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ASSAY FOR ADENYLATE CYCLASE AND CYCLIC NUCLEOTIDE PHOSPHODIESTERASES AND THE PREPARATION OF HIGH SPECIFIC ACTIVITY ^{32}P-LABELED SUBSTRATES

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Summary

Simple one step assay methods for adenylate cyclase (ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1) and cyclic nucleotide phosphodiesterases (3′,5′-cyclic nucleotide 5′-nucleotidohydrolase EC 3.1.4.17) have been developed. [α-^{32}P] ATP is used as the substrate for adenylate cyclase. Acid-heat destruction of [^{32}P] ATP remaining after the cyclase reaction followed by Zn-Ba treatment quantitatively leaves cyclic [^{32}P] AMP in the supernatant essentially free from other ^{32}P-containing compounds. This assay method requires no corrections for recovery and routinely yields blank values less than 0.03%. If higher sensitivity is desired, a simple 5 min alumina column step can be introduced into the procedure which quantitatively elutes cyclic [^{32}P] AMP directly into a liquid scintillation vial and lowers the blank values to less than 0.002%. This method is rapid and easily performed, without sacrificing high reliability, specificity, or sensitivity.

One step phosphodiesterase assays are easily accomplished using ^{32}P-labeled cyclic nucleotides as substrates. Descending paper chromatography of the reaction mixture on individual 2 cm wide paper strips gives a complete and quantitative separation of all possible products including [5′-^{32}P] AMP and [5′-^{32}P] GMP from their respective ^{32}P-labeled 3′,5′-cyclic nucleotides in 1—2 h. The paper strips are cut, inserted in scintillation vials without scintillant and the ^{32}P-products determined by Cerenkov counting. Low blank values of less than 0.5% and the use of high specific activity ^{32}P-labeled cyclic nucleotide substrates make this method the most reliable and most sensitive phosphodiesterase assay described to date. Because of the simplicity, specificity, and

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Abbreviations: MIX, 1-methyl-3-isobutyl xanthine; DCC, dicyclohexylcarbodiimide.

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high sensitivity obtainable with these assay methods using ^{32}P-labeled substrates, we have also devised simple conditions for the preparation and purification of [α-^{32}P] ATP, cyclic [^{32}P] AMP and cyclic [^{32}P] GMP with specific activities in excess of 100 Ci/mmol. These high specific activity ^{32}P-labeled cyclic nucleotides are important for these new assay methods and are also useful to follow purification recovery of endogenous cyclic AMP and cyclic GMP from biological materials before protein binding or radioimmunological isotope displacement assays when performed in the femtomole range.

Introduction

The assay of adenylate cyclase (EC 4.6.1.1) is intrinsically difficult. The low in vitro enzymatic activity necessitates a highly sensitive method for determination of minute amounts of cyclic AMP in the presence of a large quantity of ATP. While protein binding methods [1,2] are reliable and specific for quantitation of cyclic AMP, their use to determine adenylate cyclase activity is somewhat troublesome due to endogenous cyclic AMP present in enzyme preparations, and because these methods require two biochemical assay steps to obtain adenylate cyclase data, the first being the incubation of the enzyme preparation with substrate followed by either purification prior to determination or determinations at several dilutions to assure reliability of the protein binding assay [1,3].

The use of radioactive substrate and determination of radioactivity in cyclic AMP allows for a one step assay if a simple elimination of all radioactive substances other than cyclic AMP is possible. Because crude preparations are used as the enzyme source and since ATP is also utilized by other contaminating enzymes which give rise to other products, assay sensitivity and reliability are largely dependent upon the efficiency with which cyclic AMP can be separated from ATP and other products. While much study on this enzyme has been done with the methods of Krishna and coworkers [4,5], White and Zenser [6] and Ramanchandran [7], these methods are limited in sensitivity to some extent because of high blank values. In order to overcome this problem, Salomon et al. [8] recently developed a combined method of the above. High sensitivity was achieved by making use of [α-^{32}P] ATP and purification of the cyclic [^{32}P] AMP formed by two column steps. However, even this method involves time-consuming procedures including the use of columns and monitoring of cyclic AMP recovery. In view of this, we have developed a new one step method which is based upon acid-heat destruction of [α-^{32}P] ATP followed by isolation of cyclic [^{32}P] AMP by Zn-Ba treatment. The method requires no column steps and no corrections for loss of cyclic [^{32}P] AMP and is so simple that it permits a single investigator to easily process a large number of samples without sacrificing sensitivity, specificity, or reliability. Even higher sensitivity can be easily achieved by the quantitative filtration of the Zn-Ba supernatant through a small alumina column.

Currently many methods are being used for the assay of cyclic nucleotide phosphodiesterase (EC 3.1.4.17). While high sensitivity has been achieved with various methods [9—13], the disadvantages of high blanks, time-consuming

separation procedures, and additional enzyme steps beyond the phosphodiesterase reaction make these methods cumbersome and less than ideal. Problems with these assays are due to the use of ^{3}H-labeled substrates (cyclic AMP or cyclic GMP) which routinely lose tritium to water, the use of heat to stop the reaction, and the use of snake venom or 5′-nucleotidase preparations which are contaminated with small quantities of phosphodiesterase. In order to circumvent these problems, a new assay method for phosphodiesterase has been developed in which the quantitative separation of the product(s) from the substrate is accomplished by a two hour descending paper chromatography step. The method is more sensitive and reliable than widely used methods [9—13] because it uses high specific activity cyclic [^{32}P] AMP (or cyclic [^{32}P]-GMP) as substrate, yields blank values of less than 0.5%, and because the assay's reliability is not interfered with by further degradation or alteration of the 5′-monophosphate nucleotide products.

Because the simplicity, specificity, and high sensitivity of these new assay methods are dependent upon the use of high specific activity [α-^{32}P] ATP and ^{32}P-labeled cyclic nucleotides and because the commercial supply of these compounds is costly and not readily available, a simple inexpensive technique for the synthesis of these compounds is described.

Materials

Ion exchange resins (Dowex AG50W-X8, 100—200 mesh, H^+ form and Dowex AG1-X2, 50—100 mesh, Cl^- form) were purchased from Bio-Rad Laboratories. Carrier free $^{32}P_i$, [α-^{32}P] ATP (20—120 Ci/mmol), [α-^{32}P] GTP (10—100 Ci/mmol), [^{14}C] adenosine (50 Ci/mol), [^{14}C] AMP (400 Ci/mol) and [^{14}C] ATP (50 Ci/mol) were supplied by New England Nuclear Corporation. Non-radioactive nucleotides, 2′,3′-*O*-isopropylidene adenosine, dicyclohexylcarbodiimide, phosphoenol pyruvate, creatine phosphate, myokinase (1,450 U/mg), pyruvate kinase (350 U/mg), creatine phosphokinase (50 U/mg) and neutral alumina (WN-3) were obtained from Sigma Chemical Company. 1-Methyl-3-isobutyl xanthine, dimethylsulphoxide, trichloroacetonitrile, acetonitrile and triethylamine were obtained from Aldrich Chemical Company. Chromatography paper (Whatman 31ET), cellulose thin-layer chromatography sheets (Eastman Chromatogram No. 13255) and other chemicals were purchased locally. All reagent chemicals were ACS analytical grade or better and all water was glass distilled deionized water. TTX 127 liquid scintillation fluid is 1 part Triton X-100, 2 parts Triton X-114, 7 parts xylene (Fisher Scientific Co. X-5) and 7 g 2,5-diphenyloxazole (J.T. Baker Chemical Co.) in 1 l fluid. Triton X-100 and X-114 were obtained from Rohm and Haas.

Methods

Purification of ^{32}P-labeled nucleotides and chromatography

^{32}P-Labeled nucleotides (made by the methods described in this paper or purchased from New England Nuclear) were routinely purified in order to lower blank values in enzyme assays. [α-^{32}P] ATP and ^{32}P-labeled cyclic nucleotides were purified on anion exchange columns or by descending paper chromatography as described below.

Descending paper chromatography was run in solvent A (chloroform / methanol/concentrated ammonium hydroxide, 2 : 2 : 1) or in solvent B (chloroform/methanol/concentrated ammonium hydroxide, 2 : 6 : 3) using Whatman 31ET chromatography paper. Width of the paper was dependent upon the need of each experiment. It usually takes 2—2.5 h to run 35—40 cm at room temperature. However, good separations were observed in only 1 h (20 cm). Authentic compounds (either radioactive or non-radioactive) were routinely run side by side or as carriers with unknown samples. Cellulose thin-layer chromatography was performed in solvent C (*n*-butanol/glacial acetic acid/-water, 2 : 1 : 1). It usually takes 3—4 h to run 16—17 cm at room temperature.

For cation exchange column chromatography, Dowex AG50W-X8 (100—200 mesh, H^+ form) was packed in a Pasteur pipet (0.5 cm × 5 cm) and the packed column was thoroughly washed with 60 ml of water before use. Samples were neutralized and applied in a small volume (<1 ml), then eluted by water. Anion exchange chromatography was in Pasteur pipet columns (0.5 cm × 5 cm) packed with Bio-Rad AG1-X2 (50—100 mesh) which were previously treated with 30 ml 1 M HCl and 60 ml water before use [14]. Samples were neutralized and diluted with water to lower the ionic strength of the samples before application to the columns. Following a water wash, elution with HCl of successively lower pH's eluted the desired compounds.

Preparation of [α-^{32}P] ATP from $^{32}P_i$

[5′-^{32}P] AMP was synthesized with slight modifications by the method of Symons [15]. Acetonitrile, dimethyl sulphoxide, triethylamine and trichloroacetonitrile were distilled from and stored over calcium hydride. Working amounts of triethylamine and trichloroacetonitrile were kept in screw-capped tubes in a dessicator in the dark. To a reaction vessel (40 ml conical centrifuge glass tube) were added 5 μmol of 2′,3′-*O*-isopropylidene adenosine, 0.1 ml of ethanol, 0.1 μmol H_3PO_4 and 5 mCi carrier-free $^{32}P_i$ in 20 mM HCl. 12 drops of triethylamine were added and the tube contents taken to dryness on a rotary evaporator at 30° C. The tube contents were further dried by the addition and evaporation of three 1 ml lots of acetonitrile. Dimethyl sulphoxide (60 μl) and triethylamine (3 μl) were added and the contents of the tube dissolved by shaking. Finally, 3 μl of trichloroacetonitrile was added and the tube incubated at 30° C for 40—60 min during which time the reaction vessel was sealed with a silica-gel moisture trap. Dimethyl sulphoxide and excess volatile reagents were removed on the rotary evaporator. To the residue was added 2 ml of 2 M acetic acid and the tube heated at 100° C (1—1.5 h) to hydrolyze the isopropylidene groups. The acetic acid was removed on the rotary evaporator after the addition and re-evaporation of two 1 ml lots of water. The tube contents of [5′-^{32}P] AMP, unreacted $^{32}P_i$ and adenosine were dissolved in 1 ml H_2O and separated on a Dowex AG50W-X8 (100—200 mesh, H^+ form) column which was eluted with water. $^{32}P_i$ elutes first from the column followed by [5′-^{32}P] AMP. The column eluate containing [5′-^{32}P] AMP was taken to dryness in a 40 ml conical centrifuge tube on a rotary evaporator at 30° C. The purified [5′-^{32}P]-AMP was not converted to cyclic [^{32}P] AMP by DCC because poor yields were routinely experienced in our hands by the described DCC reaction [15]. Instead [5′-^{32}P] AMP was enzymatically converted into [α-^{32}P] ATP which could

be used for the adenylate cyclase assay or could be chemically converted to high specific activity cyclic [^{32}P] AMP.

[α-^{32}P] ATP was generated by enzymatic cycling procedures [16]. The reaction mixture contained (as final concentrations) 50 mM Tris · HCl (pH 7.7), 50 mM KCl, 2.5 mM $MgCl_2$, 2.0 mM $MgSO_4$, 0.10 mM EDTA, 5.0 mM dithiothreitol, 1.6 mM phosphoenolpyruvate, $5 \cdot 10^{-9}$ M ATP, 10 μg/ml myokinase, 50 μg/ml pyruvate kinase and the purified [5'-^{32}P] AMP (250 μCi/$5 \cdot 10^{-9}$ mol in the 100 μl reaction mixture). The reaction was allowed to proceed at 30° C for 0.5—1 h. The [α-^{32}P] ATP thus formed was purified on a Bio-Rad AG1-X2 column as described below. The acid (0.25 M HCl) eluate containing [α-^{32}P] ATP was neutralized with Tris (final 10 mM) and KOH, then kept at —20° C until use.

Preparation of cyclic [^{32}P] AMP from [α-^{32}P] ATP and cyclic [^{32}P] GMP from [α-^{32}P] GTP

Cyclic [^{32}P] AMP was generated by alkaline treatment of [α-^{32}P] ATP with modifications of the method of Cook et al. [17]. The reaction mixture (1.0 ml) contained 0.1 ml 0.50 M Tris · Cl (pH 8.0), 0.40 ml 0.15 M $Ba(OH)_2$ and 0.5 ml [α-^{32}P] ATP (1 mCi of 20—120 Ci/mmol). Incubation was routinely carried out at 37° C for 4 h). To each ml of the reaction mixture 2 ml of 0.15 M $Ba(OH)_2$ and 2 ml of 5% $ZnSO_4$ was added at the end of the incubation. The mixture was shaken well and was slightly alkaline. The white precipitate was removed by centrifugation (1500 × *g* for 10 min). This Zn-Ba treatment removed practically all ATP, ADP, AMP and P_i by precipitation with the resultant isolation of cyclic AMP in the supernatant [4,5,18]. The supernatant was diluted 5-fold with water and applied to a Bio-Rad AG1-X2 (50—100 mesh, Cl^- form) column through Whatman No. 42 filter paper as previously described [19]. ^{32}P-Labeled cyclic AMP was further separated from small amounts of other radioactive compounds by this anion exchange column chromatography. The pH 2.0 fractions which contained essentially pure cyclic [^{32}P] AMP was lyophilized and the radioactive residue taken up in a small volume of water and kept at —20° C until use.

When the size of the conversion reaction mixture was small, descending paper chromatography was employed in solvent A for isolation and purification of cyclic [^{32}P] AMP. ^{32}P-Labeled cyclic AMP was eluted with water from the chromatogram and concentrated by lyophilization as above.

Cyclic [^{32}P] GMP was generated by heat treatment of [α-^{32}P] GTP under alkaline conditions similar to those described by Kimura and Murad [20]. The reaction mixture (0.10 ml) contained 10 μl 0.50 M Tris · Cl (pH 8.0), 1 μl 0.20 M potassium phosphate (pH 6.0), 20 μl 0.15 M $Ba(OH)_2$, 10 μl 0.20 M $CaCl_2$, 20 μl 50 mM creatine phosphate and 40 μl [α-^{32}P] GTP (0.1 mCi of 10—100 Ci/mmol). The reaction mixture was heated in a boiling water bath for 5 min, then spotted on Whatman 31ET chromatography paper. Descending paper chromatography (solvent B) was used for isolation and purification of cyclic [^{32}P] GMP. ^{32}P-Labeled cyclic GMP was eluted with water from the chromatogram, concentrated by lyophilization, and taken up in a small volume of water. Purified cyclic [^{32}P] GMP was then kept at —20° C until use.

Preparation of rabbit heart homogenates

The heart from a New Zealand white rabbit was obtained rapidly after cervical dislocation. The heart was thoroughly rinsed in Krebs-Ringer bicarbonate solution at room temperature. Then the heart was rinsed with ice cold 50 mM Tris · Cl (pH 7.5) to remove as much blood as possible. A part of the ventricle was homogenized in 5 volumes (w/v) of the same cold buffer in a teflon pestle glass homogenizer for 30—40 strokes. The homogenate was filtered through a loose plug of glass wool to remove large pieces of tissue and then diluted 2-fold with the same buffer and further homogenized for 10 strokes. The homogenate was made each day 0.5—1 h just before use.

Assay for adenylate cyclase

Method I: The assay mixture (50 μl) contained (final concentrations) 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.20 mM [α-^{32}P] ATP ($1 \cdot 10^6$—$4 \cdot 10^6$ cpm), 10 mM KF, 0.8 mM MIX, 0.50 mM cyclic AMP, 0.10 mM EDTA, 20 mM creatine phosphate, 50 U/ml (or 1 mg/ml) creatine phosphokinase and diluted heart homogenate as a source of cyclase. The assay was started by adding preincubated (30° C, 3 min) homogenate to the preincubated reaction mixture (minus homogenate) and the reaction was allowed to proceed for 5 min at 30° C unless otherwise stated. The reaction was stopped by the addition of 1 ml of 1 M HCl. Assay blanks were prepared by omitting homogenate or by adding homogenate after addition of HCl. Both methods gave practically equivalent blank values. The acidified mixture was heated at 90° C for 8—10 min with a rubber stopper on the tube, then cooled in an ice water bath. To each assay tube was added 0.50 ml 2 M KOH, 0.5 ml 5% $ZnSO_4$ and 0.5 ml 0.15 M $Ba(OH)_2$. The slightly alkaline mixture was shaken well and allowed to stand for 2—5 min at room temperature. The white precipitate was removed by centrifugation in a bench top centrifuge with a multi-place swinging bucket rotor at 3000 rev./min for 10 min. One ml of the resultant supernatant was carefully transferred to a new tube, to which 0.5 ml 5% $ZnSO_4$ and 0.5 ml 0.15 M $Ba(OH)_2$ were added. The tube was mixed well again and centrifuged as above. One ml of the supernatant was gently removed and mixed with 6.5 ml of TTX 127 scintillation fluid, in a scintillation mini-vial. Radioactivity (^{32}P) in the supernatant was counted in a liquid scintillation spectrometer. The values were corrected for volume and blanks. Cyclic [^{32}P] AMP formed was a measure of adenylate cyclase activity in the heart homogenate. Unless otherwise stated, Method I was used in the present studies.

Method II: This procedure is identical to Method I through the second Zn-Ba treatment. One ml of the second supernatant was gently removed and mixed with 0.1 ml 1.0 M imidazole · HCl (pH 7.5) or 1.0 M Tris · Cl (pH 7.5). The buffered mixture was applied to a column (0.5 cm × 2.5 cm) packed with neutral alumina (Sigma WN-3) that had been washed previously with 15 ml of 0.1 M imidazole · HCl (pH 7.5) or Tris · Cl (pH 7.5). The column was eluted with 4 ml of the same buffer and the eluate (total 5 ml) was directly collected into a liquid scintillation vial, to which 10 ml of TTX 127 was added. The used alumina columns could be reused after treatment with 20—50 ml of buffer.

Assay for cyclic nucleotide phosphodiesterase

The assay mixture (50 μl) contained (final concentrations) 50 mM Tris ·

Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.10 mM EDTA, 0.50 μM or 0.50 mM cyclic [^{32}P] AMP ($0.5 \cdot 10^6$—$2 \cdot 10^6$ cpm) and diluted heart homogenate as a source of phosphodiesterase. The assay mixture (minus homogenate) and the homogenate were separately preincubated at 30° C for 3 min. The assay was initiated by adding the homogenate to the assay mixture. The reaction was allowed to proceed 5 min at 30° C unless otherwise indicated, and was stopped by adding 10 μl of a saturated solution of cyclic AMP and AMP in 2 M HCl and the acidified assay mixture was kept on ice. Blank controls were always run with each experiment by omitting homogenate or by adding homogenate after HCl addition. Both methods gave practically the same blank values. 30 μl (or less, depending upon the total radioactivity used) of the acidified assay mixture was spotted on Whatman 31ET paper (2 cm × 40 cm) across the strip with width of 0.5 cm (area 2 cm × 0.5 cm) and descending chromatography was run in solvent A.

In order to handle many assays at one time, we have developed a device to hold many individual paper chromatography strips. Fig. 1 shows this device which consists of two parts, the top being a stainless steel plate with 16 paper clips which can hold 16 paper strips and the bottom a stainless steel trough which holds about 250 ml of solvent. Paper strips were firmly held on the plate, and samples spotted on the strips. Descending paper chromatography was started by immersing the plate portion in the trough containing solvent A. Three of these devices were positioned in a sealed chromatography jar so that 48 strips could be developed at one time. After chromatography the plates with the attached strips were hung in a hood and the chromatograms dried under a stream of warm air. Spots of AMP and cyclic AMP were located under a UV lamp and regions corresponding to $^{32}P_i$ (origin: A), [5′-^{32}P] AMP (B) and cyclic [^{32}P] AMP (E) were cut out (see Fig. 2) and the Cerenkov radioactivity counted in a liquid scintillation spectrometer without scintillant in the ^{3}H channel. Combined radioactivity of $^{32}P_i$ and [5′-^{32}P] AMP was taken as a measure of the heart homogenate phosphodiesterase activity*. Since the achievement of a good separation between the substrate and product(s) is important in this phosphodiesterase assay the chromatography solvent should be routinely replaced with fresh solvent.

When cyclic GMP was used as substrate, the procedure was the same as described above except solvent A was replaced by solvent B, and corresponding $^{32}P_i$, [^{32}P] GMP and cyclic [^{32}P] GMP were counted.

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* During our early studies, ultraviolet spots were located first and radioactive regions on each chromatogram were detected by use of a Varian-Berthold TLC Radiochromatogram Scanner as shown in Fig. 2. Similar profiles were obtained with cyclic [^{32}P] GMP as substrate. The six regions indicated in the figure were carefully cut out and counted. The total radioactivity spotted (30 μl of acidified assay mixture) on a chromatogram varied between 5—15%, so relative amounts of radioactivity in $^{32}P_i$ and 5′-[^{32}P] AMP regions were calculated as a percentage of total radioactivity on the whole strip. Then, absolute amounts of $^{32}P_i$ and 5′-[^{32}P] AMP formed were computed on the basis of the concentration of cyclic [^{32}P] AMP in the incubation. After our techniques became highly reproducible, it turned out that direct calculations of the products formed based upon actual counts in regions (A, B and E) and known specific activity of the substrate were accurate enough for the assay. Counts in region C were negligible.

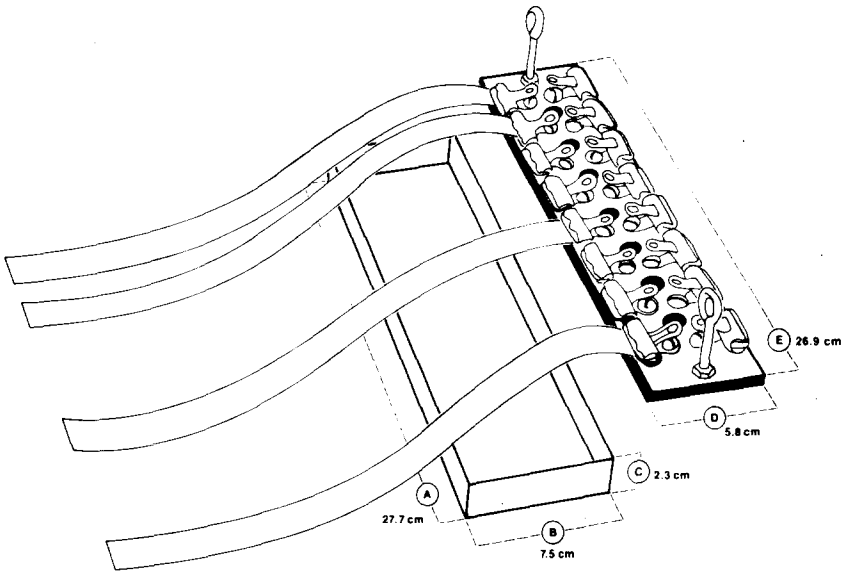


Fig. 1. A diagrammatic scheme of a trough set used for descending paper chromatography in the cyclic nucleotide phcsphodiesterase assays. At the upper right is a plate with clips which holds the chromatography papers. A trough is shown at lower left.

Other methods

Amounts of [α-^{32}P] ATP remaining at the end of adenylate cyclase assays were determined by anion exchange column chromatography. After sample application on a AG1-X2 column, ^{32}P-labeled compounds other than ATP were successively eluted with 10 ml H_2O, 30 ml pH 2.7 HCl and 20 ml pH 1.5 HCl. Then, [α-^{32}P] ATP was eluted from the column with 15 ml 0.25 M HCl. Radioactivity in each fraction was determined in a liquid scintillation spectrometer. Cyclic nucleotides were assayed either by high pressure liquid chromatography [14] or in the case of cyclic AMP by the protein binding assay [1,21] as modified [22]. Protein was determined by the method of Lowry et al. [23] with bovine serum albumin as standard.

Results

Chromatography

The solvent systems used for descending paper chromatography in the present studies provided excellent separation of cyclic nucleotides from corresponding nucleotides as shown in Fig. 2. The cyclic nucleotides moved 21—32 cm in a 40 cm run (R_F 0.53—0.80) far ahead of the other nucleotides which stayed near or moved 5—8 cm from the origin (R_F 0.14—0.20). Inosine nucleotides stayed at or near the origin under these conditions. Orthophosphate did not move at all. The results are essentially comparable to those originally reported by Kulbert et al. [24] for cellulose thin-layer chromatography. These paper chromatography systems were routinely used for purification of the cyclic nucleotides and for the phosphodiesterase assay.

R_F values obtained with thin-layer chromatography (cellulose sheet in

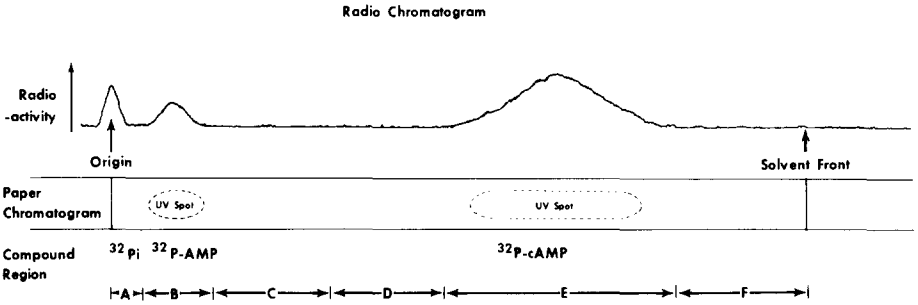


Fig. 2. Chromatographic patterns of $^{32}P_i$, 5'-[^{32}P] AMP and cyclic [^{32}P] AMP on Whatman 31ET paper. Descending paper chromatography of acidified phosphodiesterase assay mixture was run on Whatman 31ET chromatography paper in solvent A as described in Methods. 5'-AMP and cyclic AMP were located by detecting spots (shown by circles with dotted line) under a UV lamp and the radiochromatogram was obtained by use of the Varian TLC radiochromatogram scanner.

solvent C) are 0.49 for P_i, 0.41 for cyclic AMP, 0.31 for AMP and 0.08 for ATP.

When a sample containing $^{32}P_i$ and 5'-[^{14}C] AMP was loaded onto a Dowex AG50W-X8 cation exchange column, (0.4 cm × 4 cm) $^{32}P_i$ passed through and practically all $^{32}P_i$ was in the first 6 ml water with hardly any 5'-[^{14}C] AMP. Then, 5'-[^{14}C] AMP started to elute upon addition of more water with the peak at the second 6 ml and 90% of the 5'-[^{14}C] AMP was eluted in a total of 30 ml water added to the column.

After application of samples (radioactive and non-radioactive) to Bio-Rad AG1-X2 anion exchange columns, the columns were washed with 10 ml of water and successively eluted with 30 ml of pH 2.7 HCl, 30 ml of pH 2.0 HCl (twice), 15 ml of pH 1.5 HCl (twice), and 10 ml of 0.25 M HCl (twice). The elution was monitored by radioactivity measurements and/or ultraviolet absorption. Most adenosine was washed off with the initial water wash and pH 2.7 HCl removed residual adenosine from the columns. The first 30 ml of pH 2.0 HCl eluted most AMP and cyclic AMP while the second 30 ml pH 2.0 HCl completed the removal of these compounds including $^{32}P_i$ from the columns. Approximately 90% of ADP came off the columns with the first 15 ml of pH 1.5 HCl and the second 15 ml pH 1.5 HCl completely washed off ADP and a very small portion of ATP. Most of the ATP was eluted with the first 10 ml of 0.25 M HCl and the second wash eluted the rest.

Preparation of [α-^{32}P] ATP from $^{32}P_i$

Chemical synthesis of 5'-[^{32}P] AMP from $^{32}P_i$ was repeated many times in our laboratory using the method of Symons [15] as well as modified procedures in an attempt to obtain a specific activity of 5—50 Ci/mmol. The best yield (for 50 Ci/mmol) was about 20% of the total $^{32}P_i$ used. We never obtained the high yields reported by Symons [15]. Conversion of 5'-[^{32}P] AMP into [α-^{32}P] ATP was almost always quantitative in 0.5 h and is a very simple way to make high specific activity [α-^{32}P] ATP. Descending paper chromatography and thin-layer chromatography of heated and unheated samples with added [^{14}C] ATP as a monitor indicated that the 0.25 M HCl eluate contained only [α-^{32}P] ATP.

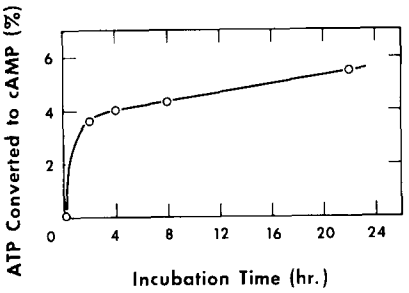


Fig. 3. Conversion of [α-^{32}P] ATP into cyclic [^{32}P] AMP. Incubation for the conversion reaction was carried out as described. Small aliquots were removed at several time points as indicated in the figure and spotted on Whatman 31ET chromatography paper. Descending paper chromatography was run in solvent A and radioactive products formed were analyzed by the procedure used for the phosphodiesterase assay. Amounts of cyclic [^{32}P] AMP formed are expressed as percent of original radioactivity ([α-^{32}P] ATP) present in the reaction mixture.

Preparation of cyclic [^{32}P] AMP from [α-^{32}P] ATP and cyclic [^{32}P] GMP from [α-^{32}P] GTP

In several experiments when [α-^{32}P] ATP was incubated at 37° C in the presence of barium hydroxide, 2—10 percent of the ATP was converted into cyclic [^{32}P] AMP during the first several hours. Fig. 3 shows a typical progress curve for the formation of cyclic [^{32}P] AMP from [α-^{32}P] ATP. Generation of cyclic [^{32}P] AMP reached almost maximum levels (4%) in 4 h and longer incubation did not significantly increase cyclic AMP formation. The identity and purity of cyclic [^{32}P] AMP thus prepared was verified by the following six criteria: (1) descending paper chromatography on Whatman 31ET paper in solvent A; (2) thin-layer chromatography on Eastman cellulose sheet in solvent C; (3) full recovery after Zn-Ba treatment followed by anion exchange column chromatography on Bio-Rad AG1-X2; (4) cyclic AMP binding and displacement assays; (5) high-pressure liquid column chromatography; (6) hydrolysis by cyclic nucleotide phosphodiesterase with resultant formation of 5′-[^{32}P]-AMP. The specific activity of radioactive cyclic AMP thus prepared was considered to be the same as that of [α-^{32}P] ATP used. In the example shown in Fig. 3, the specific activity of cyclic [^{32}P] AMP was 50 Ci/mmol. This procedure has several advantages over Symons' method [15] which is the only other method currently used for the preparation of cyclic [^{32}P] AMP. Our method is a one step method being simpler and easier with much less radiation exposure. In addition, the present method takes much less time. Whenever one needs cyclic [^{32}P] AMP and [α-^{32}P] ATP is available, the whole procedure can be completed in a single day without sacrificing time for other work. Although the yield in this method is not as high as that of Symons' [15], the 5% yield is high enough for practical purposes and works every time. In addition, unconverted [α-^{32}P] ATP and/or side product (5′-[^{32}P] AMP) [17] can be saved and easily isolated if paper chromatography is used instead of column chromatography. Thus [5′-^{32}P] AMP can be recovered by conversion to [α-^{32}P] ATP by the enzymatic cycling method.

When [α-^{32}P] GTP was heated at 100° C in the presence of barium hydroxide and phosphate, 1—2 percent of [α-^{32}P] GTP was converted into cyclic

TABLE I

CONVERSION OF [α-^{32}P] GTP INTO CYCLIC [^{32}P] GMP

Incubation for the conversion reaction was carried out as described. Small aliquots were removed at several time intervals indicated in the table and spotted on Whatman 31ET chromatography paper. Descending paper chromatography was run in solvent B as described, and radioactive compounds formed were analyzed by the procedure used for the phosphodiesterase assay.

Incubation time (min)	Cyclic [^{32}P] GMP formed (% GTP converted to cyclic GMP)
0	0.02
0.5	0.77
5.0	1.97
10	1.05
18	0.90

[^{32}P] GMP during the first several minutes. A typical time course for this conversion reaction is shown in Table I. Generation of cyclic [^{32}P] GMP reached a maximum level in 5 min and the cyclic [^{32}P] GMP formed was degraded with time by longer incubation. The identity and purity of cyclic [^{32}P] GMP thus prepared was verified by the following methods: (1) descending paper chromatography on Whatman 31ET paper in solvent B; (2) hydrolysis by cyclic nucleotide phosphodiesterase with resultant formation of 5′-[^{32}P]-GMP and (3) high pressure liquid column chromatography. The specific activity of radioactive cyclic GMP thus prepared was considered to be the same as that of [α-^{32}P] GTP used. In this example cyclic [^{32}P] GMP was produced with a specific activity of 20 Ci/mmol. However, up to 95 Ci/mmol cyclic [^{32}P] GMP has been produced in other experiments.

Quantitation of cyclic [^{32}P] AMP

Since our method for isolation of cyclic [^{32}P] AMP formed by the action of adenylate cyclase involves Zn-Ba treatment and since cyclic AMP is known to be generated in appreciable amounts by barium hydroxide treament of ATP [14,17 and this report], it is an absolute necessity to remove ATP from the assay mixture prior to this procedure to prevent high blank values. The method used in our assays was to destroy [α-^{32}P] ATP by acid (1 M HCl) and heat (90° C) treatment. Fig. 4 shows a typical time course of this process and that more than 99.5% of [α-^{32}P] ATP was destroyed in 10 min, whereas more than 90% of cyclic [^{32}P] AMP remained intact. Longer treatment destroyed cyclic AMP. Reliability of this treatment in the adenylate cyclase assays is further demonstrated in the following experiment.

Efficiency of separation and isolation of cyclic AMP from products formed by acid-heat hydrolysis of ATP was determined using cyclic [^{32}P] AMP and [α-^{32}P] ATP in separate tubes which contained every component (minus enzyme) for the cyclase assay except the designated ^{32}P-labeled compound. Table II shows that cyclic [^{32}P] AMP was quantitatively recovered in the supernatant after the second Zn-Ba treatment following the acid-heat procedure. Degraded products of [α-^{32}P] ATP were almost completely (99.98%) removed from the supernatant. The small loss of cyclic [^{32}P] AMP can be attributed to the hydrolysis of cyclic [^{32}P] AMP by acid-heat, contamination of cyclic

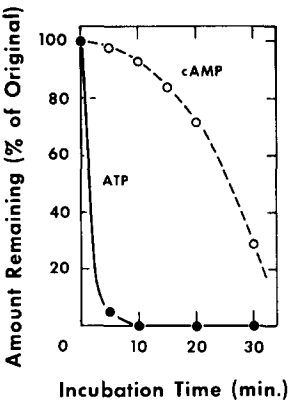


Fig. 4. Time course of acid-heat hydrolysis of ATP and cyclic AMP. Each test tube contained approximately 10 pmol (10^6 cpm) of [α-^{32}P]ATP or cyclic [^{32}P]AMP in 100 μl 1 M HCl. The tubes were heated at 90°C in a hot water bath. At several time points indicated in the figure, 1 μl of the heated mixture was removed and spotted on a Eastman chromatogram sheet (13255 Cellulose) together with authentic nucleotides. Thin-layer chromatography was run in solvent C. Nucleotide spots were detected under UV light and by a Varian TLC radiochromatogram scanner. The plate was cut into pieces corresponding to these areas and to inorganic phosphate. Radioactivity in the strips was counted in a liquid scintillation counter. Total radioactivity in the sample at each time point was determined and the radioactivity remaining in the ATP or cyclic AMP spots was calculated.

TABLE II

SEPARATION OF CYCLIC [^{32}P]AMP FROM [α-^{32}P]ATP AND ITS HYDROLYTIC PRODUCTS BY Zn-Ba TREATMENT

Tubes in group I contained 50 μl of the adenylate cyclase assay mixture (minus homogenates) as described, and tubes in group II contained the same except that the ^{32}P-labeled compound was cyclic AMP. Tube I was treated only with Zn-Ba. One ml of 1 M HCl was added to each of tubes 2–8. The tubes were heated at 90°C for 5 or 10 min, then neutralized with KOH, followed by Zn-Ba treatment as described for the adenylate cyclase assay Method I. Radioactivity in the resultant supernatant after removal of precipitates was corrected for volume and the recovery reported as percent of the total radioactivity initially present in a tube. The supernatant from tubes 7 and 8 were further processed through the alumina column (assay Method II) and radioactivity in the 5 ml eluate was determined.

Compound	Group	Tube number	Treatment					^{32}P
			Acid	Heat (min)	Zn-Ba		Alumina column	
					Once	Twice		
								Blank %
[α-^{32}P]ATP	I	1	—	—	+			2.0
		2	+	5	+			0.85
		3	+	5		+		0.02
		4	+	10	+			0.93
		5	+	10		+		0.03
		6	+	10		+		0.03
		7	+	10		+	+	0.003
		8	+	10		+	+	0.002
								Recovery %
Cyclic [^{32}P]AMP	II	1	—	—	+			97.9
		2	+	5	+			96.8
		3	+	5		+		93.7
		4	+	10	+			93.9
		5	+	10		+		94.5

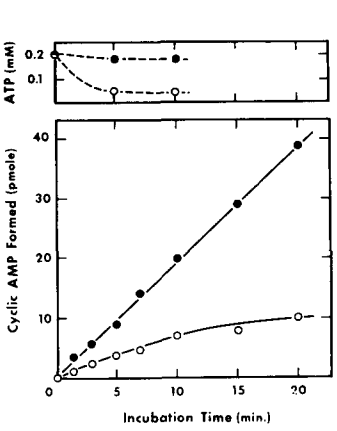
[^{32}P] AMP by 5'-[^{32}P] AMP which is precipitated by the Zn-Ba treatment, and/or technical errors. The results indicate that two Zn-Ba treatments are necessary to reduce blank values (from [α-^{32}P] ATP) to 0.02%, which is sufficiently low for most assay purposes.

These results clearly demonstrate that the procedure is reliable for the quantitative determination of cyclic [^{32}P] AMP and that it can be used for the assay of adenylate cyclase activity. If there is concern about a small loss of cyclic [^{32}P] AMP through this procedure, one can add cyclic [^{14}C] AMP at the end of the cyclase assay to accurately monitor recovery of cyclic [^{32}P] AMP. However, use of ^{3}H-labeled cyclic AMP should be avoided because acid-heat treatment will cause exchange of ^{3}H in the cyclic nucleotide with water. Since our results were highly reproducible, we did not make any efforts to monitor for recovery. However, if the activity of adenylate cyclase is very low, and the blank values (0.02%) used under our routine assay conditions become significant, the alumina column in Method II can be used. As can be seen in Table II, the alumina column removed more than 90% of any degraded products of [α-^{32}P] ATP remaining (0.02% of original) in the supernatant after the second Zn-Ba treatment, while cyclic AMP (monitored by use of cyclic [^{32}P] AMP or cyclic [^{3}H] AMP) was quantitatively recovered in the 5 ml eluate. Final blank values became insignificant (0.002%).

Assay for adenylate cyclase activity in rabbit heart homogenates

We tested the reliability of our assay method for adenylate cyclase using rabbit heart homogenates. Fig. 5 depicts the time course for the cyclase-catalyzed reaction. With the ATP-regenerating system the reaction was linear for 20 min and the enzyme activity was 72 pmol cyclic AMP formed/min/mg of protein. Without the ATP-regenerating system, however, linearity was observed only up to 10 min and the activity was 30 pmol cyclic AMP formed/min/mg of protein. Analysis of the assay mixtures by anion exchange column chromatography indicated that approximately 90% of [α-^{32}P] ATP remained intact at the end of the 10 min assay period in the presence of the regenerating system, while less than 25% of the [α-^{32}P] ATP was present after 5 min when creatine phosphate and creatine phosphokinase were not present (Fig. 5). Thus, the differences in the time course and the enzyme activity under these two conditions appear to be the result of differences in the substrate levels, that is, in [α-^{32}P] ATP concentrations. Data in Table III further supports this notion. The enzyme activity without addition or with creatine phosphokinase alone was only 1/5 that with creatine phosphate or with both creatine phosphate and creatine phosphokinase. Likewise the ATP concentrations at the end of assays under the former conditions were 1/3 of those under the latter conditions. It is interesting that the rabbit heart homogenate contained sufficient creatine phosphokinase activity to regenerate ATP when only creatine phosphate was added.

Fig. 6 demonstrates the dependency of the heart adenylate cyclase activity on amounts of homogenate protein added. It can be seen that higher enzymatic activity and ATP levels are observed with the ATP-regenerating system than without it. It is also of interest to note that a wider range of homogenate protein is linearly proportional to enzyme activity in the presence of the regenerating system. These results clearly demonstrate that our adenylate cyclase



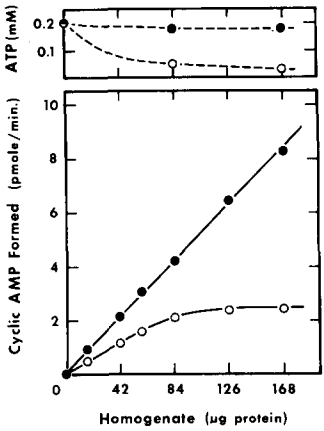


Fig. 5. Time course of heart adenylate cyclase reaction and substrate stability. The reaction mixture (400 µl) contained 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.20 mM [α-^{32}P] ATP ($3 \cdot 10^7$ cpm), 0.50 mM cyclic AMP, 10 mM KF, 0.8 mM MIX, 0.10 mM EDTA and rabbit heart homogenate (336 µg protein), with or without an ATP regenerating system (20 mM creatine phosphate and 50 U/ml creatine phosphokinase). The reaction was initiated by adding preincubated (30° C, 3 min) heart homogenate to the preincubated reaction mixture (minus homogenate), and incubation continued at 30° C. At the time points indicated 30 µl aliquots of the reaction mixture were removed for the determinations of cyclic [^{32}P] AMP by Method I and of [α-^{32}P] ATP by anion exchange column chromatography. Values of cyclic AMP refer to the amount in the 30 µl aliquot (25.2 µg protein). Symbols are: (———) cyclic [^{32}P] AMP formed and (- - - - - -) [α-^{32}P] ATP concentrations at the time samples taken in the presence (•) and absence (○) of the ATP-regenerating system.

Fig. 6. Adenylate cyclase activity as a function of heart homogenate protein. The assay mixture (50 µl) contained 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.20 mM [α-^{32}P] ATP ($2 \cdot 10^6$ cpm), 0.50 mM cyclic AMP, 10 mM KF, 0.8 mM MIX, 0.10 mM EDTA and various amounts of rabbit heart homogenate as indicated, with or without an ATP-regenerating system (20 mM creatine phosphate and 50 U/ml creatine phosphokinase). Assays were performed at 30° C for 5 min and the cyclic AMP formed and [α-^{32}P] ATP remaining at the end of the incubation were determined in aliquots and corrected back to the initial assay volume. Symbols are: (———) cyclic [^{32}P] AMP formed and (- - - - -) [α-^{32}P] ATP concentrations in the presence (•) and absence (○) of the ATP-regenerating system.

method is not interfered with by the presence of the ATP-regenerating system and in fact better kinetics are observed in its presence. Although the enzyme was assayed in the presence of F^- in the studies shown here, some determinations in the absence of F^- showed 40—50% of the activity with F^-.

TABLE III

EFFECT OF CREATINE PHOSPHATE AND CREATINE PHOSPHOKINASE ON ADENYLATE CYCLASE ACTIVITY AND SUBSTRATE LEVELS

Adenylate cyclase assays were carried out at 30° C for 5 min and cyclic [^{32}P] AMP formed was determined by Method I. Levels of [α-^{32}P] ATP were determined by anion exchange column chromatography.

Addition	Adenylate Cyclase Activity (pmol cyclic AMP formed/ min/mg of protein)	ATP concentration as [α-^{32}P] ATP (mM)
None	8.0	0.05
20 mM creatine phosphate	37	0.15
50 U/ml creatine phosphokinase	7.4	0.05
20 mM creatine phosphate and 50 U/ml creatine phosphokinase	39	0.15

Our assay mixture as well as other widely used systems contains a phosphodiesterase inhibitor or a cold cyclic AMP "trap" in order to protect newly formed cyclic [^{32}P] AMP from hydrolysis. We tested the efficiency of this technique. The assay mixture used was the same as that for adenylate cyclase except [α-^{32}P] ATP was replaced with non-radioactive ATP and cyclic [^{32}P]-AMP was included. Disappearance of cyclic [^{32}P] AMP and appearance of 5'-[^{32}P] AMP and $^{32}P_i$ were followed with time. Since amounts of cyclic AMP formed by adenylate cyclase are too small (Fig. 5) to decrease the original specific activity of cyclic [^{32}P] AMP, amounts of cyclic AMP hydrolyzed under these conditions were calculated using the original specific activity of cyclic [^{32}P] AMP. Although a significant amount of cyclic AMP was hydrolyzed even in the presence of 0.7 mM MIX, our calculations indicate that the activity of rabbit heart adenylate cyclase reported in Fig. 5 is under-estimated by only 5%, indicating the efficacy of our combination phosphodiesterase inhibitor-cold cyclic AMP trap. However, care should be taken to determine cyclic AMP phosphodiesterase activity in the specific adenylate cyclase preparation under study so that proper steps can be taken to eliminate or correct for phosphodiesterase activity.

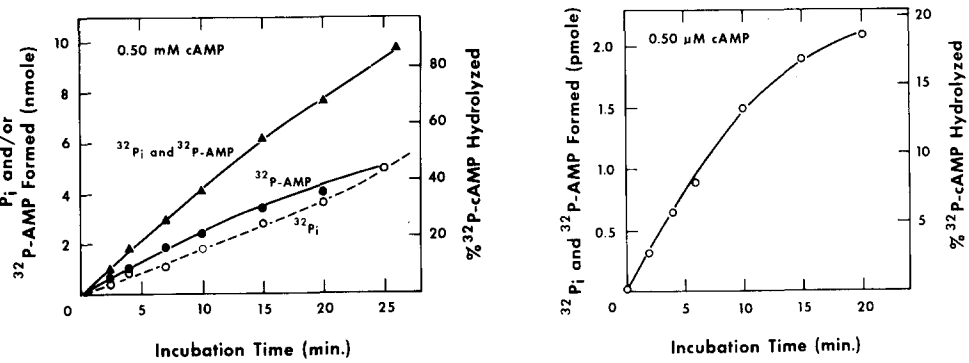


Fig. 7. Time course of heart phosphodiesterase-catalyzed reaction at high substrate (cyclic AMP) concentration. The assay mixture (600 μl) contained 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.50 mM cyclic [^{32}P] AMP ($2 \cdot 10^6$ cpm), 0.10 mM EDTA and rabbit heart homogenate (0.72 mg). The reaction was started by the addition of preincubated (30° C, 3 min) homogenate and the incubation continued at 30° C. At several time points, as indicated, 30 μl aliquots of the assay mixture were removed and mixed with 10 μl of a saturated solution of cyclic AMP and AMP in 2 M HCl. Amounts of $^{32}P_i$ (○— — —○) and 5'-[^{32}P] AMP (●————●) formed were determined in a 30 μl aliquot of the acidified reaction mixture by descending paper chromatography on Whatman 31ET with solvent A. The sum of $^{32}P_i$ and [^{32}P] AMP formed or the total amount of cyclic [^{32}P] AMP hydrolyzed is also shown (▲————▲) in the figure. Each time point represents the average of duplicate determinations.

Fig. 8. Time course of heart phosphodiesterase reaction at low substrate (cyclic AMP) concentration. The assay mixture (600 μl) contained 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.50 μM cyclic [^{32}P] AMP ($2 \cdot 10^6$ cpm), 0.10 mM EDTA and rabbit heart homogenate (0.21 mg). The reaction was started by the addition of the preincubated (30° C, 3 min) homogenate to the preincubated assay mixture (minus homogenate) and was allowed to proceed at 30° C. At several time points as indicated, 30 μl aliquots of the assay mixture were mixed with 10 μl of a solution of saturated cyclic AMP and AMP in 2 M HCl. The sum of the $^{32}P_i$ and 5'-[^{32}P] AMP formed in 30 μl of the acidified mixture and the percent of cyclic [^{32}P] AMP hydrolyzed was determined by descending paper chromatography. Each time point represents the average value of duplicate determinations.

Assay for cyclic nucleotide phosphodiesterase activity in rabbit heart homogenates

In order to validate the use of descending paper chromatography for assaying cyclic nucleotide phosphodiesterase, the activity of cyclic AMP phosphodiesterase in heart homogenates was determined by our new method. The time course of this enzymatic reaction was linear up to 13 min at high concentration (0.5 mM, Fig. 7) and up to 6 min at low concentration (0.5 μM, Fig. 8) of the substrate. Since our procedure is specific enough to allow us to quantitate formation of $^{32}P_i$ and 5′-[^{32}P] AMP separately, we also followed the time course for appearance of these compounds. The two time courses (Fig. 7) are somewhat different. It is important to notice the presence of a very high activity of 5′-nucleotidase in the crude preparations. This implies that any phosphodiesterase assay method based upon the determination of 5′-AMP alone [25] may give rise to erroneous measurement of the enzymatic activity. In the new method presented here, any phosphodiesterase activity followed by other enzymatic activity upon 5′-AMP results in products such as $^{32}P_i$, 5′-[^{32}P] IMP in addition to 5′-[^{32}P] AMP, etc. which are separable from cyclic [^{32}P] AMP and can be counted altogether as a measure of the phosphodiesterase activity.

Fig. 9 demonstrates the proportionality of the enzymatic activity to the amounts of homogenate added at both high (*a*) and low (*b*) concentrations of the substrate. Enzymatic activity with cyclic GMP as substrate (data not shown) are very similar to those obtained with cyclic AMP. The heart enzyme hydrolyzed 20 pmol substrate/min/mg protein with 0.5 μM cyclic AMP and 9 pmol/min/mg protein with 0.5 μM cyclic GMP. The blank values (without homogenate) were usually much less than 0.5% of the total radioactivity present and the phosphodiesterase activity was linear even though 10—20% of the substrate was utilized. Consistent results were obtained at two different substrate concentrations which varied over three orders of magnitude.

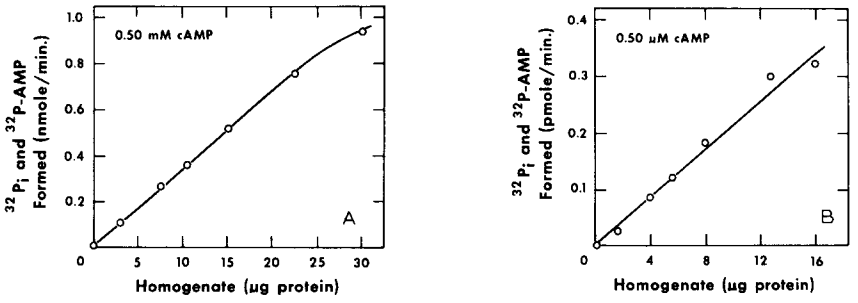


Fig. 9. Cyclic AMP phosphodiesterase activity as a function of heart homogenate protein at high (0.50 mM; *a*) and low (0.50 μM; *b*) substrate concentrations. The assay mixture (50 μl) contained 50 mM Tris · Cl (pH 7.5), 4.0 mM $MgCl_2$, 0.50 mM or 0.50 μM cyclic [^{32}P] AMP ($2 \cdot 10^5$ cpm), 0.10 mM EDTA and various amounts of rabbit heart homogenate per 25 μl as indicated in the figure. Assays were carried out at 30° C for 5 min and terminated with 10 μl of a saturated solution of cyclic AMP and AMP in 2 M HCl. The sum of $^{32}P_i$ and 5′-[^{32}P] AMP formed was determined in 30 μl of the acidified reaction mixture by paper chromatography. Each point represents the average value of duplicate determination.

Discussion

The adenylate cyclase method described here has several distinct advantages over other widely used methods [4—7]. Among all, the method is very simple and requires no complicated manipulations such as column steps or corrections for cyclic AMP loss. Thus, our one step assay method is much less time consuming. Secondly, the sensitivity is as high as those already used [4—7]. A very high sensitivity such as that attained by the method of Salomon et al. [8] can also be easily achieved by our Method II which includes a neutral alumina column step. This step can be done within 5 min, and requires no corrections for cyclic AMP loss. Thirdly, the method is very reliable and specific because [α-^{32}P] ATP and not [^{3}H] ATP or [^{14}C] ATP is used as substrate, and because a specific Zn-Ba treatment is employed for the isolation of cyclic [^{32}P] AMP. For these reasons we feel that our Method I is very feasible for routine assays of adenylate cyclase activity.

The use of [α-^{32}P] ATP as a substrate for this enzyme is not new and is becoming a popular practice because it has several obvious advantages. Contaminating enzymes in adenylate cyclase preparations may give rise to various radioactive nucleosides and bases from ^{3}H- or ^{14}C-labeled ATP but not from α-^{32}P-labeled ATP. It is logical then that isolation procedures for the radioactive product (cyclic AMP) would be much easier and blank values would be much lower with [α-^{32}P] ATP as the substrate than with [^{3}H] ATP or [^{14}C]-ATP. The same is also true, as discussed below, with cyclic nucleotide phosphodiesterase assays. One problem, however, is that commercially available [α-^{32}P] ATP and ^{32}P-labeled cyclic AMP and cyclic GMP are presently rather expensive. In order to overcome this we have developed and described an inexpensive, simple method for the synthesis of these radioactive substrates. Since the specific activity of ^{32}P-labeled nucleotides thus made is very high, these compounds are usable for a reasonably long period of time as enzyme substrates and could be very useful to determine purification recovery in assays for cyclic AMP and cyclic GMP. Because of the ultra high specific activities obtainable frequent preparation of these isotopically labeled compounds is not necessary.

When assays for cyclic nucleotide phosphodiesterase are based upon measurement of radioactive nucleoside(s) (^{3}H- or ^{14}C-labeled) or inorganic phosphate ($^{32}P_i$) [9—12], there exist inevitable disadvantages. For example, the need for snake venom or 5′-nucleotidase incubation of heated or unheated assay mixtures not only makes the assay time-consuming and complicated but also gives rise to high blank values. (Incidentally, snake venoms contain variable amounts of cyclic nucleotide phosphodiesterase activity.) The new method described in this paper requires no such second incubation, but a straightforward one step isolation procedure. Since we use thick Whatman 31ET paper with 2 cm-width, spotting samples on the papers is easy and can be done in seconds while chromatography time is relatively short (1—2 h). The ability to count by Cerenkov radiation without scintillant is a very useful feature of this new assay method. The sensitivity and reliability of the method is much higher than that of other methods [9—12] because available specific activities (up to 100 Ci/mmol) of the ^{32}P-labeled substrate(s) is much higher than that of

^{3}H-labeled compound(s) (at highest 38 Ci/mmol), and because blank values are considerably lower (less than 0.5% as compared to 4—5%). This permits reliable assays of cyclic nucleotide phosphodiesterases at only 1% reaction so that low enzyme concentrations or early time points in kinetic studies can be performed. If assays of phosphodiesterase activity involve the determination of radioactive AMP (or GMP) formed, steps should be taken to correct for contaminating 5′-nucleotidase activities, which give rise to erroneous estimations of AMP (or GMP), depending upon the types of methods and radioactive substrates (^{3}H- or ^{32}P-labeled) used [26,27]. Since we use cyclic [^{32}P]AMP (or cyclic [^{32}P]GMP) as substrate, any radioactive products (AMP, IMP, GMP and P_i) formed by phosphodiesterase preparations remain at or near the origin in our systems and are separable from the substrates. We count the radioactivity of this whole region as a measure of phosphodiesterase activity. Thus, this new method eliminates the need for corrections for possible nucleotidase and deaminase activities, and the reliability of this method is not interfered with by further degradation of 5′-nucleoside-monophosphate products.

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