The Assay of Adenosine 3′,5′-Cyclic Monophosphate and Guanosine 3′,5′-Cyclic Monophosphate in Biological Materials by Enzymatic Radioisotopic Displacement*

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ABSTRACT: A simple yet precise microassay for as little as 5 μμmoles of 3′,5′-cyclic adenosine monophosphate or 3′,5′-cyclic guanosine monophosphate has been developed through a modification of the isotope dilution principle. The hydrolysis of tritium-labeled cyclic nucleotides by a uniform phosphodiesterase reaction is diminished by the addition of nonlabeled cyclic nucleotides. Cyclic nucleotides are isolated from tissue samples by perchloric acid extraction, absorption and elution from small anion-exchange columns, and lyophilization. A simple preparation from rat brain contains both 3′,5′-cyclic adenosine monophosphate and 3′,5′-cyclic guanosine monophosphate phosphodiesterase activities. Enzyme reaction mixtures include snake venom to provide an excess of 5′-nucleotidase activity. The nucleosides produced in the coupled enzyme system are separated from unreacted substrate by treatment with anion-exchange resin. The entire assay procedure of coupled enzyme incubation, resin treatment, and liquid scintillation counting is carried out in a standard plastic counting vial. Unreacted nucleotide substrates, which are absorbed by the resin, do not interact with the *p*-dioxane–naphthalene scintillation mixture and therefore do not interfere with the detection of unbound labeled nucleosides. The reaction is carried out to an extent previously determined to be about 30% of completion with a low concentration of tritium-labeled 3′,5′-cyclic nucleotide. Addition of unknown or standard samples of the unlabeled form of the same nucleotide diminishes the radioactivity detected in the assay. Numerical values are assigned by reference to a standard curve. The assay has been used to confirm the epinephrine-induced increase in skeletal muscle levels of 3′,5′-cyclic adenosine monophosphate.

A major problem in biochemical endocrinology and pharmacology has been the difficulty in measuring the low concentrations of cyclic nucleotides occurring in tissues. A number of hormones appear to act on target tissues by affecting the concentration of 3′,5′-cyclic AMP (Sutherland *et al.*, 1965) and recently the involvement of 3′,5′-cyclic GMP has been suggested (Hardman *et al.*, 1966).

Presently available assays for 3′,5′-cyclic AMP rely upon its activation of glycogen phosphorylase (Rall and Sutherland, 1958; Posner *et al.*, 1964; Butcher *et al.*, 1965), double-isotope derivative dilution (Pauk and Reddy, 1967), or conversion into and subsequent determination of 5′-AMP (Breckenridge, 1964; Turtle and Kipnis, 1967). The measurement of 3′,5′-cyclic GMP in urine by the latter method has been reported (Hardman *et al.*, 1966). Methods employing the activation of phosphorylase for 3′,5′-cyclic AMP measurement require preparation of complex enzyme systems which are sensitive to activation by a variety of substances other than this nucleotide. The conversion of 3′,5′-cyclic AMP into 5′-AMP can introduce difficulties since the latter nucleotide is much more abundant in nature.

The present communication describes a very simple rapid assay for 3′,5′-cyclic AMP and 3′,5′-cyclic GMP in tissue samples or biological fluid. The assay is based upon the fact that the rate of conversion of labeled 3′,5′-cyclic AMP will be reduced by the introduction of nonradioactive 3′,5′-cyclic AMP (Scheme I) (Brooker and Appleman, 1968). A convenient one-step method has been developed based on this principle. Excess 5′-nucleotidase activity (snake venom) is included to convert 5′-mononucleotide produced by the phosphodiesterase into its respective nucleoside which is more readily separated from the original substrate (DeLange *et al.*, 1968). The reaction is carried out in a liquid scintillation vial and terminated by direct addition of anion-exchange resin. *p*-Dioxane scintillation fluid is added. The unreacted substrate is absorbed by the resin and quenched while the nucleoside produced is not bound by the resin and is detected in the liquid scintillation process. Results can be obtained within 1 hr after purification of the tissue sample.

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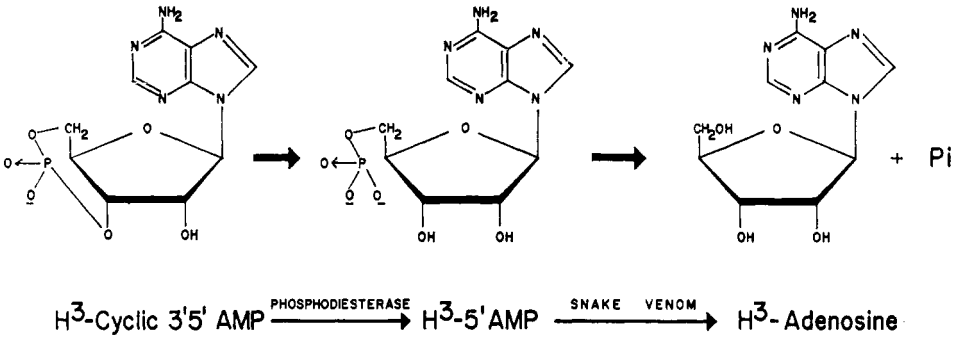
SCHEME I



Materials and Methods

Tritiated 3′,5′-cyclic AMP (2.35 Ci/mmole) and 3′,5′-cyclic AMP were purchased from Schwartz BioResearch. The 3′,5′-cyclic GMP was kindly supplied by Dr. Robert G. Kemp and by Calbiochem, Inc. Tritiated 3′,5′-cyclic GMP (2.6 Ci/mmole was synthesized by Calbiochem, Inc. Snake venom (*Ophiophaqus hanna*, king cobra) was obtained from Dr. Findlay E. Russell or from Sigma Chemical Co. The anion-exchange resins were BioRad AG-1-X-2, Cl^-, 200–400 mesh or −400 mesh. Before use they were washed with 1 N NaOH, repeatedly with water, then with 1 N HCl, and finally with water to neutrality. The resins were used in this form for all operations. The scintillation solvent consisted of 375 g of naphthalene, 22.5 g of 2,5-diphenyloxazole, and 1.13 g of 1,4-bis-[2-(4-methyl-5-phenyloxazole)]benzene dissolved in 3 l. of *p*-dioxane. Polyethylene scintillation vials were used throughout and all pipetting into the vials was performed with automatic pipets and disposable plastic tips. All other chemicals used were reagent grade obtained from commercial sources.

Preparation of Nucleotide 3′,5′-Phosphodiesterase. The following steps were carried out at 0–4°. A male Sprague–Dawley rat was killed by decapitation and the brain was removed and homogenized in five volumes of water and centrifuged for 30 min at 30,000*g*. The supernatant was adjusted to 50% saturation with a saturated solution of enzyme grade $(NH_4)_2SO_4$ and centrifuged for 10 min at 30,000*g*. The pellet was taken up with five volumes per gram original wet weight in 60 mM Tris-HCl (pH 8.0) and 5 mM 2-mercaptoethanol and dialyzed overnight against two changes of an excess of this buffer. Protein concentration varied from 5 to 8 mg per ml (Lowry *et al.*, 1951). This preparation could be kept at 0° or frozen without appreciable loss of activity. Enzymatic activity was determined under conditions of the 3′,5′-cyclic AMP or 3,′5′-GMP assay without the addition of nonradioactive nucleotides. Specific activity ranged from 50 to 300 μμmoles of 3′,5′-cyclic nucleotide hydrolyzed per mg of protein per min.

Tissue Preparation. Weighed frozen tissue (50–1000 mg) is powdered in a stainless steel pulverizer precooled in liquid nitrogen. The powdered tissue is extracted and homogenized with 5 ml of 0.6 N perchloric acid containing tracer amounts of [^{3}H]cyclic nucleotide (250 cpm/ml). The sample is centrifuged and the supernatant is removed and neutralized with 2 N KOH to the bromothymol blue end point (pH 7.5). After cooling at 0° for 1 hr, the $KClO_4$ is removed by centrifugation. The supernatant is applied to a 5 × 80 mm (AG 1-X-2, 200–400 mesh), anion-exchange column prepared in a Pasteur pipet fitted with a plastic funnel to increase the volume above the resin. The columns are eluted sequentially with water, HCl (pH 2.40), and HCl (pH 1.40) (Figure 1). 3′,5′-Cyclic AMP appears in the pH 2.40 elution following the adenine nucleotide monophosphates; 3′,5′-cyclic GMP is eluted by the pH 1.40 acid solution. In each case the cyclic nucleotide emerges when the eluate reaches the pH of the eluting acid. The tracer added to the perchloric acid extract is used to determine elution volume and recovery. Fractions of interest are lyophilized and taken up in 60 mM Tris-HCl (pH 8.0) (0.5 ml/g of original tissue). Some samples require additional neutralization and are adjusted to pH 8.0 with 2 N KOH before being assayed.

Assay Procedure. The entire assay is conducted in a plastic liquid scintillation vial. A 0.05-ml sample containing 5–320 μμmoles of cyclic nucleotide in 60 mM Tris-HCl (pH 8.0) is placed in the vial. An equal volume of substrate containing 120 mM Tris-HCl (pH 8.0), 2.5 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, 120 mM $MgCl_2$, 0.12 mM 5′-AMP, and 1.6×10^{-7} M [^{3}H]3′,5′-cyclic AMP or [^{3}H]3′,5′-cyclic GMP (about 10,000 cpm/0.05 ml) is added. The reaction is started by the addition of 0.05 ml of an enzyme mixture which contains the brain phosphodiesterase preparation (10–40 μg of protein/ml), lyophilized snake venom (1 mg/ml), bovine serum albumin (2 mg/ml), 60 mM Tris-HCl (pH 8.0), and 5 mM 2-mercaptoethanol. The amount of phosphodiesterase is sufficient to hydrolyze 30–40% of the [^{3}H]cyclic nucleotide without the addition of nonradioactive cyclic nucleotide. The reaction is stopped after 10-min incubation at 30° by addition of 0.8 ml of a slurry (50% settled volume) of AG-1-X-2 −400 mesh anion-exchange resin in water. After the mixture has been equilibrated for 10 min, 10 ml of scintillation fluid is added, and the samples are counted. The values for unknown samples are obtained by reference to standard curves such as those shown in Figure 2 for 3′,5′-cyclic AMP and Figure 3 for 3′,5′-cyclic GMP.

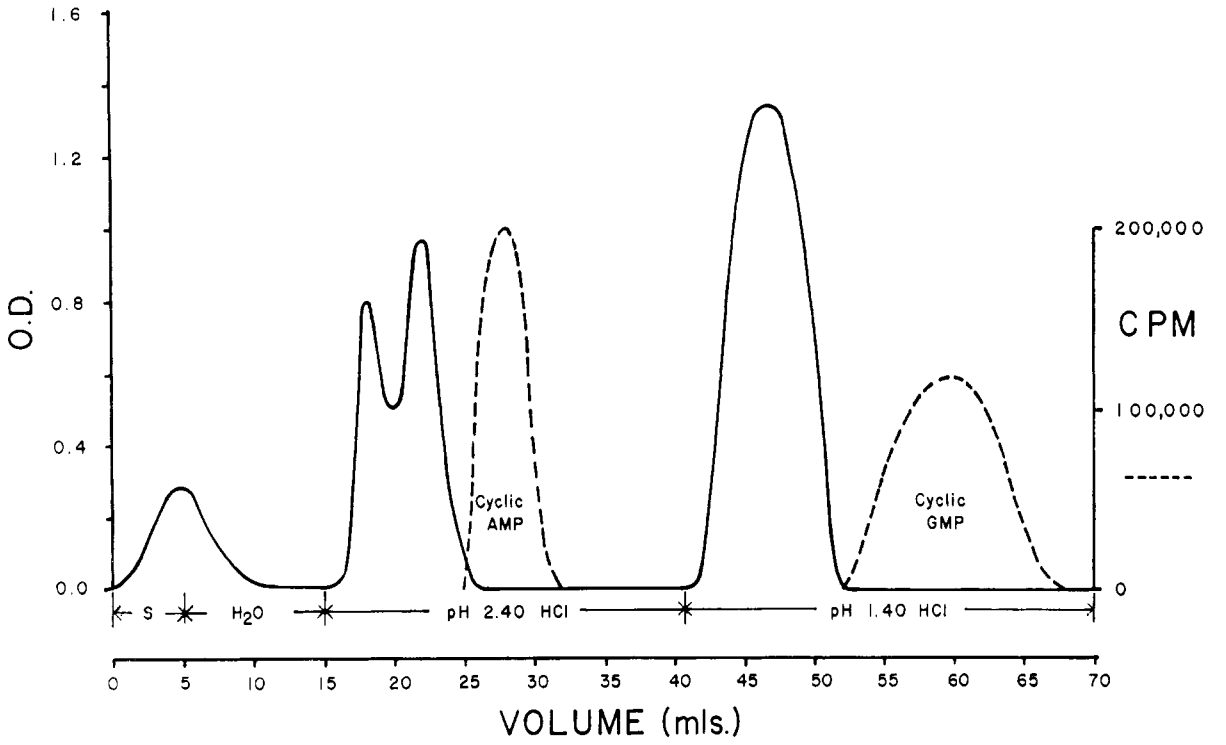


FIGURE 1: Elution profile for the purification of cyclic nucleotides by anion-exchange chromatography. Rat skeletal muscle (1 g) was prepared as described in the text with the exception that perchloric acid extracting solution contained 10^6 cpm each of [^{3}H]3′,5′-cyclic AMP and [^{3}H]cyclic GMP. Conditions of chromatography are those indicated in the text. Optical density at 260 mμ is indicated by (——) and counts per minute per milliliter by (- - - - -)

Results and Discussion

This assay for cyclic nucleotides is easily set up and maintained and is sensitive enough for the measurement of 3′,5′-AMP at the low levels which appear to be physiologically significant. A theoretical analysis of the general technique of enzymatic isotope displacement for the analysis of enzymatic substrates is to be found in the following paper (Brooker and Appleman, 1968).

A simple column anion-exchange procedure is used for the separation of 3′,5′-cyclic AMP and 3′,5′-cyclic GMP from tissues extracts. It has been found that the elution volume for 3′,5′-cyclic AMP varies somewhat depending on the type of tissue from which the cyclic AMP is being extracted. Tracer amounts of labeled 3′,5′-cyclic AMP and labeled 3′,5′-cyclic GMP added during the tissue extraction procedure permit the determination of their recoveries after purification. The small amount of isotope used is two orders of magnitude smaller than the amount of isotope present in the assay and therefore does not interfere. Recovery of added cyclic nucleotides ranged from 75 to 95%. The specific activity of cyclic nucleotides formed from either ^{14}C- or ^{32}P-labeled precursors can be determined using this assay since these isotopes can be differentiated from tritium in the liquid scintillation process.

Conditions for the phosphodiesterase reaction have been chosen empirically to provide maximum repro-

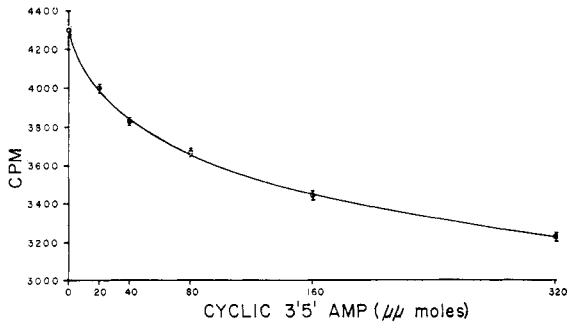


FIGURE 2: Standard curve for measurement of 3′,5′-cyclic AMP. Assay conditions as described in the text. Vertical bars are the standard error of the mean for five determinations.

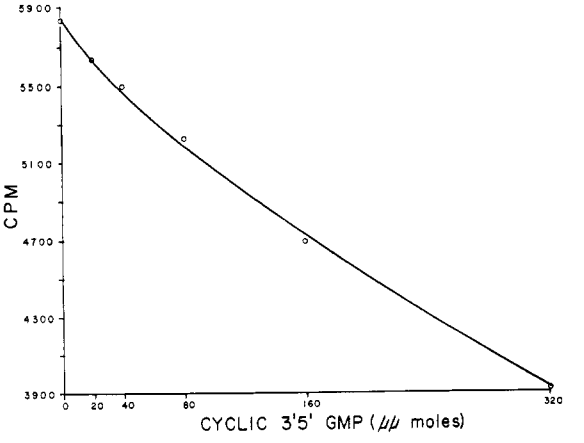


FIGURE 3: Standard curve for measurement of 3′,5′-cyclic GMP. Assay conditions as described in text.

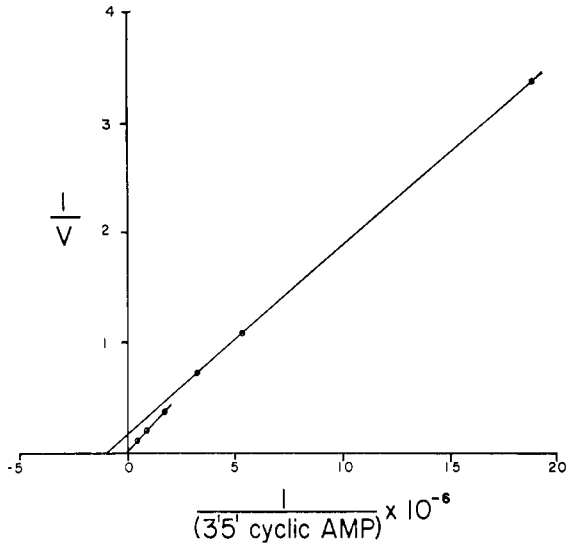


FIGURE 4: Lineweaver–Burk presentation of the kinetics of rat brain 3′,5′-cyclic AMP phosphodiesterase. Points are derived from data in Figure 2 by conversion into velocity terms based on the total number of counts present.

ducibility in the assay of the cyclic nucleotide. With the brain phosphodiesterase preparation, an optimal 30% of total reaction (measured in the absence of nonradioactive cyclic nucleotide) can be achieved with 0.5–2.0 μg of protein/assay for 10 min at 30°. The 5′-mononucleotide formed by the action of the phosphodiesterase is converted quantitatively to the corresponding nucleoside by an excess of king cobra venom 5′-nucleotidase. The snake venom used shows negligible phosphodiesterase activity toward the cyclic nucleotides under the conditions of this assay. 5′-AMP is included in the assay mixture to act, after hydrolysis by the venom, as a carrier for the minute quantities of labeled adenosine produced in the reaction. While the amount of 5′-AMP used inhibits the diesterase to some extent it apparently is sufficient to saturate an inhibitory site on the enzyme. As much as 10^{-4} M additional 5′-AMP has no further effect on this assay system and interference by any residual 5′-nucleotide from the purified tissue extract is eliminated.

TABLE I: Validity Tests of the Assay for 3′,5′-Cyclic AMP in Skeletal Muscle.

	μμmoles of Cyclic AMP
1. 100 mg of tissue	80
2. 50 mg of tissue	40
3. 50 mg of tissue + 125 μμmoles of 3′,5′-cyclic AMP	180
4. 100 mg of tissue pretreated with phosphodiesterase[a]	0
5. 100 mg of tissue + 125 μμmoles of 3′,5′-cyclic AMP pretreated with phosphodiesterase[a]	0
6. As in 5 except add back 125 μμmoles of 3′,5′-cyclic AMP after phosphodiesterase treatment[a]	115

[a] Brain phosphodiesterase preparation (50 μg) incubated 5 min with the sample and then destroyed by boiling 5 min.

TABLE II: Effect of Epinephrine on Rat Skeletal Muscle 3′,5′-Cyclic AMP Levels.

Experiment	% Recov of Cyclic AMP	3′,5′-Cyclic AMP (μmoles/kg wet wt)
1. Control	80	0.48
Epinephrine	80	2.50
2. Control	91	0.44
Epinephrine	81	1.60
3. Control	92	0.44
Epinephrine	77	1.28

The procedure of adding resin to the vial to differentiate substrate from product leaves a background in control assays which is about 10% of the 10,000 cpm of substrate added. It was found that this background could be eliminated by removing the resin from the vial but could not be eliminated by raising the cutoff energy of the lower discriminator level in the scintillation counter. There is no observable shift in scintillation spectra between the isotope with and without the addition of resin. Since a similar phenomenon was observed with a higher energy isotope, $^{35}SO_4^{2-}$, the mechanism of this effect on the counting process must be that of a physical barrier created by the resin such that the scintillation fluid cannot come into contact with those compounds absorbed.

Various experiments were conducted to confirm the validity of this assay procedure for 3′,5′-cyclic AMP (Table I). Observed values are proportional to the amount of tissue sample; prior destruction of 3′,5′-AMP with phosphodiesterase reduced the assayed value to zero; readdition of 3′,5′-cyclic AMP to an unknown tissue sample is accounted for by the procedure.

During the course of this investigation it was found that as much as 320 μμmoles of 3′,5′-cyclic GMP did not interfere with the assay of 3′,5′-cyclic AMP and conversely this quantity of 3′,5′-cyclic AMP gave a value of less than 8 μμmoles in the 3′,5′-cyclic GMP assay. This is an indication that there are distinct 3′,5′-cyclic AMP and 3′,5′-cyclic GMP phosphodiesterases in the brain preparation. The data of Hardman *et al.* (1966) with respect to the levels in urine would support the concept that these two cyclic purine nucleotides are under separate metabolic control.

One of the requirements for this type of assay is that the enzyme used must have a Michaelis constant for the substrate of interest which is not appreciably higher than the concentrations which are to be measured. The reported constant for rat brain 3′,5′-cyclic AMP phosphodiesterase is about 2×10^{-4} M (Cheung, 1967). Use of a more sensitive assay indicates the presence of two 3′,5′-cyclic AMP phosphodiesterase activities with apparent K_m's of 1.3×10^{-4} and 1×10^{-6} M (Figure 4). A similar phenomenon has been found in skeletal muscle (R. G. Kemp, 1966, personal communication). It is the lower K_m activity which is essential for the assay reported here. The 3′,5′-cyclic GMP phophodiesterase displays no such anomaly; the Michaelis constant is about 5×10^{-6} M.

This assay procedure is capable of detecting physiological changes in 3′,5′-cyclic AMP concentration (Table II). These experiments on anethetized rats were carried out essentially as those described by Posner *et al.* (1964). Skeletal muscle levels of 3′,5′-cyclic AMP were determined before and after injection of epinephrine. The control was obtained by clamping one hind leg with a Wollenberger clamp precooled in liquid nitrogen. Epinephrine (10 μg/kg) was injected into the heart and 60 sec later the other hind leg was clamped. The control values from the four rats were quite constant and agree with published reports for resting levels of 3′,5′-cyclic AMP (Posner *et al.*, 1964; Sutherland *et al.*, 1965). In all cases injection of epinephrine caused a dramatic increase in 3′,5′-cyclic AMP while injection of saline was ineffective.

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