Sepharose was synthesized as previously described (21). In the present experiments, a column containing 50 mM cAMP derivative was used. Fifty to one hundred milligrams of the 0-50% ammonium sulfate fraction in TMG buffer was chromatographed on a 2 ml column at 4°. After collecting the unadsorbed protein (> 98% of total protein applied), the column was washed sequentially with 0.1 M NaCl, 0.4 M NaCl, and 1 M urea containing 1.5 mM methylamine. The cAMP receptor protein was then eluted with 7 M urea containing 12 mM methylamine.

Cyclic Nucleotide Binding Activity. The binding of [^{3}H]cGMP to receptor protein was measured by retention of the [^{3}H]cGMP : receptor protein complex on cellulose ester filters (20,22-24). Reaction mixtures of 0.1 ml contained 50 mM sodium acetate-acetic acid buffer (pH 4.0), 8 mM theophylline, 6 mM 2-mercaptoethanol, [^{3}H]cGMP to the final concentrations indicated and binding protein. Complete reaction mixtures were incubated for 60 min at 0°. Equilibrium was reached by 15 min and the complex formed was stable for at least 120 min. The amount of cGMP bound increased linearly to 350 µg protein per filter. After incubation 0.5 ml of cold 20 mM phosphate buffer (pH 6.2) was added and the reaction rapidly filtered on cellulose ester filters (Millipore, 0.45 µ). Filters were washed with three 5 ml portions of cold phosphate buffer and radioactivity was determined by liquid scintillation counting in Bray's solution (25).

The binding of [^{3}H]cAMP to receptor protein was measured in a similar manner (23, 24). [^{3}H]cAMP binding was determined at either pH 4.0 using 50 mM sodium acetate-acetic acid as above or at pH 7.4 using 50 mM Tris-HCl as indicated in the text. Bovine serum albumin (100 µg) was added to assays which contained small amounts of highly purified receptor protein.

RESULTS. The initial determination of [^{3}H]cGMP binding to the 0-50% ammonium sulfate fraction of bovine adrenal cortical cytosol yielded a Scatchard plot with two slopes corresponding to a high affinity binding site with KD = 3.8×10^{-8} M and to a lower affinity binding site with KD = 7.0×10^{-7} M. The high affinity site appeared specific for cGMP

since 10-fold higher concentrations of unlabeled cAMP than unlabeled cGMP were required for comparable displacement of [^{3}H]cGMP from binding sites. The lower affinity binding site presumably represents cGMP binding to the cAMP receptor site. Characterization of cGMP binding in crude protein preparations was difficult because of the presence of excess high affinity cAMP binding sites compared to high affinity cGMP binding sites. An estimate of the number of high affinity binding sites for each nucleotide in crude protein preparations from a number of tissues was obtained by using a concentration of nucleotide (9.4×10^{-8} M [^{3}H]cAMP or [^{3}H]cGMP) sufficient to saturate the high affinity binding site for each nucleotide. An equal amount of the other unlabeled nucleotide was added to suppress low affinity binding. The results shown in Table I indicate that cAMP binding sites are present in greater than 10-fold excess. Since bovine adrenal cortical

TABLE I: Quantitation of cAMP and cGMP Receptor Sites in the Cytosol of Various Tissues

Tissue	[^{3}H]cAMP bound (pmoles/mg)	[^{3}H]cGMP bound (pmoles/mg)	Ratio $\frac{[^3\text{H}]\text{cAMP bound}}{[^3\text{H}]\text{cGMP bound}}$
Adrenal cortex	10.3	0.80	12.9
Adrenal medulla	10.0	0.62	16.1
Brain	6.2	0.46	13.1
Heart	21.1	0.60	35.1
Liver	2.8	0.24	11.7
Lung	6.4	0.18	35.5
Uterus	14.4	1.10	13.1

Bovine adrenals were separated into cortical and medullary fractions. Pooled tissues from 5 guinea pigs were homogenized and fractionated as described under "Methods." Receptor activity was determined using 9.4×10^{-8} M [^{3}H]cGMP or cAMP and an equal amount of the other unlabeled nucleotide to measure specific binding sites. Values for whole guinea pig adrenal glands resembled those for bovine adrenal cortex.

tissue contains high concentrations of cGMP receptor sites, this has been used for the present studies.

In order to determine whether the cGMP and cAMP receptor sites were present on the same or different proteins, the crude protein preparation was interacted with C8-NC_6NH_2-cAMP-Sepharose. This chromatography medium has previously been shown to preferentially retain the cAMP receptor protein (21). cGMP binding activity was unadsorbed whereas the bulk of the cAMP binding activity was retained (Table II). The cAMP receptor was subsequently eluted in highly purified form (21). The separation of the cGMP binding activity from the bulk of the cAMP receptor indicated that two distinct receptor protein species were present.

TABLE II: Separation of cGMP Receptor from cAMP Receptor Protein by Chromatography on C8-NC_6NH_2-cAMP-Sepharose

	Specific activity [^{3}H]cAMP bound (pmoles/mg)	Specific activity [^{3}H]cGMP bound (pmoles/mg)	Ratio $\frac{[^3H]cAMP\ bound}{[^3H]cGMP\ bound}$
0-50% ammonium sulfate fraction	10.30	0.80	12.9
Unadsorbed onto C8-NC_6NH_2-cAMP-Sepharose			
a) through column	1.06	0.76	1.4
b) shaken overnight	0.38	0.68	0.56
Urea elution	3000.00	0.00	∞

Bovine adrenal cortical cytosol protein (0-50% ammonium sulfate fraction) was chromatographed on a 2 ml column of C8-NC_6NH_2-cAMP-Sepharose (50 mM). The protein was either passed through the column (a) or passed through the column and then reacted with the chromatography medium for an additional 12 hours by continuous shaking (b). The unadsorbed fraction was collected, the column was washed as described under "Methods," and the retained protein was eluted with 7 M urea. Binding to each fraction was determined using either 6.3×10^{-8} M [^{3}H]cGMP or [^{3}H]cAMP as described under "Methods." No binding activity was detectable in the intermediate wash fractions. Similar results were obtained using protein from other tissues.

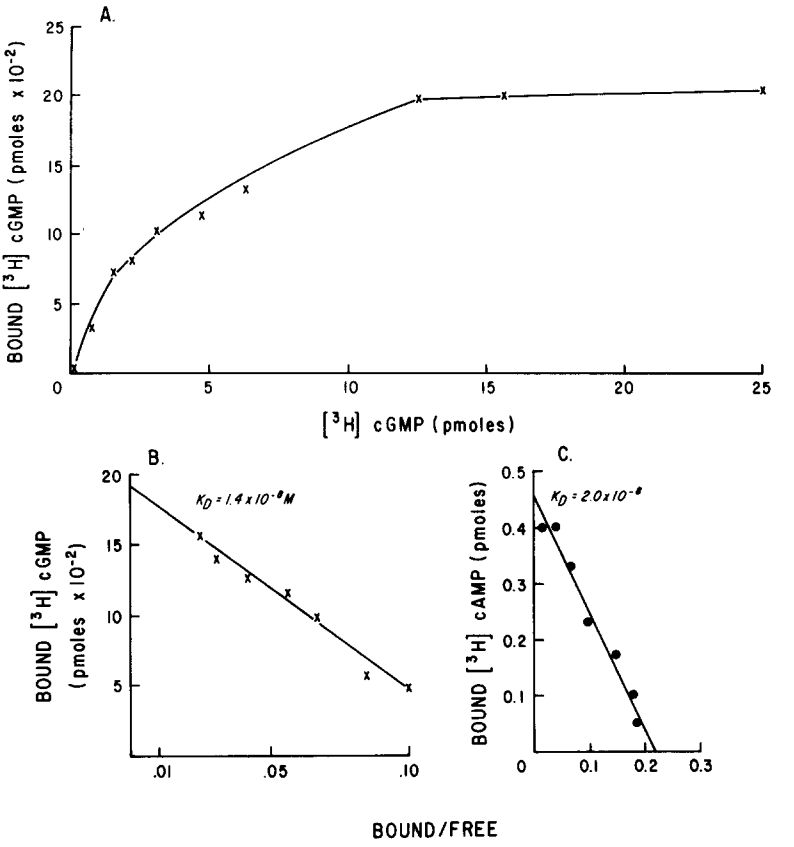

Figure 1. Interaction of [^{3}H]cGMP with partially purified receptor protein. Bovine adrenal cortical cytosol protein (0-50% ammonium sulfate fraction) was chromatographed on a 2 ml column of C8-NC_6NH_2-cAMP-Sepharose (50 mM) to remove cAMP receptor protein. Increasing quantities of [^{3}H]cGMP were added to a constant amount of the unadsorbed protein (250 µg) in a final reaction volume of 0.1 ml and binding assayed in duplicate as described under "Methods."

A. The amount of [^{3}H]cGMP bound is plotted as a function of added nucleotide.

B. Scatchard analysis of the interaction of [^{3}H]cGMP with receptor protein. The concentration of free [^{3}H]cGMP was obtained by subtracting the [^{3}H]cGMP bound from the total [^{3}H]cGMP initially added. Single site binding kinetics follow the equation: amount bound = number of sites - KD (bound/free). The observed KD = 1.4 X 10^{-8} M; the specific activity of binding calculated from the intercept on the Y axis = 0.75 pm/mg protein.

C. Scatchard analysis of the interaction of [^{3}H]cAMP with the cAMP receptor protein. Protein (0.4 µg) prepared by urea elution from the C8-NC_6NH_2-cAMP-Sepharose column was incubated with increasing quantities of [^{3}H]cAMP in a final reaction volume of 0.1 ml and binding assayed in duplicate as described under "Methods." The observed KD = 2 X 10^{-8} M.

The interaction of [^{3}H]cGMP with this receptor protein preparation was determined. Saturation of the high affinity site is now observed (Fig. 1A). Scatchard analysis of cGMP binding indicated a single affinity class of receptor sites with KD = 1.4 X 10^{-8} M (Fig. 1B).

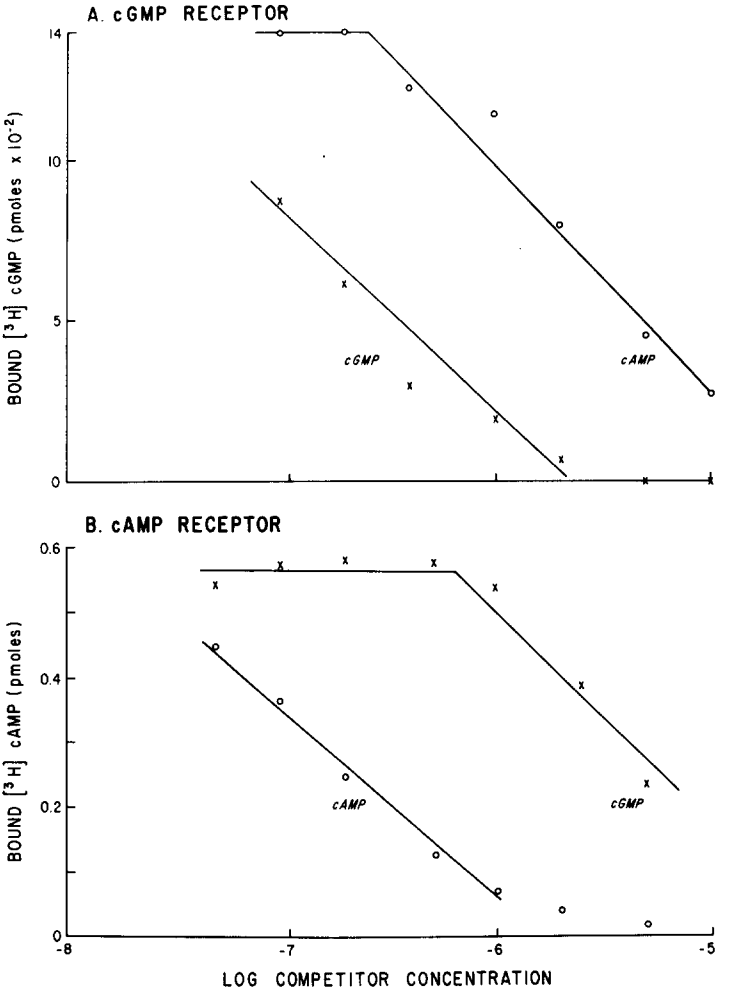

Figure 2. Specificity of binding.

A. cGMP receptor protein was prepared by chromatography on C8-NC_6NH_2-cAMP-Sepharose as described under "Methods." Increasing concentrations of unlabeled cGMP or cAMP were added to reaction mixtures of 0.1 ml containing saturating concentrations of [^{3}H]cGMP (9.4 X 10^{-8} M). The reaction was initiated by adding the binding protein solution. Binding of [^{3}H]cGMP was determined by retention of bound nucleotide on cellulose ester filters. Each point represents an average of duplicates. X———X, unlabeled cGMP as competitor; O———O, unlabeled cAMP as competitor.

B. cAMP receptor protein was prepared by elution from C8-NC_6NH_2-cAMP-Sepharose (21). Similar results were obtained using cAMP receptor protein prepared as previously described (26). Increasing concentrations of unlabeled cAMP or cGMP were added to reaction mixtures of 0.1 ml containing saturating concentrations of [^{3}H]cAMP (9.4 X 10^{-8} M) and binding was determined as described under "Methods."

The KD for cGMP binding is similar to the KD for cAMP binding to the cAMP receptor protein (KD = 2 X 10^{-8} M) (Fig. 1C).

Because the protein, which was unadsorbed to C8-NC_6NH_2-cAMP-Sepharose, con-

tained residual cAMP binding activity, and because cGMP has been reported to bind to certain forms of cAMP-dependent protein kinase at lower pH (27), it was necessary to demonstrate nucleotide specificity. Increasing concentrations of unlabeled cGMP or cAMP were added to saturating concentrations of [^{3}H]cGMP (9 X 10^{-8} M) and the displacement of radiolabeled cGMP from binding sites determined (Fig. 2A). Approximately 10-fold higher concentrations of cAMP than cGMP are required to achieve parallel displacement of [^{3}H]cGMP from binding sites. The converse experiment using cAMP receptor protein confirms the specificity of the cAMP receptor protein for cAMP (Fig. 2B). The specificity of cAMP binding to its receptor can be demonstrated using protein purified by elution from C8-NC_6NH_2-cAMP-Sepharose or by previously reported procedures indicating that interaction with the column does not alter the receptor protein (21,26). Each of the separated receptor proteins demonstrates nucleotide specificity. The specificity of the cGMP receptor site was further defined by using a number of nucleotides as competitors for cGMP binding (Table III). Of the compounds tested only purines containing a 3',5' cyclic phosphate function as effective competitors. Inosine 3',5'-monophosphate which structurally most resembles cGMP is the most effective competitor for cGMP binding sites. The C8 derivative of cAMP, C8-NC_6NH_2-cAMP, does not function as a competitor of cGMP binding. Since this compound is an excellent competitor of cAMP binding to the cAMP receptor protein, it serves as an effective affinity chromatography agent to separate the two receptor proteins.

Characteristics of the cGMP Receptor. cGMP receptor activity is precipitated by ammonium sulfate, non-dialyzable, heat labile (100% loss of activity after 30 min at 60°), and pronase sensitive. Receptor activity is not affected by RNase or DNase treatment. [^{3}H]Nucleotide recovered from receptor protein migrated with authentic cGMP indicating that cGMP was not metabolized upon binding.

The cGMP receptor protein sedimented at 7S in glycerol density gradients. The peak fraction bound 6 pm of [^{3}H]cGMP per mg protein; [^{3}H]cGMP binding specificity was con-

TABLE III: Nucleotide Specificity

Nucleotide competitor	Competitor Concentration 10^{-6} M Bound [^{3}H]cGMP (pmoles)	10^{-5} M
None	0.198	
cGMP	0.020	0.000
cIMP	0.050	0.002
cAMP	0.114	0.027
cCMP	0.139	0.120
GTP	0.130	0.115
GDP	0.217	0.174
GMP	0.187	0.175
ATP	0.132	0.110
ADP	0.168	0.152
AMP	0.201	0.198
C8-NC_6NH_2-cAMP	0.236	0.233

Reaction mixtures of 0.1 ml contained [^{3}H]cGMP at a final concentration of 9.4 X 10^{-8} M and unlabeled nucleotides to a final concentration of 10^{-6} M or 10^{-5} M. Reactions were initiated by the addition of cGMP receptor protein (250 µg) prepared by interaction with C8-NC_6NH_2-cAMP-Sepharose as described under "Methods." Binding of [^{3}H]cGMP was determined in duplicate.

firmed in experiments similar to those shown in Fig. 2. In previous experiments, the cAMP receptor : protein kinase complex has been shown to sediment at an identical 7S position; the free cAMP receptor sediments at 4.6S (26,28).

DISCUSSION. The 8-(6-aminohexyl)-amino derivative of cAMP is a competitive inhibitor of the binding of cAMP to the cAMP receptor site (21). The cAMP derivative does

close to the distance between 2 N atoms attached to the 2' and 4' positions of the reagent, i.e. 7.5 Å.[2] There remains the possibility of a cross-link between the N-terminal amino group of one β chain and the 82 lysine of the other, but this seems less likely since the distance is 11 Å.[3]

The available evidence therefore points to a cross-link between the β-82 lysines. The flexibility of lysine side chains would create a cross-link with minimum rigidity which would allow the considerable degree of conformational change observed on ligand binding. The introduction of this cross-link also demonstrates that a hemoglobin tetramer can bind ligand cooperatively without subunit exchange (10).

HbXL appears to be the first cross-linked hemoglobin with significant retention of allosteric properties. Previous attempts to cross-link hemoglobin with bifunctional reagents (11 - 14) have resulted in derivatives which had lost the linked functions associated with conformational change.

Acknowledgements. We are deeply indebted to Dr. Anna Pocker, whose generous gift of 2-nor-2-formylpyridoxal 5'-phosphate has made this work possible. The study was supported by U.S. Public Health Service Grants HL-05791 and HL-06787 from the National Heart and Lung Institute and Grant GB-39288 from the National Science Foundation; Grant CRBS-331 from the National Foundation and a grant from the Merck Institute for Therapeutic Research. R.B. is a recipient of a U.S. Public Health Research Career award.

References

1. Benesch, R. E., Benesch, R. & Yu, C. I. (1969) Fed. Proc., 28:604.
2. Renthal, R., Benesch, R. E., Benesch, R. & Bray, B. A. (1970) Fed. Proc., 29 #2:732.
3. Benesch, R. E., Benesch, R., Renthal, R. D. & Maeda, N. (1972) Biochemistry, 11:3576-3582.

[2] Reaction with lysine side chains is quite plausible, since this is, in fact, the site of attachment of PLP to numerous enzymes (9).

[3] These distances were calculated from the atomic coordinates of human deoxyhemoglobin, kindly supplied by Dr. Arthur Arnone.

REFERENCES

1. Hadden, J. W., Hadden, E. M., Haddox, M. K., and Goldberg, N. D. (1972) Proc. Nat. Acad. Sci. USA 69, 3024-3027.
2. Seifert, W. E., and Rudland, P. S. (1974) Nature 248, 138-140.
3. Rudland, P. S., Gospodarowiez, D., and Seifert, W. (1974) Nature 250, 741-742, 773-774.
4. Ryan, W. L., and Heidrick, M. L. (1968) Science 162, 1484-1485.
5. Hsie, A. W., and Puck, T. T. (1971) Proc. Nat. Acad. Sci. USA 68, 358-361.
6. Johnson, G. S., Friedman, R. M., and Pastan, I. (1971) Proc. Nat. Acad. Sci. USA 68, 425-429.
7. Sheppard, J. R. (1971) Proc. Nat. Acad. Sci. USA 68, 1316-1320.
8. Masui, H., and Garren, L. D. (1971) Proc. Nat. Acad. Sci. USA 68, 3206-3210.
9. Smith, J. W., Steiner, A. L., and Parker, C. W. (1971) J. Clin. Invest. 50, 442-448.
10. Goldberg, N. D., Haddox, M. K., Dunham, E., Lopez, C., and Hadden, J. W. (1974) Control of Proliferation in Animal Cells, pp. 609-625, Cold Spring Harbor Conferences on Cell Proliferation, Volume I.
11. Emmer, M., deCrombrugghe, B., Pastan, I., and Perlman, R. (1970) Proc. Nat. Acad. Sci. USA 66, 480-487.
12. Riggs, A. D., Reiness, G., and Zubay, G. (1971) Proc. Nat. Acad. Sci. USA 68, 1222-1225.
13. deCrombrugghe, B., Chen, B., Anderson, W., Nissley, P., Gottesman, M., Pastan, I., Perlman, R. (1971) Nature New Biol. 231, 139-142.
14. Anderson, W. B., Schneider, A. B., Emmer, M., Perlman, R. L., and Pastan, I. (1971) J. Biol. Chem. 246, 5929-5937.
15. Kuo, J. F., and Greengard, P. (1970) J. Biol. Chem. 245, 2493-2498.
16. Hofmann, F., and Sold, G. (1972) Biochem. Biophys. Res. Commun. 49, 1100-1107.
17. Van Leemput-Coutrez, M., Camus, J., and Christophe, J. (1973) Biochem. Biophys. Res. Commun. 54, 182-190.
18. Kuo, J. F. (1974) Proc. Nat. Acad. Sci. USA 71, 4037-4041.
19. Casnellie, J. E., and Greengard, P. (1974) Proc. Nat. Acad. Sci. USA 71, 1891-1895.
20. Sold, G.,and Hofmann, F. (1974) Eur. J. Biochem. 44, 143-149.
21. Ramseyer, J., Kaslow, H. R., and Gill, G. N. (1974) Biochem. Biophys. Res. Commun. 59, 813-821.
22. Murad, F., Manganiello, V., and Vaughan, M. (1971) Proc. Nat. Acad. Sci. USA 68, 736-739.
23. Gilman, A. G. (1970) Proc. Nat. Acad. Sci. USA 67, 305-312.
24. Gill, G. N., and Walton, G. M. (1974) Meth. Enzymol. 38, 376-382.
25. Bray, G. A. (1960) Anal. Biochem. 1, 279-285.
26. Gill, G. N., and Garren, L. D. (1971) Proc. Nat. Acad. Sci. USA 68, 786-790.
27. Haddox, M. K., Nicol, S. E., and Goldberg, N. D. (1973) Biochem. Biophys. Res. Commun. 54, 1444-1450.
28. Gill, G. N., and Garren, L. D. (1969) Proc. Nat. Acad. Sci. USA 63, 512-519.
29. Gill, G. N., and Garren, L. D. (1970) Biochem. Biophys. Res. Commun. 39, 335-343.
30. Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971) J. Biol. Chem. 246, 1986-1995.
31. Kuo, J. F., Miyamoto, E., and Reyes, P. L. (1974) Biochem. Pharm. 23, 2011-2021.
32. Kuo, J.-F., Lee, T.-P., Reyes, P. L., Walton, K. G., Donnelly, Jr., T. E., and Greengard, P. (1972) J. Biol. Chem. 247, 16-22.