Dische, Z., and Schettles, L. B. (1948), *J. Biol. Chem. 175*, 595.
Edelman, G. M., Cunningham, B. A., Gall, W. E., Gottlieb, P. D., Rutishauser, U., and Waxdal, M. J. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 78.
Edelman, G. M., and Gall, W. E. (1969), *Annu. Rev. Biochem. 38*, 415.
Edmunson, A. B., Sheber, F. A., Ely, K. R., Simonds, N. B., Hutson, N. K., and Rossiter, J. L. (1968), *Arch. Biochem. Biophys. 127*, 725.
Fahey, J. L. (1963), *J. Clin. Invest. 42*, 111.
Fahey, J. L., and McLaughlin, C. (1963), *J. Immunol. 91*, 484.
Franklin, E. C. (1960), *J. Clin. Invest. 39*, 1933.
Gray, W. R. (1967), *Methods Enzymol. 11*, 469.
Grey, H. M., and Abel, C. A. (1970), *Protides Biol. Fluids, Proc. Colloq.* (in press).
Grey, H. M., and Kunkel, H. G. (1967), *Biochemistry 6*, 2326.
Grey, H. M., and Mannik, M. (1965), *J. Exp. Med. 122*, 619.
Hilschmann, N. (1967), *Physiol. Chem. 348*, 1077.
Hood, L., Grant, J. A., and Sox, H. C. (1969), *Acta Pathol. Microbiol. Scand. 76*, 1969.
Kohler, H., Shimizu, A., Paul, C., van Dalen, A., and Putnam, F. W. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 257.
Konigsberg, W. (1967), *Methods Enzymol. 11*, 461.
Langer, B., Steinmetz-Kayne, M., and Hilschmann, N. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 945.
Marshall, R. (1967), *Proc. Int. Congr. Biochem., 7th, 1967.*
McConahey, P. J., and Dixon, F. J. (1966), *Int. Arch. Allergy Appl. Immunol. 29*, 185.
Melchers, F. (1969), *Biochemistry 8*, 938.
Milstein, C. (1967), *Nature 216*, 330.
Pink, J. R. L., and Milstein, C. (1969), *Fed. Eur. Biochem. Soc. Symp. 15*, 177.
Pink, J. R. L., and Milstein, C. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 703.
Poulik, M. D. (1960), *Biochim. Biophys. Acta 44*, 390.
Prostingl, H., Hess, M., and Hilschmann, N. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 867.
Rosevear, J. W., and Smith, E. (1961), *J. Biol. Chem. 236*, 425.
Spiegelberg, H. L., and Weigle, W. O. (1968), *J. Immunol. 101*, 377.
Svennerholm, L. (1956), *J. Neurochem. 1*, 42.
Swenson, R. M., and Kern, M. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 546.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Wikler, M., Kohler, G., Shinoda, T., and Putnam, F. W. (1969), *Science 163*, 75.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.

An Assay for Adenosine 3′,5′-Cyclic Monophosphate Based on the Association of the Nucleotide with a Partially Purified Binding Protein*

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ABSTRACT: The binding properties of a cyclic AMP receptor protein are utilized to provide a sensitive and rather specific assay for this nucleotide in biological tissues. A fivefold purification of the cyclic AMP binding protein from beef adrenal glands provides a preparation which allows the determination of as little as 20 pmoles of cyclic AMP isolated from tissue extracts. The assay is based upon the isotopic dilution of cyclic [^{3}H]AMP by the cyclic AMP which is being measured and the subsequent attachment of the cyclic nucleotide to the receptor protein. The rapid separation of free ligand from that bound to the protein is accomplished by passing the mixture through a membrane filter with a demonstrated affinity for the protein–ligand complex. At levels of cyclic AMP determined by the assay, a high specificity for the cyclic nucleotide was observed in the presence of similar concentrations of other nucleotides. The assay was used to determine the levels of cyclic AMP isolated from various tissues of the rat.

The binding of adenosine 3′,5′-cyclic monophosphate[1] to a protein fraction from beef adrenal cortex has been previously reported by this laboratory (Gill and Garren, 1969). Utilizing the equilibrium dialysis technique, the binding activity was found to be present in both the soluble and microsomal fractions of the adrenal cortex. Although the role of this protein in metabolism is as yet unknown, an association with a cyclic AMP dependent protein kinase activity has been suggested through partial purification of the two activities from

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[1] Cyclic AMP, adenosine 3′,5′-cyclic monophosphate; cyclic IMP, inosine 3′,5′-cyclic monophosphate; cyclic GMP, guanosine 3′,5′-cyclic monophosphate; cyclic CMP, cytidine 3′,5′-cyclic monophosphate; AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.

the soluble fraction of the adrenal cortex (Gill and Garren, 1970). The strong affinity and high degree of specificity of the binding protein for the nucleotide suggested properties which were applicable to a convenient and sensitive method for the determination of cyclic AMP in extracts of biological tissues.

The development of the assay for cyclic AMP, and the determination of binding activity was further enhanced by a rapid and quantitative separation of the ligand-bound complex from the free ligand. This was accomplished with the use of a cellulose membrane filter which demonstrated an affinity for the cyclic AMP binding protein.

Experimental Section

Materials. Cyclic [^{3}H]AMP was a product of Schwarz Bio-Research, Inc., and had a specific activity of 2.35 Ci/mmole. Cyclic IMP, cyclic GMP, and cyclic CMP were obtained from Boehringer Mannheim Corp., ACTH from Armour Pharmaceutical Co., and hydrocortisone hemisuccinate and prednisone acetate from Mann Research Laboratories. Beef adrenal glands were obtained fresh from a local meat packing company. Cellulose ester membrane filters (HA 0.45 μ, 24 mm) were from Millipore Corp.

Determination of Cyclic AMP Binding Activity. Duplicate reaction mixtures were prepared by rapidly adding and mixing, up to 200 μg of protein in a 0.25-ml reaction mixture, containing 5.3×10^{-8} M cyclic [^{3}H]AMP (31 mμCi) in various buffers as described in the legends of the text. Theophylline (5–8 mM) was added to inhibit possible cyclic AMP phosphodiesterase activity except where indicated in the text. The mixture was incubated for 30 min at 30° and then cooled in an ice bath. A Millipore filter apparatus was used to separate the free from protein-bound cyclic AMP. The membrane filter was initially immersed in 0.025 M Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$. Then the reaction mixture was quantitatively transferred to the reservoir situated above the moistened membrane filter which contained 5 ml of the same cold buffer. A mild vacuum was applied and the solution was pulled through the filter. Following this, the filter was washed thoroughly with cold buffer and dried. The duplicate filters were dissolved together in a scintillation vial by the addition of 10 ml of Bray's scintillator (Bray, 1960) and assayed for radioactivity in the liquid scintillation counter.

Purification of the Binding Protein from Beef Adrenal Glands. The cyclic AMP binding protein was purified by modification of the method described by Gill and Garren (1969). All the procedures used in the purification of the protein were carried out in a cold room at 4° or in an ice bath. Fresh beef adrenal glands were stripped of medulla and the cortex was homogenized in two volumes of 0.25 M sucrose, 50 mM KCl, and 1 mM $MgCl_2$ in 0.05 M Tris-HCl (pH 7.4). The homogenate was centrifuged at 500*g* for 10 min followed by a 12,000*g* centrifugation of the supernatant for 10 min. The supernatant was further centrifuged at 105,000*g* for 90 min. This postmicrosomal supernatant (S-1) was diluted with the homogenizing media to a protein concentration of approximately 10 mg/ml and treated with 0.32 g/ml of ammonium sulfate; the pH was adjusted to 7.4 with dilute NaOH. The precipitate formed was resuspended in 0.05 M Tris-HCl (pH 7.4) containing 6 mM mercaptoethanol and dialyzed against 0.01 M Tris-HCl (pH 7.4) containing 6 mM mercaptoethanol. The dialyzed protein solution (S-11) was diluted to a concentration of 5 mg/ml or less and applied to a DEAE-cellulose column (2.5 × 46 cm) equilibrated with 5 mM Tris-HCl (pH 7.4) at a ratio of 1 mg of protein/0.14 cm^3 of column. The column was washed with four to five volumes each of 0.01 M Tris-HCl (pH 7.4), and 0.01 M Tris-HCl (pH 7.4) containing 0.05 M NaCl. The activity was eluted with 0.01 M Tris-HCl (pH 7.4) containing 0.2 M NaCl. The active fractions were pooled and dialyzed against 5 mM Tris-HCl (pH 7.4) containing 6 mM mercaptoethanol (S-III). The binding activity was adsorbed onto calcium phosphate gel, previously washed with 5 mM Tris-HCl (pH 7.4), by stirring the gel into the S-III fraction at a ratio of 10 mg of gel/mg of protein. The gel was washed initially with 0.5 M Tris-HCl (pH 7.4) and the binding activity eluted with 0.5 M potassium phosphate (pH 7.8) buffer (S-IV). The protein solution was immediately concentrated in dialysis tubing to 5 mg/ml or greater with the aid of polyethylene glycol (mol wt 15,000–20,000) and subsequently dialyzed against 0.02 M potassium phosphate buffer (pH 7.5).

Protein estimation during purification was based on the optical density observed at 280 and 260 mμ (Colowick and Kaplan, 1957). In calculating specific activity, however, the protein concentration was determined by the method of Lowry *et al.* (1951). A unit of specific activity was defined as 1 pmole of cyclic AMP bound per mg of protein.

Cyclic 3′,5′-Nucleotide Phosphodiesterase. The isolation and preparation of brain cyclic 3′,5′-nucleotide phosphodiesterase was by the method of Brooker *et al.* (1968).

Tissue Preparation for the Determination of Cyclic AMP Levels. Five-month-old female rats (Sprague–Dawley) were used in these studies. The animals were anesthetized with sodium pentobarbital (40 mg/kg) administered by intraperitoneal injection. Thirty minutes later the tissues were removed and immediately frozen in liquid nitrogen.

Twenty rats were used in studying the effect of ACTH on cyclic AMP levels in the adrenal glands. Each animal was injected intraperitoneally with 1 ml of 0.9% NaCl containing 6 mg each of hydrocortisone hemisuccinate and prednisone acetate at 16 hr and again at 2 hr prior to being anesthetized to suppress the secretion of ACTH. The adrenal glands of ten of the animals (controls) were removed 30 min after the administration of anesthesia. The remaining ten animals each had 4 units of ACTH injected into the femoral vein 10–15 min before the adrenal glands were removed. The glands were quickly freed of fat, and frozen in liquid nitrogen.

Extraction of Cyclic AMP from Tissue. Cyclic AMP was isolated from tissue by the method of Aurbach and Houston (1968) with the following minor modifications. The frozen tissues were pulverized and 0.4–0.6 g of the powdered tissue was homogenized in 6% trichloroacetic acid at a ratio of 6 ml/g of tissue. Cyclic [^{3}H]AMP (1.25 mμCi/ml of 6% trichloroacetic acid was added to the homogenate to allow quantitation of the recovery of cyclic AMP through the entire extraction procedure. The level of cyclic [^{3}H]AMP added was 3.2×10^{-3} nmole/g of tissue and represented a level approximately 0.01–0.001 of that found in most tissues. The trichloroacetic acid was extracted with ether and the residual ether was expelled with a stream of air. The extract was neutralized to pH 7.5 with a 1 M solution of Tris-OH, adjusted to a volume of 3 ml with water, treated with 0.2 ml of 5% $ZnSO_4$, neutralized with 0.3 N $Ba(OH)_2$, and kept in ice for 10 min to allow the precipitate to form. The precipitate was removed by centrifugation and 1.5 ml of the supernatant was placed on a 7 ×

TABLE I: Summary of Cyclic AMP Binding Protein Purification.[a]

Purification Procedure	Sp Act. (pmoles/mg of Protein)	Act. Recovd (%)
S-I postmicrosomal supernatant	3.18	100
S-II ammonium sulfate precipitation	4.40	98
S-III DEAE-cellulose column	12.6	55
S-IV calcium phosphate gel	15.2	50

[a] The reaction mixtures were prepared in 0.05 M potassium phosphate buffer (pH 6.5) and binding activity determined as described in the text.

80 mm Dowex 50W-X8 (100–200 mesh) column in the H^+ form. Cyclic AMP was eluted with water in the 5–8-ml fractions. The fractions were pooled and lyophilized to dryness. The residue was taken up in 0.4 ml of water and the radioactivity of a 0.1-ml aliquot was determined and calculated to yield recoveries of 45–55%.

Cyclic AMP was similarly extracted from rat adrenal glands with the exception that the frozen glands, without pulverization, were directly homogenized in the 6% trichloroacetic acid.

Cyclic AMP Assay. Approximately 200 μg of binding protein (S-IV) was added to thoroughly mixed reaction mixtures of 0.25 ml containing 0.05 M potassium phosphate buffer (pH 6.5), 5.3×10^{-9} M cyclic [^{3}H]AMP (3.1 mμCi), and an aliquot of cyclic AMP isolated from tissue or standard solution of unlabeled cyclic AMP such that the final concentration of the nucleotide was $5–40 \times 10^{-8}$ M. A standard was similarly prepared without unlabeled cyclic AMP in order to determine the optimum binding of cyclic [^{3}H]AMP when no competing nucleotide was present. A sample was also prepared which contained only cyclic [^{3}H]AMP in the same buffer to determine the nonspecific adsorption of the nucleotide to the membrane filter when the binding protein was not present. In the reported experiments, this background was approximately 20 cpm. Duplicate samples were prepared, incubated, and the cyclic [^{3}H]AMP–protein complex was isolated as previously described. A standard curve was performed for each determination of cyclic AMP isolated from tissue extracts.

Results

Purification of the Cyclic AMP Binding Protein. A summary of the results of the fractionation procedure as described in the text is presented in Table I. The specific activity of the S-IV fraction was enriched approximately 5-fold over that observed in the postmicrosomal supernatant, with a recovery of 50% of the total activity. The procedure provided a stable preparation for the assay. The cyclic AMP binding activity of the S-IV fraction was not significantly diminished even after freezing for as long as 2 months, provided a protein concentration of greater than 5 mg/ml was maintained. Storage of dilute preparations, however, resulted in a rapid decrease in binding activity.

Cyclic AMP Binding Activity in Various Tissues. To ascertain whether cyclic AMP binding protein was present in tissues other than the adrenal cortex, a survey of this activity was performed on postmitochondrial supernatant fractions from various tissues of the rat and beef adrenal gland (Table II). Since the amount of endogenously bound cyclic AMP was not determined in the various tissues assayed, the binding activity reported represents only that afforded by residual cyclic AMP binding sites. The nucleotide specificity of the receptor protein was tested only in adrenal cortical tissue. Nevertheless, the association of the ligand–protein complex to the membrane filter should afford some specificity to the assay. In the following studies, the beef adrenal cortex was used as the source of the cyclic AMP binding protein.

TABLE II: Survey of Cyclic AMP Binding Activity in Various Tissues.[a]

Tissue	Sp Act. of Cyclic AMP Binding Protein (pmoles/mg of Protein)
Adrenal gland	7.9
Heart	4.7
Muscle	3.0
Spleen	2.6
Brain	2.3
Liver	2.1
Kidney	1.7
Beef adrenal cortex	4.4
Beef adrenal medulla	2.9

[a] Weighed rat tissues and beef adrenal glands were diced and homogenized in glass with a Teflon pestle in approximately four volumes of 0.05 M Tris-HCl (pH 7.4). The homogenates were centrifuged at 12,000g for 30 min and the supernatants were dialyzed against the same buffer. All procedures were performed at 5°. The reaction mixtures contained 67 mM Tris-HCl (pH 7.4), 6.7 mM theophylline, 10 mM $MgCl_2$, 5.3×10^{-8} M cyclic [^{3}H]AMP (31 mμCi), and tissue extract containing 90–180 μg of protein. Binding activity was determined as described in the text.

Properties of the Binding Activity. The finding that the cyclic AMP binding protein adhered to the membrane filter led to a quick and convenient method for the separation of unbound nucleotide from the cyclic AMP–protein complex. The linear relationship of the affinity of the protein complex for the filter as a function of protein concentration is shown in Figure 1. This partially purified binding protein preparation demonstrated a specific activity of 8.9 pmoles of cyclic AMP bound per mg of protein when saturated with the nucleotide. A linear relationship up to 250 μg of protein was demonstrated for the association of the cyclic AMP–protein complex to a single membrane filter and saturation occurred at approximately 500 μg of protein. The total amount of cyclic [^{3}H]AMP present in the reaction mixture was not at limiting concentrations at the higher protein levels since two membrane filters together provided twice the binding capacity for the protein complex and a linear response was observed up to 480 μg of protein. In the assay for cyclic AMP, approximately 200 μg of the protein containing binding activity was used.

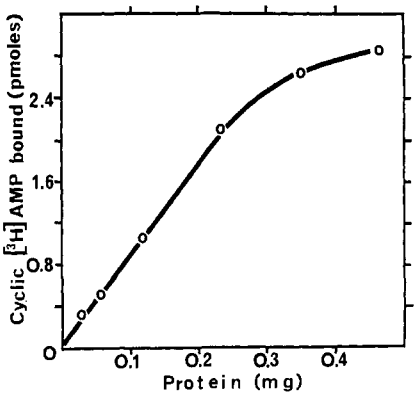

FIGURE 1: The affinity of cyclic [^{3}H]AMP–protein complex for a membrane filter as a function of protein concentration. The incubation media for the binding assays contained 0.05 M potassium phosphate (pH 7.5), 5.3×10^{-8} M cyclic [^{3}H]AMP (31 mμCi), and binding protein (S-III) as indicated.

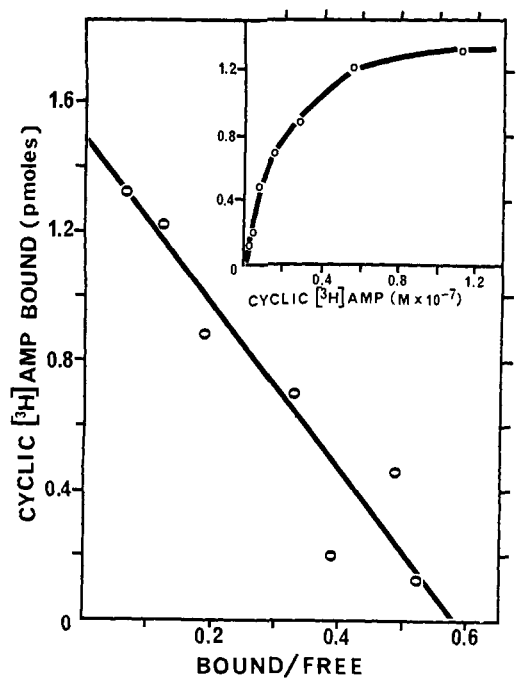

FIGURE 2: Inset: the interaction of cyclic [^{3}H]AMP with binding protein as a function of the total concentration of nucleotide added. The amount of nucleotide bound is also plotted as a function of the ratio of the concentration of bound to free cyclic AMP. The concentration of free cyclic [^{3}H]AMP was obtained by subtracting the cyclic [^{3}H]AMP bound from the total cyclic [^{3}H]AMP concentration initially added to the reaction mixture. Duplicate assays were performed by incubating 0.05 M Tris-HCl (pH 7.4), cyclic [^{3}H]AMP as indicated, 5 mM theophylline, and 10 mM $MgCl_2$ with 400 μg of S-II binding protein in a total volume of 0.20 ml. Isolation of the cyclic AMP-bound protein complex was performed as described in the text, with the exception that two filters were used together in order to provide the capacity needed to bind this amount of protein. Duplicate assays were separately dissolved in Bray's scintillator and counted. To evaluate k the molar concentration was determined from the total amount of ligand bound in 0.2 ml of reaction mixture.

Heating of the protein fraction at 100° for 3 min or the addition of 5% trichloroacetic acid to the ligand–protein complex resulted in a complete loss of binding activity. The nature of the association of cyclic AMP to the binding protein is not known but the lack of binding activity observed following denaturation of the ligand-bound complex rules out a covalently bound moeity, unless of course such treatment destroys the ability of the complex to bind to or be retained by the membrane filter. The results of Gill and Garren (1969) suggested that the cyclic AMP molecule remained intact upon removal from the complex which also supports the noncovalent association of cyclic AMP with the protein.

A measure of the amount of cyclic AMP bound as a function of total ligand concentration is shown in Figure 2 (insert). A Scatchard plot (Scatchard, 1949) of these data was performed to determine the intrinsic association constant, k, of the protein complex. The k of 1.3×10^{-8} agrees closely with the value of 5×10^{-8} obtained previously for the binding of cyclic AMP utilizing equilibrium dialysis (Gill and Garren, 1969).

The affinity of cyclic AMP for the protein, as evidenced by the low intrinsic constant, was further demonstrated by the fact that, even after extensive washing of the bound complex on the membrane filter, little cyclic AMP was removed. As shown by Gill and Garren (1969), dialysis of such a ligand bound complex for as long as 96 hr resulted in a loss of only 25% of the total cyclic AMP bound.

The binding of cyclic AMP as a function of pH revealed a broad range of activity. From pH 4.5 to 8.5, the binding activity was at least 85% of that observed at the optimum pH of 5.5 at a cyclic AMP concentration of 5.3×10^{-8} M. The activity in a citrate–sodium phosphate buffer was comparable to that found in potassium phosphate at the same pH. However, the activity observed in Tris-HCl buffer (pH 7.5) was variable and appeared to be less than that with potassium phosphate at the same pH. However, the activity could be restored to levels observed in potassium phosphate buffer with the addition of 8 mM theophylline to the assay mixture. Although the difference in binding activity observed with Tris-HCl buffer is not entirely clear, presumably the loss of binding of cyclic AMP in this buffer occurred through the action of cyclic AMP phosphodiesterase which is known to be inhibited by theophylline (Butcher and Sutherland, 1962). At levels of cyclic AMP considered to be saturating, the cyclic AMP–protein complex formation was found to be relatively independent of Mg^{2+}, but a slight stimulation of binding activity (15%) was observed with $MgCl_2$ at a concentration of 10 mM. Under similar assay conditions, 1 mM EDTA showed no significant effect on binding activity.

Binding Specificity. Although cyclic AMP binding activity was shown to exist in other tissues as described above, in the present study, the nucleotide specificity of binding activity was determined only in beef adrenal cortical tissue and the results are consistent with those presented previously (Gill and Garren, 1969). Various concentrations of nucleotides were tested as possible competitors of cyclic AMP for binding sites on the protein at a nonsaturation concentration of cyclic [^{3}H]AMP in the mixture. The specificity of the binding of cyclic AMP to the protein in the presence of other nucleotides is shown in Figure 3. In the presence of 2.4×10^{-7} M cyclic AMP, the binding of cyclic [^{3}H]AMP was reduced to 27% of the control; *i.e.*, the control demonstrated the binding activity observed when no competing nucleotide

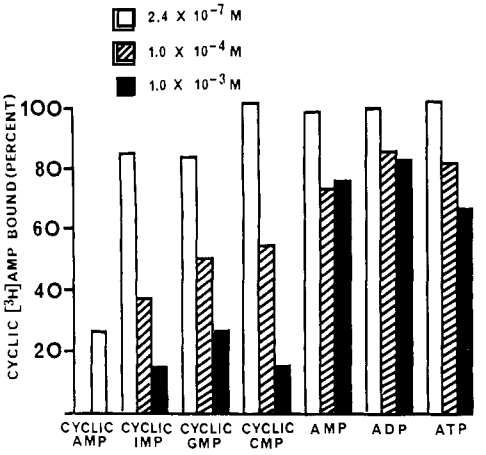

FIGURE 3: Effects of various concentrations of nucleotides on the binding of cyclic [³H]AMP. The reaction mixture contained 0.05 M potassium phosphate (pH 6.5), 5.3 × 10^{-9} M cyclic [³H]AMP (3.1 mμCi), and 210 μg of S-IV protein preparation (Table I), and unlabeled nucleotide as indicated. The control was the same but no unlabeled nucleotide was added. In the control 1570 cpm was bound which is represented as 100% of the cyclic [³H]AMP bound.

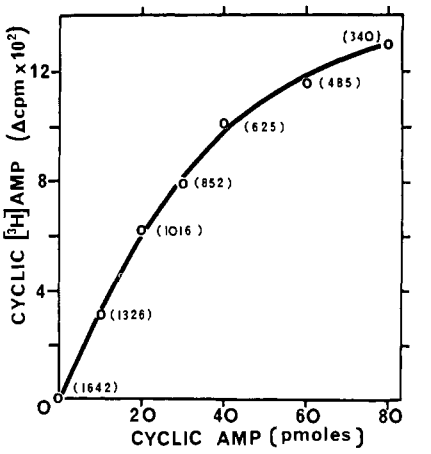

FIGURE 4: A typical standard curve for the measurement of cyclic AMP isolated from tissue extracts. Assays were performed with the S-IV preparation of Table I as described in the text. Nonradioactive cyclic AMP was added as indicated. The concentration of cyclic AMP added is plotted as a function of the difference in counts per minute of cyclic [³H]AMP bound in the presence and absence of the nonradioactive cyclic AMP. The counts per minute bound are indicated in parentheses.

was present. At the same low concentration of 2.4 × 10^{-7} M, cyclic IMP and cyclic GMP decreased the binding of cyclic [³H]AMP to only 84%, at most, of the control. An increase in the concentration of cyclic IMP to 10^{-4} M and again to 10^{-3} M, further reduced the binding of cyclic [³H]AMP to 38 and 15% of the control, respectively. Cyclic GMP and cyclic CMP at these high concentrations produced a similar degree of inhibition of binding activity. No significant inhibition of cyclic [³H]AMP binding was caused by the addition of either AMP, ADP, or ATP at 2.4 × 10^{-7} M. However when AMP was added at 10^{-4} and 10^{-3} M, the binding of cyclic [³H]AMP was reduced to 73 and 76%, respectively, of the binding obtained without the addition of competing nucleotides. Similar findings were obtained with ADP and ATP as competing nucleotides. The small increase in the inhibition of binding activity observed with the 10-fold increase in the concentration of the adenosine nucleotides added (*i.e.*, from 10^{-4} to 10^{-3} M) differed from the inhibition observed with the cyclic nucleotides; *i.e.*, the binding of cyclic [³H]AMP fell progressively as increasing amounts of either cyclic AMP, cyclic GMP, or cyclic CMP were added to the reaction mixture. This suggested the possibility that the small inhibition of cyclic [³H]AMP binding, observed by the addition of the noncyclic adenosine nucleotides to the reaction, may have resulted from an interaction of these nucleotides at a site other than the binding site of cyclic AMP, while the cyclic nucleotides appeared to compete directly for the cyclic AMP binding site. Because the degree of specificity of the binding protein for cyclic AMP was not absolute, in determining the amount of cyclic AMP of tissue extracts, cyclic AMP was first isolated from possible competing nucleotides. It is to be emphasized, however, that the concentration of cyclic [³H]AMP used in these binding specificity studies was less than a saturation level, and resembled the conditions under which the assay for cyclic AMP is performed. Even so, when the various nucleotides were tested for competition at reasonable tissue levels, only unlabeled cyclic AMP significantly reduced the binding of cyclic [³H]AMP.

Assay of Cyclic AMP. The methodology for the determination of cyclic AMP isolated from tissue was based on the isotopic dilution of cyclic [³H]AMP in the reaction mixture prior to the addition of binding protein. The subsequent binding of the cyclic AMP–protein complex to a membrane filter was then used to determine levels of the nucleotide. As increasing amounts of unlabeled cyclic AMP were added to the reaction mixture, less cyclic [³H]AMP was bound to the receptor protein. A typical standard curve for the determination of cyclic AMP, as depicted in Figure 4, represents the difference in the binding of cyclic [³H]AMP to the protein in the absence of nonradioactive cyclic AMP from that observed with the addition of increasing concentrations of cyclic AMP to the reaction mixture. While the formation of cyclic [³H]AMP–protein complex was not a linear function of the nonradioactive cyclic AMP concentration, this relationship approached linearity at levels of cyclic AMP that were less than saturating for the amount of protein used. The cyclic [³H]AMP concentration used was 5.3 × 10^{-9} M and was slightly less than the half maximal saturation concentration of 1.3 × 10^{-8} M observed under the conditions described in Figure 2.

Cyclic AMP in Tissue. The levels of cyclic AMP obtained in various tissues of a rat by this method are summarized in Table III. The observations are similar to previously published levels for cyclic AMP (Aurbach and Houston, 1968; Brooker *et al.*, 1968; Posner *et al.*, 1964; Pauk and Reddy, 1967; Turtle and Kipnis, 1967; Steiner *et al.*, 1969).

The levels of cyclic AMP that occurred in rat adrenal glands in response to the administration of ACTH is shown in Table IV. In line with previous work (Grahame-Smith *et al.*, 1967; Haynes, 1958), where it was shown that cyclic AMP levels in adrenal glands increased rapidly and markedly after the injection of the hormone, the present study showed

TABLE III: Cyclic AMP in Rat Tissues.

Expt	Tissue	No. of Samples	Cyclic AMP[a] (nmoles/g of Tissue)
1	Adrenal gland	4	1.5 ± 0.3[b]
2	Adrenal gland	4	1.6 ± 0.3
3	Brain	2	2.8 ± 0.2
4	Liver	2	1.2 ± 0.6
5	Muscle	2	0.27 ± 0.10

[a] Tissues were prepared and cyclic AMP was isolated and assayed as described in the Experimental Section. The cyclic AMP values were obtained from duplicate assays performed on each sample. [b] Average of samples plus and minus standard deviation.

that the concentration of cyclic AMP increased to 40 times that observed in the adrenals of untreated animals.

The level of cyclic AMP in the adrenal glands of control animals differed in the experiments recorded in Tables III and IV. Since marked increases in the level of cyclic AMP in the adrenal gland have been reported (Grahame-Smith *et al.*, 1967) these differences may reflect normal physiological variations.

Since the assay for cyclic AMP depends upon the binding of cyclic [^{3}H]AMP to the receptor protein, it was necessary to insure that the cyclic AMP samples isolated from tissue extracts for assay did not also contain any competitors or nonspecific inhibitors of cyclic AMP binding that would result in spuriously high estimates of the nucleotide by the method. Thus, the extracted cyclic AMP was hydrolyzed with cyclic nucleotide phosphodiesterase and the effect of this treatment on the assay for the nucleotide was observed. As shown in Table V, the decrease in binding of cyclic [^{3}H]-AMP to the binding protein by the addition of the nucleotide to the reaction was completely reversed by prior treatment with the cyclic nucleotide phosphodiesterase. The cyclic AMP isolated from adrenal gland, brain, and muscle demonstrated a significant decrease in the binding of cyclic [^{3}H]AMP due to the presence of the unlabeled nucleotide in the extracts. The addition of cyclic nucleotide phosphodiesterase to the tissue extracts containing cyclic AMP resulted in binding of cyclic [^{3}H]AMP that was indistinguishable from that demonstrated by the control which contained no extracted cyclic AMP or nucleotide phosphodiesterase. Thus the cyclic AMP extracts isolated from these tissues as described contained no material which interfered with the absolute determination of the cyclic nucleotide by the method.

It is to be pointed out, however, that a rather crude preparation from brain was used as the source of the cyclic nucleotide phosphodiesterase, and although unlikely the possibility existed that the enzyme preparation inactivated competing substances in addition to cyclic AMP.

TABLE IV: Cyclic AMP in the Adrenal Glands of Rats.

Treatment	Cyclic AMP[a] (nmoles/g of Tissue)
Control	2.6 ± 0.3[b]
ACTH	97 ± 5

[a] Ten animals were used in each study. Duplicate samples of cyclic AMP isolated from the adrenals of each group were prepared. Duplicate assays were performed on each sample as described in the Experimental Section. [b] Average of two samples plus and minus standard deviation.

TABLE V: Hydrolysis of Cyclic AMP in Tissue Extracts with Cyclic Nucleotide Phosphodiesterase.

Additions[a]	Cyclic [^{3}H]AMP Bound (cpm) Cyclic Nucleotide Phosphodiesterase −	+
None	1471	1433
Cyclic AMP (1.6×10^{-7} M)	177	1419
Adrenal gland	552	1409
Brain	545	1550
Muscle	644	1602

[a] The additions consisted of either commercial unlabeled cyclic AMP or cyclic AMP extracted from the indicated tissues as described in the Experimental Procedure. Each sample was incubated with and without 64 μg of the brain cyclic nucleotide phosphodiesterase in the presence of 4 mM Tris-HCl (pH 8.0) and 1.5 mM $MgCl_2$ at 30° for 3 hr. The samples were boiled for 5 min and a 0.05-mM aliquot was assayed for cyclic AMP as described in the Experimental Section. The reaction mixtures contained 50 mM Tris-HCl (pH 7.4), 5 mM theophylline, 5.3×10^{-9} M cyclic [H^3]AMP (3.1 mμCi), and 66 μg of binding protein (S-IV).

Discussion

The sensitivity of the presented assay for cyclic AMP appears to be in line with those previously reported (Posner *et al.*, 1964; Breckenridge, 1964; Butcher *et al.*, 1965; Pauk and Reddy, 1967; Turtle and Kipnis, 1967; Aurbach and Houston, 1968; Brooker *et al.*, 1968; Steiner *et al.*, 1969) as approximately 20 pmoles of cyclic AMP was conveniently measured by this method. The assay utilizes a stable and easily obtainable protein preparation with a high affinity and specificity for cyclic AMP. The attachment of the binding protein to a membrane filter to accomplish the separation of free from protein-bound ligand provides a rapid and accurate method for the determination of the nucleotide.

There are two obvious limitations to the sensitivity of the assay—the specific activity of the radioactive ligand (cyclic AMP) and the purity of the binding protein. In this study the specific activity of the protein preparation used for the assay of cyclic AMP was about 15 pmoles/mg of protein and represented less than a 5-fold degree of purifica-

tion. The cyclic AMP binding protein has been purified approximately 200-fold in this laboratory (Gill and Garren, 1970), and with the availability of higher specific activity of cyclic [^{3}H]AMP, the sensitivity of the assay can be markedly enhanced.

References

Aurbach, G. D., and Houston, B. A. (1968), *J. Biol. Chem. 243*, 5935.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Breckenridge, B. M. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 1580.

Brooker, G., Thomas, L. J., Jr., and Appleman, M. M. (1968), *Biochemistry 7*, 4177.

Butcher, R. W., Ho, R. J., Meng, H. C., and Sutherland, E. W. (1965), *J. Biol. Chem. 240*, 4515.

Butcher, R. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237*, 1244.

Colowick, S. P., and Kaplan, N. D. (1957), *Methods Enzymol. 3*, 454.

Gill, G. N., and Garren, L. D. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 512.

Gill, G. N., and Garren, L. D. (1970), *Biochem. Biophys. Res. Commun. 39*, 335.

Grahame-Smith, D. G., Butcher, R. W., Ney, R. L., and Sutherland, E. W. (1967), *J. Biol. Chem. 242*, 5535.

Haynes, R. C., Jr. (1958), *J. Biol. Chem. 233*, 1220.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Pauk, G. L., and Reddy, W. J. (1967), *Anal. Biochem. 21*, 298.

Posner, J. B., Hammermeister, K. E., Bratvold, G. E., and Krebs, E. G. (1964), *Biochemistry 3*, 1040.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 55*, 660.

Steiner, A. L., Kipnis, D. M., Utiger, R. and Parker, C. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 367.

Turtle, J. R., and Kipnis, D. M. (1967), *Biochemistry 6*, 3970.

Peroxidase-Catalyzed Formation of Indole-3-carbaldehyde and 4-Hydroxyquinoline from Indole-3-acetaldehyde*

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ABSTRACT: Spectrophotometric studies on horseradish peroxidase (HRP) catalyzed oxidation of indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), and IAAld–$NaHSO_3$ have shown that oxidation of each compound led to different products and that the pH of the reaction mixture had a profound effect on the final oxidation product of IAAld. In the pH range 3.7–4.5, 3-methyleneoxindole was the major product of HRP-catalyzed oxidation of IAA, whereas oxidation of IAAld under the same conditions gave as high as 50% yield of indole-3-carbaldehyde (IAld). At pH near 7 HRP-catalyzed oxidation of IAAld plus equimolar $NaHSO_3$ gave a new product which was subsequently identified as 4-hydroxyquinoline (4-HQ) by the direct isolation of the oxidation product in crystalline form. However, at acidic pH IAld was the major end product in HRP-catalyzed oxidation of IAAld–$NaHSO_3$.

Kenten and Mann (1950) studied the oxidation of β-phenylethylamine by pea seedling extracts and found that the total oxygen uptake was in excess of that required for the oxidation of the amine to β-phenylacetaldehyde. Further studies on the pea seedling "aldehyde oxidase" activity by Kenten (1953) indicated that the proposed aldehyde oxidase was not a simple flavoprotein but a heme-containing peroxidase. The peroxidase-catalyzed oxidation of β-phenylacetaldehyde did not yield phenylacetic acid but benzaldehyde; hence Kenten suggested that the oxidation of indole-3-acetaldehyde (IAAld)[1] by plant peroxidase might cause the formation of indole-3-carbaldehyde (IAld). Evidence obtained in the present work supports Kenten's view that the oxidation of IAAld by peroxidase results in the formation of IAld as one of the major products.

The possibility that 4-hydroxyquinoline (4-HQ) can be produced as an oxidation product of IAA was not previously confirmed (Manning and Galston, 1955). However, our preliminary investigations indicate that 4-HQ was formed from horseradish peroxidase (HRP) oxidation of IAAld in

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[1] Abbreviations used are: IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAld, indole-3-carbaldehyde; 4-HQ, 4-hydroxyquinoline; and HRP, horseradish peroxidase.