Measurement of Adenosine 3':5'-Cyclic Monophosphate by Competitive Binding to Salt-Dissociated Protein Kinase

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An assay for cyclic AMP is described which takes advantage of the high affinity of the dissociated receptor moiety of cyclic AMP-dependent protein kinase I for the nucleotide. The kinase is kept dissociated by salt (800mM-NaCl/30mM-EDTA). In the presence of a simply prepared heat-stable protein fraction the binding reagent is stable for the time needed to reach equilibrium of binding. A simple procedure [precipitation with poly-(ethylene glycol) followed by DEAE-cellulose chromatography] is described for the separation of protein kinase I from other binding proteins for cyclic AMP in rabbit skeletal muscle. The sensitivity, precision, reproducibility and specificity of the assay compared favourably with those of other cyclic AMP assays. The main advantage of the present assay is its resistance towards non-specific interference from a number of salts, tissue-culture media and substances found in crude tissue extracts. The reliability of cyclic AMP measurement directly in crude tissue extracts was ensured by removal of the assayable cyclic AMP with cyclic nucleotide phosphodiesterase digestion or adsorption with antibody against cyclic AMP, by comparison with measurement in tissue extracts purified by chromatography on QAE-Sephadex or sequentially on Dowex 50, and aluminium oxide as well as by dilution and recovery experiments.

The most widely used assays for cyclic AMP are based on the competition between cyclic AMP and a known amount of radioactively labelled nucleotide for a high-affinity binding site. The binding reagent is either an immunoglobulin (Steiner *et al.*, 1969, 1972; Cailla *et al.*, 1973) or naturally occurring binding protein for cyclic AMP (Gilman, 1970; Walton & Garren, 1970; Brown *et al.*, 1971). Naturally occurring binding proteins combine ease of preparation with a generally high specificity and assay sensitivity (Brown *et al.*, 1972; Gilman & Murad, 1974). The regulatory moiety of cyclic AMP-dependent protein kinase (EC 2.7.1.37), isoenzyme type I according to the nomenclature of Corbin *et al.* (1975), is the binding protein with the highest affinity for cyclic AMP in bovine adrenal cortex. At high ionic strength (800mM-NaCl/30mM-EDTA) the catalytic subunit of protein kinase did not affect the high-affinity binding of cyclic AMP to the regulatory moiety of that isoenzyme (Døskeland & Ueland, 1977). The present paper describes an assay for cyclic AMP where salt-dissociated protein kinase I (from rabbit skeletal muscle) is the binding reagent.

In the presence of a heat-stable protein fraction as well as the agents (EDTA, 2-mercaptoethanol, serum albumin) previously found to protect the binding reagent against inactivation (Døskeland *et al.*, 1977), the binding protein was completely stable for the time needed for equilibrium of binding of cyclic AMP to occur.

The performance of the assay for the measurement of cyclic AMP in samples containing various salts, proteins, tissue-culture media and ether-extracted trichloroacetic acid solutions was studied because interference with the determination of cyclic AMP in such samples has been reported for several competitive binding assays (Weinryb, 1972; Cooper *et al.*, 1972; Albano *et al.*, 1974; Brostrom & Kon, 1974; Arner & Østman, 1975; Wright & Price, 1975). Several tests were performed to validate the direct measurement of cyclic AMP in crude tissue extracts by the present method.

Finally, the influence on the cyclic AMP standard curve of competitive inhibition of cyclic AMP binding and of lowered affinity of the binding protein for the nucleotide was investigated.

Experimental

Materials

Cyclic [8-³H]AMP (24–30 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 (medium grade) and QAE [diethyl-(2-hydroxypropyl)aminoethyl]-Sephadex (A-25) were from Pharmacia, Uppsala, Sweden. Dowex 50W (X4; 200–400 mesh; H⁺ form) and neutral chromatographic aluminium oxide (AG7, 37–44 µm) were

obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. *N*⁶-Phenylisopropyladenosine, *N*⁶-cyclohexylallyladenosine and *erythro*-9-(2-hydroxynon-3-yl)adenine were from Dr. H. Storck, Boehringer, Mannheim, Germany. Other purine derivatives, crystallized bovine serum albumin, 3':5'-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Poly(ethylene glycol) 6000 (synthetic grade), trichloroacetic acid, HClO₄ (analytical grade) and most other chemicals were from Merck, Darmstadt, Germany. The tissue-culture media were from Gibco Bio-Cult, Paisley, Scotland, U.K.

Buffers

Buffer A is 50mM-Hepes/20mM-EDTA/800mM-NaCl/20mM-2-mercaptoethanol/bovine serum albumin (0.5 mg/ml), adjusted to pH 7.2 at 0°C with NaOH. Except where otherwise indicated adjustment of pH was carried out at the temperature at which the buffer solutions were used.

Preparation of tissue specimens and extraction of cyclic AMP from tissue

Liver tissue was obtained from normally fed, male NMRI mice (about 25g) injected intraperitoneally with 0.1mg of glucagon (Novo Industries A/S, Copenhagen, Denmark) 3min before they were killed by cervical dislocation. The pieces of liver were clamped with forceps precooled in liquid N₂ and plunged into the same liquid within 40s of the animal's death. Tissue from human renal-cell carcinoma and adjacent renal cortex was obtained in the operating theatre from kidneys removed for therapeutic reasons. The tissue specimens were powdered under liquid N₂, the powder was transferred to a glass/Teflon homogenizer containing 2ml of ice-cold 5% (w/v) trichloroacetic acid with 0.1M-HCl, and swiftly homogenized by 15 strokes at a pestle speed of 1740 rev./min. The homogenate was transferred to a centrifuge tube and the homogenizer rinsed with 2×0.5ml of the trichloroacetic acid solution. The pellet obtained by centrifuging at 10000g_{av} for 10min was resuspended in 0.5ml of the trichloroacetic acid solution and re-pelleted. The final pellet was dissolved in 1M-NaOH, and the protein content measured. The combined supernatants were extracted with 5×2vol. of water-saturated diethyl ether and neutralized by the addition of about 50μl of a solution of 1M-Tris base and 5M-NaOH. In some cases liver tissue was extracted by homogenizing with ice-cold aq. 0.6M-HClO₄ or in aq. 60% or 96% (v/v) ethanol at -20°C.

Determination of protein

This was measured by the method of Klungsøyr (1969), with bovine serum albumin as the standard. Two dilutions of the sample (containing between 40 and 300μg of protein) were measured.

Purification of cyclic AMP from tissue extracts

For QAE-Sephadex chromatography, 1ml of a concentrated trichloroacetic acid extract of tissue (representing 30–50mg of tissue protein) was diluted to 10ml with 20mM-ammonium formate buffer, pH 9.0, containing 5pmol of cyclic [³H]AMP, and applied to a QAE-Sephadex column (0.9cm×15cm) equilibrated with the same buffer. After washing with 15ml of water and 5ml of 20mM-ammonium formate, pH 6.0, the column was developed with 200ml of a linear NaCl gradient (0–160mM) in 20mM-ammonium formate, pH 6.0, and fractions (6ml) were collected. The peak fraction contained 35–40% of the cyclic [³H]AMP applied, and was completely free of cyclic GMP as judged from a preliminary experiment in which 100nmol of cyclic GMP was applied together with a trace amount of cyclic [³H]AMP (the peak of cyclic GMP was eluted four fractions after cyclic [³H]AMP).

Alternatively sequential Dowex/alumina chromatography, essentially as described by Salomon *et al.* (1974), was used. For this, 1ml of neutralized trichloroacetic acid tissue extract (containing 1pmol of cyclic [³H]AMP) was applied to a column (0.7cm×2.6cm) of Dowex 50 which had been rinsed with 1M-HCl (4ml) and water (10ml), the column washed with 2ml of water and eluted with 4ml of water. The eluate was applied to a column (0.7cm×3.3cm) of neutral alumina which had been rinsed with 0.1M-imidazole/HCl, pH 7.5 (10ml), and was eluted with 4ml of the same buffer. About 85% (range 81–90% in eight experiments) of the cyclic [³H]AMP added to the tissue extracts was recovered in the eluate from alumina.

Preparation of antibody against cyclic AMP

Cyclic AMP was succinylated (3-carboxypropionylated) in the 2'-*O*-position as described by Cailla & Delaage (1972). Succinyl-cyclic AMP was coupled to albumin by the procedure of Steiner *et al.* (1969). The conjugate (0.2mg) was emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) and injected into the foot pads of three rabbits at 5-week intervals, the animals being bled 1 week after the third injection. The γ-globulin fraction of the serum with the highest capacity for high-affinity binding of cyclic AMP was precipitated once with 50% and twice with 33%-satd. (NH₄)₂SO₄. The last precipitate was dissolved in phosphate-buffered saline, pH 7.2 (135mM-NaCl, 8mM-

Na_2HPO_4 , 3mM-KCl and 7.5mM- KH_2PO_4), passed through a column of Sephadex G-25 equilibrated with the same buffer, and the volume adjusted to that of the original serum sample.

Removal of cyclic AMP in tissue extracts by antibody or phosphodiesterase treatment

Neutralized tissue extract (1ml) was made 5mM in EDTA and incubated at 0°C with 200 μl of the preparation of antibody against cyclic AMP (see above). After 24h, satd. $(\text{NH}_4)_2\text{SO}_4$ (1ml) was added, and the mixture centrifuged for 30min at 20000g_{av.}. The supernatant was re-centrifuged as above, and a sample (1.1ml) was removed from the second supernatant, mixed with water (0.9ml), heated for 5min in a boiling-water bath, centrifuged once more, and the concentration of cyclic AMP was measured in the supernatant.

Treatment with cyclic nucleotide phosphodiesterase was at 37°C. 1ml of neutralized tissue extract was mixed with 0.2ml of 30mM-Tris/HCl (adjusted to pH7.5 at room temperature) containing 250mM-magnesium acetate and 0.4ml of phosphodiesterase in 30mM-Tris/HCl (pH7.5)/20mM-2-mercaptoethanol. After 3h of incubation, another 0.4ml of phosphodiesterase solution was added, and 2h later the reaction terminated by adding 1ml of 50mM-Hepes/NaOH (pH7.2)/40mM-EDTA and heating for 5min in a boiling-water bath. The phosphodiesterase solution was either a commercial preparation (from Sigma; 0.11unit/ml, where 1 unit is 1 μmol /min) or the peak fraction of phosphodiesterase activity obtained by DEAE-cellulose chromatography of human renal-cortex cytosol, which was obtained by loading 140mg of cytosol protein on to a DEAE-cellulose column (0.9cm $\times$ 15cm) and eluting with 300ml of a linear gradient of NaCl (0–0.35M) in 15mM-Tris/HCl, pH7.6. The peak of cyclic AMP phosphodiesterase activity was eluted between protein kinase isoenzyme I and isoenzyme II. The assay for cyclic AMP phosphodiesterase activity and the separation of degradation products of cyclic [^3H]AMP by chromatography on polyethyleneimine-impregnated cellulose sheets have been described by Døskeland *et al.* (1977). More than 98% degradation of cyclic [^3H]AMP added to tissue extracts was obtained with both enzyme preparations.

Preparation of binding protein for the assay of cyclic AMP

An anaesthetized rabbit was killed by bleeding, and the muscle tissue from the back and hind-limbs put into ice-cold homogenization buffer [15mM-Tris/HCl (pH8.1)/4mM-EDTA/0.25M-sucrose/20mM-2-mercaptoethanol/6.5% (w/v) poly(ethylene glycol) 6000] and chopped. Homogenization (1:5, w/v) was

performed with an emulsifier (Silverston Machineries, Waterside, Bucks., U.K.) operated at full speed for 3 $\times$ 30s with 2min of cooling in ice/water between each period. The pH of the homogenate was adjusted to 7.5 by the addition of 1.5 M-Tris/HCl, pH8.8, and the homogenate spun for 15min at 12000g_{av.}. To the supernatant was added poly(ethylene glycol) (7.5g/100ml) with continuous stirring. After 1h the precipitate was collected by centrifugation as above, dissolved in 200ml of 10mM-Tris/HCl (pH7.5)/0.1mM-EDTA/20mM-2-mercaptoethanol and applied to a DEAE-cellulose column (2.6cm $\times$ 30cm) equilibrated with the same buffer. After the column had been washed with 0.5litre of equilibration buffer, a linear gradient [formed from 500ml of equilibration buffer + 500ml of 30mM-Tris/HCl (pH7.5)/1mM-EDTA/200mM-NaCl/20mM-2-mercaptoethanol] was applied and fractions were collected. The fractions (20ml) with cyclic [^3H]AMP-binding activity corresponding to protein kinase I (up to and including the peak fraction) were pooled and precipitated with 55%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in a minimal volume of 15mM-Hepes/NaOH (pH7.2)/1mM-EDTA/20mM-2-mercaptoethanol and swiftly desalted by chromatography on a Sephadex G-25 column (whose bed volume was about six times the volume of the sample applied) equilibrated with the same buffer. The preparation of binding protein was frozen in liquid N_2 after albumin (final concn. 1mg/ml) had been added. The cyclic AMP-binding capacity of the preparation was determined by allowing it (at various dilutions) to incubate for 2h at 0°C with 15nM-cyclic [^3H]AMP in buffer A containing 0.3mg of heat-stable protein fraction/ml. Less than 2% of cyclic [^3H]AMP was digested when the binding protein (at a concentration corresponding to 50nM-cyclic AMP-binding sites) was tested for phosphodiesterase activity as described by Døskeland *et al.* (1977). The preparation did not bind [^3H]adenosine under conditions optimal for the binding of the nucleoside to cyclic AMP/adenosine-binding proteins (Døskeland & Ueland, 1977). No detectable decrease in binding capacity or affinity was noted during prolonged (6 months) storage of the reagent, including repeated (5–10 times) thawing and freezing. Protein kinase I from bovine adrenal cortex or mouse liver behaved like the kinase from rabbit skeletal muscle in the binding assay, but rabbit skeletal muscle was routinely used as the source of binding protein, because it was easier to obtain and gave a higher yield of protein kinase I.

Preparation of crude heat-stable protein fraction

Crude heat-stable protein fraction for use as a stabilizer in the assay for cyclic AMP was obtained by homogenizing rabbit skeletal muscle (1:4, w/v) in 30mM-Tris/HCl (pH7.8)/5mM-EDTA/20mM-2-

mercaptoethanol/0.15M-KCl, centrifuging for 30 min at 12000g_{av.}, heating the supernatant at 80–85°C for 5 min, re-centrifuging and precipitating the supernatant by addition of solid (NH₄)₂SO₄ (56 g/100 ml). The precipitate was dissolved in 15 mM-Hepes/NaOH (pH 7.2)/1 mM-EDTA/20 mM-2-mercaptoethanol and desalted by passage through a Sephadex G-25 column (bed volume six times the sample volume) equilibrated with the same buffer. The heat-stable protein fraction was kept at –20°C.

Preparation of partially purified protein kinase inhibitor

This was prepared by a modification of the procedure of Walsh *et al.* (1971), as described by Ueland & Døskeland (1976).

Routine procedure for the assay of cyclic AMP

For this 1 vol. of 0.6 μM-cyclic [³H]AMP was mixed with 99 vol. of 200 mM-Hepes/NaOH, pH 7.2 (adjusted at 0°C), containing 3.2 M-NaCl and 120 mM-Na₂EDTA. Portions (100 μl) of this solution were added to plastic tubes (polystyrene, 5 ml capacity) containing 200 μl of either cyclic AMP standard solution or the sample whose cyclic AMP concentration was to be measured. The tubes were capped, their contents mixed, and left for about 15 min at 0°C. Then was added 100 μl of binding protein (binding capacity about 2 pmol of cyclic AMP/ml) in ice-cold 15 mM-Hepes/NaOH (pH 7.2)/1 mM-EDTA/80 mM-2-mercaptoethanol/albumin (2 mg/ml)/heat-stable protein fraction (1.2 mg/ml). The tube contents were gently mixed, left for 12–18 h at 0°C, and the binding reaction terminated by the addition of 4 ml of ice-cold aq. 80%-satd. (NH₄)₂SO₄, followed by repeated inversions of the tubes. The solution of (NH₄)₂SO₄ containing the precipitated binding protein and unbound cyclic [³H]AMP was passed through membrane filters (HAWP, 0.45 μm pore size; 25 mm diameter; Millipore Corp., Bedford, MA, U.S.A.) which were washed with 3 × 3 ml of cold 65%-satd. (NH₄)₂SO₄, and put into scintillation vials containing 1 ml of aq. 2% (w/v) sodium dodecyl sulphate. After shaking for 10 min at room temperature, 7 ml of scintillation fluid [Unisolve (Koch-Light, Colnbrook, Bucks., U.K.) or Diluene (Packard, Wembley, Middx., U.K.)] was added, the contents were vortex-mixed, the vials put in a liquid-scintillation counter and counted for radioactivity for 2 × 4 min.

A decrease in c.p.m. was observed when the same plastic vial containing radioactivity was counted after various periods of storage: it varied with the type of plastic vial and the amount of scintillation fluid used, but was nearly linear with time for the first 48 h. To avoid differences in counting efficiency for samples in the first and last vials in large series,

the vials were first counted in one order and thereafter in the opposite order, and the mean c.p.m. value used for the calculations. With cyclic [³H]AMP of specific radioactivity 24 Ci/mmol the amount of radioactivity bound in the absence of unlabelled cyclic AMP corresponded to about 3500 c.p.m. When 14 ml instead of 7 ml of scintillation fluid was used the c.p.m. increased by about 7%.

Results

Stability of protein kinase I

Discrepancies between the concentration of cyclic AMP estimated from measurements in crude compared with purified tissue extracts, as well as a slight enhancement of cyclic [³H]AMP binding (at both 1.5 nM- and 15 nM-labelled nucleotide) by phosphodiesterase-treated tissue extracts, were observed when buffer A (containing 0.5 mg of albumin/ml) was used alone (results not shown). The existence in tissue extracts of factors more efficient than albumin in enhancing the binding to the dissociated protein kinase I was thus suggested. A minimally purified preparation [80%-satd.-(NH₄)₂SO₄ precipitate] of heat-stable protein from rabbit skeletal muscle was found to be an efficient stabilizer of the binding protein under conditions (36 h of incubation, the first 24 h in the absence of cyclic AMP and the last 12 h in the presence of 15 nM-cyclic AMP) in which the presence of added protein was essential for recovery of the binding activity. Whereas less than 10% of the binding activity was recovered with buffer A without added protein, 80% was recovered with buffer A, and 97% with buffer A containing 0.25 mg of the crude heat-stable inhibitor preparation/ml. The assay for cyclic AMP was therefore conducted in the presence of the above concentrations of albumin and heat-stable protein, and was then not affected by the addition of boiled commercially obtained phosphodiesterase, serum albumin, partially purified protein kinase inhibitor, or phosphodiesterase-treated tissue extracts.

Kinetics of cyclic AMP binding to protein kinase I

Whereas the amount of ligand bound to protein kinase I tended to decrease after 6 h of incubation in buffer A with 1.5 nM-cyclic [³H]AMP, a steady increase to a stable plateau value (reached after about 10 h) was noted when the heat-stable protein was included in the incubations (Fig. 1). Shorter incubation times than 10 h produced standard curves for cyclic AMP measurement that were non-linear for low concentrations of the nucleotide. Raising the concentration of cyclic [³H]AMP to 15 nM made it possible to obtain linear standard curves with 2 h of incubation, since the plateau of binding was reached earlier (Fig. 1).

Fig. 1. Time course of cyclic $[^3\text{H}]\text{AMP}$ binding to protein kinase I

Protein kinase I was incubated at 0°C in buffer A containing 1.5 nM-cyclic $[^3\text{H}]\text{AMP}$ (Δ) or in buffer A (40 ml) containing 0.3 mg of crude heat-stable protein fraction/ml and ($\circ$) 1.5 nM- or ($\square$) 15 nM-cyclic $[^3\text{H}]\text{AMP}$. Samples (0.8 ml) were removed at the time points indicated on the abscissa.

Optimization of the assay for cyclic AMP

A binding protein incubated at some concentration of radioactively labelled ligand (X^*) will bind a certain amount of radioactivity (C_0). If unlabelled ligand (X) is present, the amount of bound ligand (labelled+unlabelled) will be the same (binding protein saturated) or increase (binding protein not saturated), and the specific radioactivity of the bound ligand will be decreased by the factor $[\text{X}^*]/([\text{X}^*]+[\text{X}])$. The amount of radioactivity bound (C_x) will, in addition to X^* and X , depend on the concentration of binding reagent (R) and its affinity for cyclic AMP (represented by the equilibrium dissociation constant, K_d , when the incubations are conducted under equilibrium conditions of binding). An expression for C_0/C_x as a function of $[\text{X}^*]$, $[\text{X}]$, $[\text{R}]$ and K_d was derived essentially as described by Wundervald *et al.* (1974).

$$\frac{\text{C}_0}{\text{C}_x} = \left(\frac{[\text{X}^*]+[\text{X}]}{[\text{X}^*]} \right) \cdot \left(\frac{K_d + [\text{R}] + [\text{X}^*] - (K_d^2 + [\text{R}]^2 + [\text{X}^*]^2 + 2K_d[\text{R}] + 2K_d[\text{X}^*] - 2[\text{R}] \cdot [\text{X}^*])^{\frac{1}{2}}}{K_d + [\text{R}] + [\text{X}^*] + [\text{X}] - \{K_d^2 + [\text{R}]^2 + ([\text{X}^*] + [\text{X}])^2 + 2K_d \cdot [\text{R}] + 2K_d([\text{X}^*] + [\text{X}]) - 2[\text{R}] \cdot ([\text{X}^*] + [\text{X}])\}^{\frac{1}{2}}} \right)$$

Figs. 2 and 3 show the case when the concentration of labelled ligand $[\text{X}^*]$ is 1.5 nM. The solid lines of Fig. 2 show the shape of the standard curves obtained with 0.4 nM-cyclic AMP-binding sites (R) and various affinities of the binding reagent for cyclic AMP. A binding reagent with a K_d of 0.1 nM, which is close to the K_d for the interaction between cyclic AMP and salt-dissociated protein kinase I from rabbit skeletal muscle (S. O. Døskeland, unpublished work) and bovine adrenal cortex (Døskeland & Ueland, 1977), gives a standard curve nearly as steep as that for a

Fig. 2. Standard curve for cyclic AMP measurement superimposed on theoretical plots for various affinities of the binding protein for cyclic AMP

Values of C_0/C_x were calculated as a function of the concentration (final) of added unlabelled cyclic AMP. The calculations were based on a cyclic $[^3\text{H}]\text{AMP}$ concentration of 1.5 nM and concentrations of 0.4 nM (*a*, *b*, *c* and *e*), 0.69 nM (*d*) or 3.6 nM (*f*) of cyclic AMP-binding sites. The curves were constructed by assuming a homogeneous population of binding sites with an apparent equilibrium dissociation constant (K_d) of 0, 0.1, 1 or 10 nM. The experimental points (mean $\pm$ s.d., $n=4$) were obtained with 0.4 nM-binding sites under standard conditions for cyclic AMP measurement, except that the incubation volume was 0.8 ml, 14 ml of scintillation fluid was added and counting time was 2×10 min. The inset shows the shape of the standard curves at low concentrations of unlabelled cyclic AMP.

completely saturated binding reagent ($K_d=0$). On the other hand, binding reagents with K_d 1–10 nM, which is the range of K_d values reported for most naturally occurring binding proteins used for cyclic AMP assays (Gilman, 1970; Walton & Garren, 1970; Fisch *et al.*, 1972; Sanborn *et al.*, 1973; Brostrom &

Kon, 1974; Tovey *et al.*, 1974), produce curves that progressively become less steep. If the concentration of binding sites (R) is increased from 0.4 nM to 0.69 and 3.6 nM for binding reagents with K_d of 1 and 10 nM respectively, C_0 will be the same as for $[\text{R}]=0.4$ nM and $K_d=0.1$ nM, but the standard curves (broken lines of Fig. 2) will become even less steep than the parent curves. The standard curves of Fig. 3 were obtained by varying the concentration of binding sites $[\text{R}]$ by assuming $K_d=0.1$ nM. The slope and linearity of the curves is minimally affected by

Fig. 3. Effect of concentration of binding protein on the slope of the cyclic AMP standard curve

The plots were constructed by assuming the K_d of the interaction between binding protein and cyclic AMP to be 0.1 nM and the concentration of cyclic $[^3\text{H}]\text{AMP}$ to be 1.5 nM. The curves represent concentrations of binding sites, $[R]$, of 0.1, 0.4, 0.8, 1.2, 1.6 and 2.0 nM in descending order. The experimental points of Fig. 2 are given without the standard deviation. The inset shows the shape of the standard curves at low concentrations of unlabelled cyclic AMP.

increasing $[R]$ up to 0.4 nM, and little affected up to 0.8 nM. A considerably gentler slope as well as a significant deviation from linearity occur, however, when the concentration of binding sites approaches the concentration of labelled ligand. From the data of Figs. 2 and 3 it was decided to perform the cyclic AMP assay with 1.5 nM-cyclic $[^3\text{H}]\text{AMP}$ and about 0.5 nM-cyclic AMP-binding sites. The experimental points obtained with $[R]=0.4$ nM are plotted in Fig. 2 (with standard deviation) and in Fig. 3.

Precision and sensitivity of the present assay for cyclic AMP

The highest precision of cyclic AMP measurement (coefficient of variation for single determinations less than 5%) was for concentrations of cyclic AMP between 0.4 and 20 nM. The coefficient of variation for C_0 was 1% in the experiment of Fig. 2 and $1.6 \pm 0.3\%$ (mean $\pm$ S.D., $n=9$) in nine other experiments conducted under routine conditions (see the Experimental section) for cyclic AMP measurements. The limit of detection, as judged from the concentration of cyclic AMP corresponding to two standard deviations of C_0 , was 0.03 nM in the experiment of Fig. 2 and 0.05 nM for the routine assays.

Specificity and susceptibility to interference of the cyclic AMP assay

Several substances were tested for their ability to modulate the amount of cyclic $[^3\text{H}]\text{AMP}$ bound to

protein kinase under standard conditions for the assay of cyclic AMP. Among the tissue nucleotides likely to interfere with cyclic AMP measurement, cyclic GMP was about 200, adenosine 10^6 , ADP 4×10^6 , AMP 1.5×10^7 , adenine 2×10^7 and ATP 2×10^7 times less potent than cyclic AMP in displacing cyclic $[^3\text{H}]\text{AMP}$. The combination of Mg^{2+} and ATP inhibited the binding of cyclic $[^3\text{H}]\text{AMP}$ more efficiently than Mg^{2+} (no inhibition) or ATP alone, the inhibitory effect of MgATP rapidly disappearing on dilution. Whereas 10 mM- Mg^{2+} /1 mM-ATP produced about 50% inhibition of the cyclic $[^3\text{H}]\text{AMP}$ binding, 5 mM- Mg^{2+} /0.1 mM-ATP produced no inhibition.

Analogues of cyclic AMP and adenosine as well as inhibitors of the enzymic degradation of those substances were also tested. N^6, O^2 -Dibutyl cyclic AMP was about 300 times and 3-isobutyl-1-methyl-xanthine about 7×10^5 times less efficient than unlabelled cyclic AMP as inhibitor of cyclic $[^3\text{H}]\text{AMP}$ binding. 2-Chloroadenosine and 6-chloropurine riboside at 50 μM (final concentration in the incubations) produced an inhibition corresponding to that with 0.4 nM- and 0.2 nM-unlabelled cyclic AMP respectively, whereas 50 μM concentrations of 7-deaza-adenosine, erythro-9-(2-hydroxynon-3-yl)adenine, 3'-deoxyadenosine or 2'-deoxyadenosine, or 15 μM concentrations of N^6 -cyclohexylallyl-adenosine, 8-bromo-adenosine or N^6 -phenylisopropyladenosine, produced inhibitions corresponding to less than that by 0.1 nM-cyclic AMP.

Of common buffers and salts, including those used to elute cyclic nucleotides during column chromatography, the following caused an inhibition corresponding to less than that by 0.2 nM-unlabelled cyclic AMP at the concentrations (final) given: 100 mM-potassium phosphate, pH 7.2, 100 mM-Tris/HCl, pH 7.2, 50 mM-imidazole/HCl, pH 7.2, 10 mM- CaCl_2 , 10 mM- PbCl_2 , 1.6 M- NaCl , 1 M-sodium formate, pH 7.2, 20%-satd. $(\text{NH}_4)_2\text{SO}_4$ and 0.2% (w/v) NaN_3 : 1 M-LiCl produced an inhibition corresponding to that by 0.3 nM-cyclic AMP.

Neither Dulbecco's modified Eagle medium nor RPMI (Roswell Park Memorial Institute) medium 1640 significantly lowered the binding of cyclic $[^3\text{H}]\text{AMP}$ to protein kinase I.

The supernatant obtained from a neutralized 1 M solution of HClO_4 (the solution was adjusted to pH 7.2 by the addition of 5 M-KOH containing 1 M-KCl and 0.5 M-Tris base, left for 2 h at 0°C, and the resulting precipitate removed by spinning at 5000g for 10 min) decreased the cyclic $[^3\text{H}]\text{AMP}$ binding corresponding to that by 0.2 nM-cyclic AMP. Such an inhibition was noted also when the solution of HClO_4 was neutralized with K_2CO_3 . An aq. 5% (w/v) solution of trichloroacetic acid containing 0.1 M-HCl did not interfere with the binding after ether extraction and neutralization, and a standard

Table 1. *Reproducibility of cyclic AMP measurement in crude tissue extracts and comparison with measurement in purified extracts*

Eight portions were taken from liver powder (kept in liquid N₂) and processed separately for measurement of cyclic AMP and protein, as described in the Experimental section. The cyclic AMP content is given as pmol/mg of liver protein (mean $\pm$ s.d.). Samples (50 μ l) from each of the eight tissue extracts were pooled, 10 pmol of cyclic [³H]AMP was added, and the whole sample (containing about 1 nmol of cyclic AMP) subjected to QAE-Sephadex chromatography. Samples from tissue extracts of renal cortex and carcinoma, containing cyclic [³H]AMP as a tracer, were chromatographed sequentially on Dowex and Al₂O₃. Each tissue extract was chromatographed at two different occasions, the cyclic AMP concentration of the purified extracts measured, and the cyclic AMP content/mg of protein (mean of the two experiments) calculated, with correction for the loss of cyclic AMP during chromatography.

Source of extract	Purification steps	Cyclic AMP content (pmol/mg of protein)
Pooled livers from glucagon-injected mice	None	52.0 $\pm$ 3.1 (n=8)
	QAE-Sephadex	49.9
Human renal cortex	None	5.07
	Dowex 50 and Al ₂ O ₃	5.05
Human renal-cell carcinoma	None	15.1
	Dowex 50 and Al ₂ O ₃	14.1

curve for cyclic AMP measurement done in the presence of such a solution did not differ from that obtained with standards of cyclic AMP in water. For that reason trichloroacetic acid was routinely used for the extraction of cyclic AMP from tissues.

Extractability of cyclic AMP from tissues, and reproducibility of the determination of tissue cyclic AMP

Less than 0.5% of the cyclic AMP content of glucagon-stimulated liver could be extracted by boiling, ethanol or HClO₄ treatment of the pellet left after the tissue had been extracted with trichloroacetic acid. Extraction with 1M-HClO₄ was also complete, as judged by the failure of the other procedures to extract additional cyclic AMP from the tissue. Identical tissue concentrations of cyclic AMP were found after extraction with either HClO₄ or trichloroacetic acid. On the other hand, extraction with aq. 60 or 96% (v/v) ethanol at -20°C, 0°C or 20°C was incomplete, as 10–30% of tissue cyclic AMP remained and could be extracted with trichloroacetic acid. These results are at variance with those of Farmer *et al.* (1975).

The recovery of cyclic [³H]AMP added during homogenization of liver was 95.9 $\pm$ 0.6% (mean $\pm$ s.d., n=5). The amount of total tissue protein remaining in the trichloroacetic acid extract was 4.0 $\pm$ 0.2% (mean $\pm$ s.d., n=8).

To estimate the reproducibility of the determination of tissue cyclic AMP, eight random samples of finely pulverized liver powder were assayed. The results (Table 1) indicated a coefficient of variation of 6%.

Tests of the validity of the cyclic AMP measurement in crude tissue extracts

Linearity of cyclic AMP content versus amount of tissue extract was obtained for the whole range of cyclic AMP concentrations within the useful part of the standard curve, and accurate recovery of added cyclic AMP was noted at several dilutions of tissue extract (Fig. 4). The substance(s) in tissue extracts measured as cyclic AMP was removed by two different preparations of cyclic nucleotide phosphodiesterase and by antibody raised against conjugated cyclic AMP (Fig. 4), and could not be separated from cyclic AMP by column chromatography on QAE-Sephadex, Dowex 50 or aluminium oxide (Table 1). Results similar to those presented in Fig. 4 were obtained for tissue extracts from renal cortex and mouse liver. The removal of cyclic AMP from tissue extracts by incubation with the globulin fraction from a rabbit immunized against conjugated cyclic AMP was not due to hydrolysis of the nucleotide by contaminating cyclic nucleotide phosphodiesterase, since the cyclic AMP originally present in the tissue extract could be recovered by boiling the precipitated globulin. The recovery studies were complicated by the presence of a high concentration (corresponding to 1 μ M in the rabbit serum) of cyclic AMP bound to the globulin. The nucleotide was tightly bound to the globulin, since less than 5% was removed by dialysis against phosphate-buffered saline for 10h at room temperature with frequent changes of buffer.

Measurement of cyclic AMP in the presence of interfering substances

A competitive inhibitor of cyclic AMP binding will influence the shape of the standard curve in the same manner as if a certain concentration of unlabelled cyclic AMP were present. The concentration of cyclic AMP corresponding to a certain concentration of inhibitor depends on the relative affinity of the binding reagent for cyclic AMP and the inhibitor, and can be found experimentally by measuring the ratio C₀/C₀₁, where C₀₁ is the amount of labelled cyclic AMP bound in the presence of inhibitor, and then calculate the value of X corresponding to C₀/C_x=C₀/C₀₁ from a cyclic AMP standard curve. For the inhibitor cyclic GMP, a concentration of

Fig. 4. Tests of the reliability of cyclic AMP measurement in crude tissue extract

A trichloroacetic acid extract was prepared from human renal-cell carcinoma: 1 ml of the extract represented 14.8 mg of tissue protein. A 2-fold dilution series of the extract in water was prepared, the cyclic AMP concentrations (final) were assayed in duplicate and plotted ($\circ$) versus extract dilution, represented by the corresponding concentration of tissue protein, and a straight line was drawn through the experimental points. The concentration of cyclic AMP was also measured in two dilutions of extract after phosphodiesterase-treatment (Δ) or adsorption antibody ($\square$), and in three dilutions of untreated extract to which had been added 1.6 nM- ($\bullet$) or 12 nM- ($\bullet$) cyclic AMP. The concentration of cyclic AMP calculated in various dilutions of tissue extract to which had been added cyclic AMP (+) are given for comparison with the experimental points.

0.3 μ M corresponded to about 1.7 nM-cyclic AMP, and accurate estimates of the cyclic AMP concentrations of samples containing 0.3 μ M-cyclic GMP were obtained by subtracting 1.7 nM from the values calculated from the normal standard curve (Fig. 5a) for cyclic AMP. The experimental values of C_0/C_x for the cyclic AMP assay run in the presence of 0.3 μ M-cyclic GMP were grouped around the curve (Fig. 5e) theoretically calculated for an assay containing 3.2 nM- instead of 1.5 nM- (Fig. 5a) cyclic [3 H]AMP.

Inhibitors of cyclic AMP binding may also act by decreasing the affinity of the binding reagent for cyclic AMP. It was calculated that decreasing the K_d for cyclic AMP from 0.1 to 1.3 nM would result in the same decrease in binding as 0.3 μ M-cyclic GMP (in the absence of unlabelled cyclic AMP). The resulting standard curve (Fig. 5d) deviated from that of a competitive inhibitor, especially at high concentrations of cyclic AMP.

In the presence of 1.6 M-NaCl, which inhibits binding of cyclic AMP by lowering the affinity of protein kinase I for the nucleotide (S. O. Døskeland, unpublished work), the binding of cyclic [3 H]AMP

was decreased corresponding to an addition of 0.14 nM-unlabelled nucleotide. Standard curves for competitive inhibition (Fig. 5c) and increase of K_d (Fig. 5b) were constructed, and found to be identical for practical purposes.

From the above it follows that for modest inhibition of the binding of labelled nucleotide, it is legitimate to subtract the blanks measured at zero-added nucleotide concentration, whether the inhibition is competitive or is the result of a lowered affinity of the binding reagent. At higher degrees of inhibition only the 'zero blanks' of competitive inhibitors can be subtracted with confidence.

Standard curves run in the presence of high concentrations of Mg^{2+} and ATP (6 and 2 mM respectively) deviated from those calculated for competitive inhibition or altered affinity, and underestimation of the cyclic AMP in the samples resulted when 'zero blanks' were subtracted (results not shown).

Discussion

Interference with competitive binding assays may result from (1) competitive displacement of the labelled ligand by substances other than the one to be measured, (2) altered affinity of the binding reagent for the labelled ligand, (3) altered number of available binding sites or ligand molecules, (4) erroneous estimation of the amount of bound ligand.

The results of the specificity tests carried out with the present binding protein indicate that cyclic AMP need not be separated from other purine derivatives in tissue extracts before assay. Similarly, several analogues of cyclic AMP or adenosine and inhibitors of cyclic AMP phosphodiesterase or adenosine deaminase gave none or a modest displacement of cyclic [3 H]AMP at the concentration at which they are likely to be encountered in tissue-culture media or adenylate cyclase assay media. Furthermore, the effect of a competitive inhibitor on the determination of cyclic AMP can easily be corrected for by subtracting 'zero blanks' (see the Results section, Fig. 5).

The moderate decreases in cyclic [3 H]AMP binding observed at high concentrations of several salts, probably resulting from a lowered affinity of the binding protein for cyclic AMP, can also be corrected for by subtraction of 'zero blanks' (Fig. 5). The presence of Mg^{2+} and ATP at the concentrations likely to be encountered in tissue extracts or moderately diluted assay media for adenylate cyclase did not interfere with the binding of cyclic [3 H]AMP to protein kinase I. The interference with cyclic AMP determinations caused by very high concentrations of Mg^{2+} and ATP differed from that expected from competitive inhibition or simple alteration of the affinity of the binding protein, presumably because $MgATP$ promotes the reassociation (Brostrom *et al.*, 1971) of the high-affinity regulatory moiety and cata-

Fig. 5. Effect on the cyclic AMP assay of altered affinity of the binding protein or of adding a competitive inhibitor of cyclic AMP binding

Three parallel experimental series were set up with the concentrations (final) of unlabelled cyclic AMP indicated on the abscissa. The conditions were the standard ones (see the Experimental section) in one series ($\circ$), whereas 1.6M-NaCl was present in the second ($\square$) and 0.3 μ M-cyclic GMP in the third series ($\bullet$). The concentration of binding sites for cyclic AMP was 0.6 nM. The solid lines represent theoretically calculated standard curves when cyclic AMP and binding protein interact with a K_d of 0.1 nM, and the concentration of cyclic [3 H]AMP is 1.5 nM (a), 1.64 nM (c) or 3.2 nM (e). The broken lines are the curves predicted when the concentration of cyclic [3 H]AMP is 1.5 nM and the K_d is 0.18 nM (b) or 1.3 nM (d).

lytic moiety of protein kinase I to the low-affinity (Døskeland & Ueland, 1977) holoenzyme form, thereby producing two populations of binding sites with different affinities for cyclic AMP.

Interference with the present assay by enzymic transformation of cyclic AMP would not be a problem, because the binding reagent is devoid of cyclic AMP phosphodiesterase activity, and because the assay conditions (presence of 30mM-EDTA) are unfavourable for that enzyme activity.

One or several factors in trichloroacetic acid extracts of tissues may interfere with the measurement of cyclic AMP (Weinryb, 1972; Weller *et al.*, 1972; Arner & Østman, 1975), presumably by stabilizing the binding reagent. In the presence of EDTA, 2-mercaptoethanol, serum albumin and a crude heat-stable protein fraction from muscle the dissociated protein kinase I was stabilized so that the incubation could be performed for the time needed for the binding of cyclic [3 H]AMP to reach equilibrium (Fig. 1). Under those conditions neither phosphodiesterase-treated tissue extracts nor several proteins enhanced the binding to protein kinase.

The separation of bound and free nucleotide with charcoal or by membrane filtration of the native binding protein seems more prone to interference

(Sheppard & Tsien, 1975; Albano *et al.*, 1974) than is membrane filtration of $(\text{NH}_4)_2\text{SO}_4$ -precipitated binding protein (Døskeland *et al.*, 1977). The $(\text{NH}_4)_2\text{SO}_4$ -precipitation procedure is more efficient than membrane filtration (Ueland & Døskeland, 1977) in detecting cyclic AMP binding to cyclic AMP/adenosine-binding proteins (Yuh & Tao, 1974). The removal of such proteins from the binding reagent would thus be mandatory when using $(\text{NH}_4)_2\text{SO}_4$ precipitation, and can be easily achieved by poly-(ethylene glycol) fractionation followed by DEAE-cellulose chromatography as described in the Experimental section.

The specificity of the present assay and its resistance to non-specific interference suggest that cyclic AMP can be reliably measured directly in tissue-culture media, column-chromatographic fractions, adenylate cyclase assay mixtures and crude tissue extracts. The assay has been validated for the measurement of adenylate cyclase activity of liver plasma membranes (S. O. Døskeland & S. Kvinnsland, unpublished work). Several experiments were conducted to ensure that the material measured in crude tissue extracts was authentic cyclic AMP. Linearity of cyclic AMP content versus amount of tissue extract (Fig. 4) indicated that cyclic AMP or another competitive inhibitor of cyclic [3 H]AMP binding was measured. Alternatively one or more factors in the extract altered the stability or the affinity of the binding protein in such a manner as to mimic cyclic AMP. The accurate recovery of added cyclic AMP at several dilutions of tissue extract (Fig. 4) did not support the latter explanation. More than 98% of the material assayed as cyclic AMP was removed by phosphodiesterase digestion or adsorption with anti-(cyclic AMP) antibody of crude tissue extracts (Fig. 4). The failure to separate the material measured as cyclic AMP in the extracts from authentic cyclic AMP by chromatographic procedures (Table 1) is additional evidence that the substance measured is cyclic AMP. The measurement of cyclic AMP directly in tissue extracts eliminates the error involved in determination of nucleotide recoveries after purification procedures.

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References

- Albano, J. D., Barnes, G. D., Maudsley, D. V., Brown, B. L. & Etkins, R. P. (1974) *Anal. Biochem.* **60**, 130–141
- Arner, P. & Østman, J. (1975) *Scand. J. Clin. Lab. Invest.* **35**, 691–697
- Brostrom, C. O. & Kon, C. (1974) *Anal. Biochem.* **58**, 459–468

- Brostrom, C. O., Corbin, J. D., King, C. A. & Krebs, E. G. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2444–2447
- Brown, B. L., Albano, J. D. M., Etkins, R. P. & Sgherzi, A. M. (1971) *Biochem. J.* **121**, 561–562
- Brown, B. L., Etkins, R. P. & Albano, J. D. M. (1972) *Adv. Cyclic Nucleotide Res.* **2**, 25–40
- Cailla, H. & Delaage, M. (1972) *Anal. Biochem.* **48**, 62–72
- Cailla, H. L., Racine-Weisbuch, M. S. & Delaage, M. A. (1973) *Anal. Biochem.* **56**, 394–407
- Cooper, R. H., McPherson, M. & Schofield, J. G. (1972) *Biochem. J.* **127**, 143–154
- Corbin, J. D., Keely, S. L. & Park, C. R. (1975) *J. Biol. Chem.* **250**, 218–225
- Døskeland, S. O. & Ueland, P. M. (1977) *Biochem. J.* **165**, 561–573
- Døskeland, S. O., Ueland, P. M. & Haga, H.-J. (1977) *Biochem. J.* **161**, 653–665
- Farmer, R. W., Harrington, C. A. & Brown, D. H. (1975) *Anal. Biochem.* **64**, 455–460
- Fisch, M. U., Pliška, V. & Schwyzer, R. (1972) *Eur. J. Biochem.* **30**, 1–6
- Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305–312
- Gilman, A. G. & Murad, F. (1974) *Methods Enzymol.* **38C**, 49–57
- Klungsoyr, L. (1969) *Anal. Biochem.* **27**, 91–98
- Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Sanborn, B. M., Bhalla, R. C. & Korenman, S. G. (1973) *Endocrinology* **92**, 494–499
- Sheppard, H. & Tsien, W. H. (1975) *Life Sci.* **16**, 139–148
- Steiner, A. L., Kipnis, D. M., Utiger, R. & Parker, C. W. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 367–373
- Steiner, A. L., Kipnis, D. M., Utiger, R., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113
- Tovey, K. C., Oldham, K. G. & Whelan, J. A. M. (1974) *Clin. Chim. Acta* **56**, 221–234
- Ueland, P. M. & Døskeland, S. O. (1976) *Biochem. J.* **157**, 117–126
- Ueland, P. M. & Døskeland, S. O. (1977) *J. Biol. Chem.* **252**, 677–686
- Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1977–1985
- Walton, G. M. & Garren, L. D. (1970) *Biochemistry* **9**, 4223–4229
- Weinryb, I. (1972) in *Methods in Molecular Biology* (Chasin, M., ed.), pp. 29–79, Marcel Dekker, New York
- Weller, M., Rodnight, R. & Carrera, D. (1972) *Biochem. J.* **129**, 113–121
- Wright, S. T. C. & Price, R. (1975) *Anal. Biochem.* **67**, 342–346
- Wunderwald, P., Jurz, G. & Michael, G. (1974) *Anal. Biochem.* **59**, 468–481
- Yuh, K.-C. M. & Tao, M. (1974) *Biochemistry* **13**, 5220–5226