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A SIMPLE DIRECT ASSAY FOR CYCLIC AMP IN PLASMA AND OTHER BIOLOGICAL SAMPLES USING AN IMPROVED COMPETITIVE PROTEIN BINDING TECHNIQUE

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Summary

An improved cyclic AMP assay using a purified and activated binding protein from bovine skeletal muscle is described.

Activation of the purified binding protein by bovine serum albumin was investigated and shown to increase the assocation constant from $0.4 \cdot 10^9$ M^{-1} to $1.0 \cdot 10^9$ M^{-1}. The fully activated binding protein was not affected by the presence of large amounts of protein in biological samples such as plasma.

Using the fully activated binding protein and with optimised assay conditions it has been demonstrated that the assay has a remarkable freedom from non-specific interference by materials normally found in crude biological extracts.

This freedom from interference, coupled with a simple method of preparing plasma, permits the hitherto unreported rapid direct assay of plasma cyclic AMP without the need for extraction procedures or the use of cyclic AMP-free plasma controls, thus making the method more reliable and suitable for use in the clinical laboratory.

Development of an assay markedly insensitive to non-specific interference increases the number of potential applications of the assay.

Introduction

Although a wide variety of methods [1] have been used for the assay of cyclic adenosine monophosphate (cyclic AMP) in tissues, urine and plasma, and in the assay of adenylate cyclase activity, saturation analysis methods have become the method of choice [2].

Specificity is an important requirement for any assay; that is, the assay for the compound being measured should be free from interference from other substances [3]. Such interference can result not only from compounds similar

to that under assay but also from other materials (salts, proteins, metal ions, enzyme inhibitors, etc.) likely to be present in biological extracts.

Biological fluids, such as urine, which contain high levels of cyclic AMP can be greatly diluted before assay. This dilution markedly decreases the possibility of interference in most, but not all assays [4]. Plasma contains a much lower level of cyclic AMP and interference cannot usually be avoided by dilution. Current estimations of plasma cyclic AMP levels require either tedious blank corrections using cyclic AMP-free plasma [5,6], or extraction methods [5,8] which require correction both for recovery and for further interference by substances isolated during the extraction procedure [5–10,12]. No technique yet described has been sufficiently free from non-specific interference to permit a simple, direct assay of plasma cyclic AMP.

Differences in the composition of standards and unknowns cause environmental changes which can result in gross interference with the primary binding reaction, with the separation procedure, or with both. One method of minimising such interference is to ensure that incubation mixtures containing unknowns and standards should be as near identical as possible. This requires calibration with standard solutions containing reagent blanks prepared from identical biological media which have been treated to remove the compound being assayed. Such procedures are laborious, particularly when different plasmas exert different non-specific effects [6] thus requiring a separate reagent blank and calibration curve for each sample. Additionally, because cyclic AMP assays are predominantly a research interest, they are carried out on samples obtained from widely differing sources of biological material which necessitates the preparation of separate calibration curves for each source.

Alternative methods used to minimise non-specific interference in cyclic AMP assays have involved the chromatographic isolation of the pure nucleotide, the use of complex internal standardization methods [10], or the development of more sensitive assays. These methods are complicated and tedious. Thus an assay which is subject to minimal non-specific interference from substances likely to be found in biological extracts will have many advantages over current methods. We have developed such an assay by selecting assay conditions to minimise interference, by the use of a purified binding protein having a high specificity and affinity for cyclic AMP, by using an improved charcoal separation technique, and by using an incubation mixture containing EDTA. The assay has been shown to permit the rapid and simple direct assay of cyclic AMP in plasma and other biological samples, and has a high degree of freedom from non-specific interference.

Materials

Tritiated cyclic AMP, [8-^{3}H]adenosine 3′,5′-monophosphate with a specific activity of 29 Ci/mmole was obtained from The Radiochemical Centre (Amersham, U.K). Cyclic AMP, cyclic GMP and ATP were obtained from the Sigma (London) Chemical Co. (London, U.K.). Norit GSX charcoal was obtained from Norit Clydesdale Co. (Glasgow, U.K.) and theophylline from British Drug Houses (Poole, U.K.).

Reagents for liquid scintillation counting were obtained as follows: PCS

(Amersham/Searle) liquid scintillation cocktail for aqueous samples, from Hopkin and Williams (Chadwell Heath, U.K.); Triton X-100 detergent and toluene (sulphur-free) from British Drug Houses (Poole, U.K.); 2,5-diphenyloxazole, (PPO) and *p*-bis(2-(5-phenyloxazolyl)-benzene), (POPOP) from Nuclear Enterprises (Edinburgh, U.K.).

Fresh bovine muscle was obtained from a local abattoir.

Methods

Preparation of assay reagents

(1) Binding protein was isolated from bovine skeletal muscle and purified essentially as described by Gilman [1,11] but omitting the acid precipitation step. 400 g of muscle were homogenised in 500 ml of assay buffer, then centrifuged for 30 min at 10 000 × *g*. The supernatant was fractionated with ammonium sulphate. The 25-45% saturation fraction was dissolved in 0.005 M potassium phosphate buffer pH 7.5 containing 0.5 mM EDTA (buffer A) and dialysed overnight against 4 l of the same buffer. The dialysed protein was then applied to a DEAE column (previously equilibrated with buffer A) and eluted with buffer A. Cyclic AMP-binding activity was eluted from the DEAE-cellulose column in two peaks. Binding protein from the first peak contained 50% of the initial binding activity and after dialysis, Tris—HCl buffer pH 7.5, EDTA and bovine serum albumin (BSA) were added to give the following final concentrations: Tris—HCl buffer pH 7.5, 50 mM; EDTA, 4 mM and BSA, 0.1%. The concentration of binding protein was adjusted so that 100 μl of the solution would bind 55% of the activity (zero-dose binding) when incubated with 0.9 pmoles of tritiated cyclic AMP in a final volume of 200 μl.

The specific activity of this preparation was 0.1 pmole cyclic AMP bound/μg binding protein and when stored at −20° was stable for several months.

(2) Assay buffer. 50 mM Tris—HCl buffer pH 7.5 containing 4 mM EDTA.

(3) Tritiated cyclic AMP reagent. 180 pmoles (ca 5 μCi) in 10 ml of assay buffer (equivalent to 0.9 pmoles/50 μl sample).

(4) Standard cyclic AMP reagent. 1600 pmoles non-radioactive cyclic AMP in 5 ml of assay buffer (equivalent to 16 pmoles/50 μl sample). Four serial dilutions of this reagent with equal volumes of assay buffer provided additional calibration standards containing 8, 4, 2 and 1 pmole/50 μl sample.

(5) Charcoal adsorbent. 520 mg of Norit GSX charcoal suspended in 20 ml of assay buffer containing 2% BSA.

Preparation of toluene—Triton X-100 scintillation cocktail

4.4 g of PPO and 0.1 g of POPOP were dissolved in 1 litre of toluene; 500 ml of Triton X-100 were added, and the solution was well mixed and stored in the dark. Before adding radioactive samples for counting, vials were prepared by adding 10 ml aliquots of the scintillant cocktail and 1 ml of distilled water to each vial and shaking to obtain a clear solution.

Standard assay procedure

To each tube the components of the reaction mixture were added in the

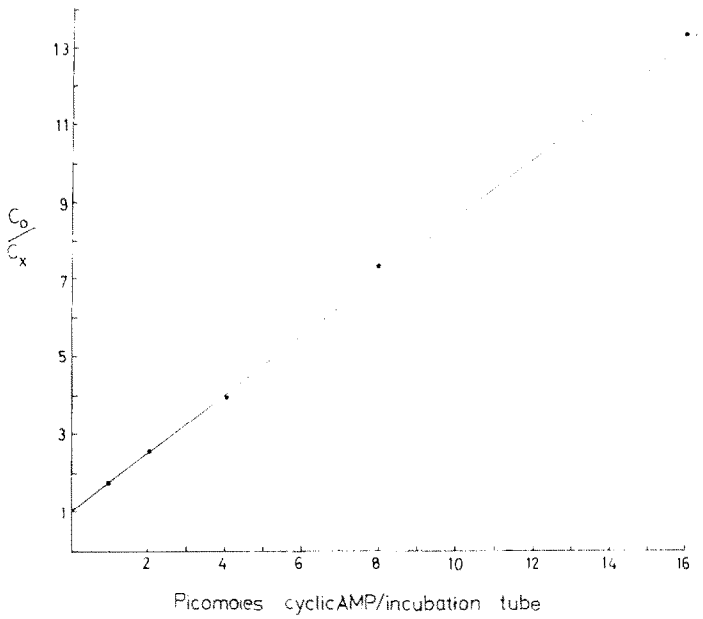

Fig. 1. A typical standard curve for the assay of cyclic AMP. Assays were carried out as described in the text.

following order: 50 µl of tritiated cyclic AMP reagent, 50 µl of unlabelled cyclic AMP (calibration standard or unknown), and 100 µl of binding protein solution. After mixing the contents, the tubes were incubated for 2 h (± 30 min) in an ice-bath. 100 µl of charcoal suspension were added to each tube which was then vortex mixed for 10 s. After centrifugation (ca 10 000 × *g* for 1 min at room temperature) 200 µl samples of supernatants (bound fraction) were removed and their radioactivity determined by liquid scintillation counting in either PCS or a toluene—Triton X-100 scintillation cocktail. A blank reading was determined by measuring the cpm in 200 µl of supernatant from a tube containing 50 µl tritiated cyclic AMP, 150 µl of assay buffer, and 100 µl of charcoal suspension. This blank reading was subtracted from all other results. The cpm bound in the absence of unlabelled cyclic AMP (i.e. the zero-dose binding) was designated C_0 and the cpm bound in the presence of unlabelled cyclic AMP (calibration standard or unknown) was designated C_x. The ratio C_0/C_x was calculated for each level of cyclic AMP. A straight line calibration curve was obtained by plotting C_0/C_x against pmoles of standard cyclic AMP on linear graph paper (see Fig. 1). The amount of cyclic AMP in the unknowns was then read off this line using the measured C_0/C_x values.

Preparation of ox brain extract

Ox cerebral cortex was homogenised at a concentration of 100 mg of tissue/ml of 0.1 M HCl and the homogenate was heated at 100° for 2 min as described elsewhere [10]. After cooling, the suspension was centrifuged, the supernatant taken, freeze-dried and dissolved in water. Two ml of this extract*

* A kind gift of Dr M. Weller, Institute of Psychiatry, London, U.K.

were adjusted to pH 7.5 with 1 M NaOH and diluted appropriately with assay buffer before assay.

Preparation of plasma

A freshly withdrawn sample of blood was added immediately to 1% of its volume of 0.5 M EDTA pH 7.5 contained in a cooled centrifuge tube and the contents were rapidly mixed. The EDTA-treated blood was centrifuged for 5 min at 4000 × *g* at room temperature. The plasma was then removed and assayed the same day or stored at −20° for assay later.

Results and Discussion

(A) Design of the assay

Four major factors must be considered in attempts to design a competitive protein binding assay which will suffer minimal interference. These are: the sensitivity of the assay; the composition of the incubation mixture; and the influence of potentially interfering factors on both the binding reaction and the separation procedure.

(1) The sensitivity of the assay

Non-specific interference can be avoided by designing an assay of very high sensitivity so that assay samples can be diluted to an extent which reduces interfering factors to insignificant levels. Maximum sensitivity, however, is not the only requirement in an assay. For optimum performance the assay should be simple, precise and rapid, and have an adequate range.

Table I and Fig. 1 show that the assay technique described fulfils these requirements: counting times of 4 min are adequate; the detection limit (2 standard deviations at zero-dose) is 0.05 pmoles/50 μl sample; the simple non-logarithmic linear calibration curve has a mid-range (i.e. the dose required to reduce the response to half its initial value) of 1.20—1.30 pmoles/50 μl sample; can be used with a within-assay coefficient of variation of less than 11% over

TABLE I

SENSITIVITY, WITHIN-ASSAY REPRODUCIBILITY AND PRECISION

Assays were carried out as described in the text. All samples were counted for 4 min; total activity was 6935 cpm; blank value was 64 cpm. Results are mean values ± S.D. (n = 6).

Dose (pmoles cyclic AMP)	% Bound	Coefficient of variation (%)	C_o/C_x	Standard deviation (pmoles cyclic AMP)	Coefficient of variation (%)
0	62.5 ± 1.3	2.1	1.00 ± 0.02	0.027	∞
0.25	51.5 ± 0.9	1.8	1.21 ± 0.02	0.027	10.8
0.50	43.1 ± 0.8	1.9	1.45 ± 0.03	0.035	7.0
1.0	34.4 ± 0.9	2.7	1.82 ± 0.05	0.061	6.1
2.0	23.0 ± 0.8	3.6	2.75 ± 0.10	0.12	6.0
4.0	14.6 ± 0.6	4.2	4.27 ± 0.18	0.23	5.7
8.0	7.9 ± 0.3	4.3	7.95 ± 0.34	0.43	5.3
16.0	4.9 ± 0.5	10.9	12.83 ± 1.40	1.75	10.9

TABLE II

BETWEEN-ASSAY REPRODUCIBILITY AND PRECISION

pmoles cyclic AMP/50 μl sample		Coefficient of variation (%)
Dose	Standard deviation	
0.5*	± 0.03	6.0
6.0*	± 0.36	6.0
12.0*	± 1.36	11.3
3.4**, ***	± 0.14	4.1
7.0**, ****	± 0.52	7.4

* Cyclic AMP standards assayed in duplicate on 10 separate occasions.
** Urine standards assayed in duplicate on 6 separate occasions.
*** = 1:100 Dilution.
**** = 1:50 Dilution.

the range 0.25—16 pmoles/50 μl sample, and with maximum precision (coefficient of variation < 7%) over the range 0.5—8 pmoles/50 μl sample. Between-assay reproducibility is excellent (see Table II).

The sensitivity achieved is adequate for the vast majority of cyclic AMP assays but, because of the high affinity of the binding protein for cyclic AMP ($K_a = 10^9$ M^{-1}), it could readily be increased further if higher sensitivity is required. This could be done by reducing the concentrations of labelled cyclic AMP and binding protein, or by staggered addition of labelled cyclic AMP. These modifications would, however, make the assay less simple.

(2) Composition of the incubation mixture

The composition of the incubation mixture can have a significant effect on the susceptibility of an assay to non-specific interference.

Assays were carried out at pH 7.5 rather than at pH 4 as in the Gilman assay [1,11], for several reasons: the binding protein's capacity was optimal at pH 7.5. A pH of 7.5 avoids the need to adjust the pH of most biological samples before assay. Many proteins precipitate at pH 4 and such precipitation would prevent simple direct assays on protein-rich samples like plasma.

EDTA was included in incubation mixtures to chelate bivalent metal ions and thus simultaneously prevent enzymatic degradation of cyclic AMP and reduce the possibility of metal ion interference in the binding reaction [13] or separation procedure [14]. The use of EDTA proved to have further marked advantages in the preparation of plasma samples (see section C, part 2).

We therefore used an incubation mixture containing 50 mM Tris buffer pH 7.5 and 4 mM EDTA. Fig. 2 shows that the assay is insensitive to large increases in the concentrations of buffer and EDTA.

(3) The binding reaction

It is advantageous to use a binding protein with a high affinity for cyclic AMP, with minimal cross-reactivity with other nucleotides, and subject to minimal interference from other proteins present in incubation mixtures. Gilman [11] reported that a purified binding protein from bovine skeletal muscle had a high affinity for cyclic AMP ($K_a = 3.3—5 \cdot 10^8$ M^{-1}) and a very low cross-

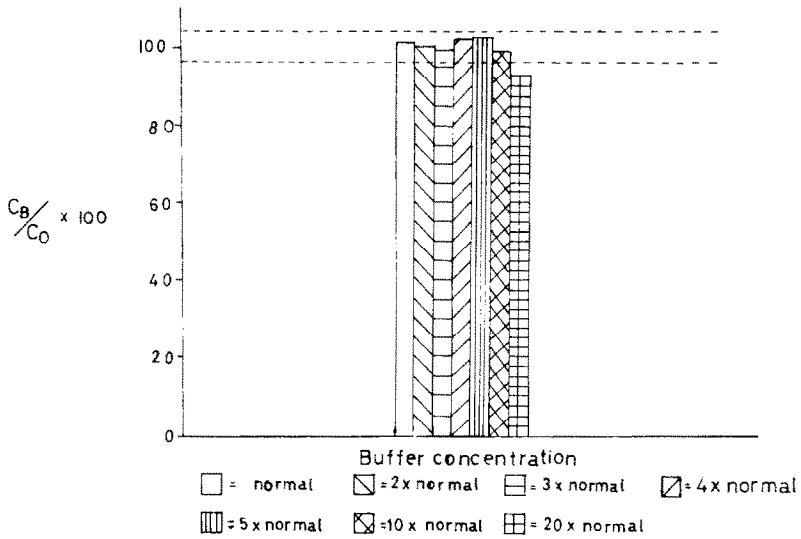

Fig. 2. The effect of increasing the buffer concentration on the binding of tritiated cyclic AMP was determined in reaction mixtures containing 100 µl binding protein, 50 µl of tritiated cyclic AMP, and 50 µl of buffer whose concentration was increased from the normal value of 50 mM Tris—4 mM EDTA, pH 7.5 to a level 20 times the normal concentration. C_0, cpm bound at zero-dose cyclic AMP in normal buffer concentration. C_B, cpm bound at zero-dose cyclic AMP in higher buffer concentration. Broken lines indicate 95% confidence limits for zero-dose binding in normal buffer.

reactivity with ATP and cyclic GMP. The addition, to the purified binding protein, of a protein kinase inhibitor fraction isolated from bovine muscle increased both the number of binding sites and the affinity for cyclic AMP ($K_a = 10^9\ M^{-1}$). Later Gilman [1] reported that the increase in total binding capacity was a non-specific effect and could be duplicated with albumin. The increase in affinity caused by the inhibitor preparations was presumed to be specific but the possible effect of albumin on the association constant was not investigated.

We have shown that the increase in binding capacity caused by the addition of bovine serum albumin (BSA) is dependent upon the concentration of albumin present. Maximal activation was achieved with a BSA concentration in the incubation mixture of 0.05%. No further effect was observed on increasing the concentration of BSA from 0.05% to 2%. Double reciprocal plots of bound cyclic AMP against free cyclic AMP were used to calculate the association constants and the numbers of available binding sites both in the absence and in the presence of BSA. Straight line plots were obtained and the results, shown in Fig. 3, indicate that maximal activation by 0.05% BSA resulted both in an increase in the association constant from $0.4 \cdot 10^9\ M^{-1}$ to $1.0 \cdot 10^9\ M^{-1}$, and in a 60% increase in the number of available binding sites. Thus we have shown that the addition of BSA gave an increase in the association constant identical to that obtained with the protein kinase inhibitor. This increase, previously presumed to be a specific effect [1] can, therefore, also be duplicated non-specifically with BSA. There have been some indications, although never investigated in detail, that similar effects are possible with other purified binding proteins [15].

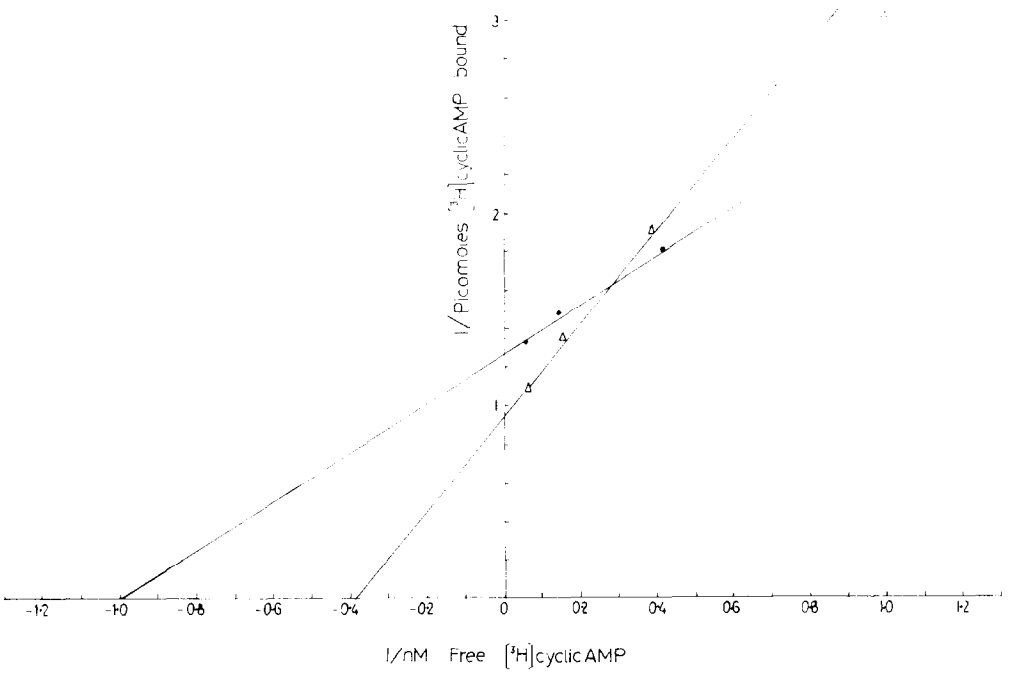

Fig. 3. Determination of the association constant for cyclic AMP at pH 7.5. ○, without BSA; binding protein concentration 15 μg/200 μl. ●, with 0.05% BSA; binding protein concentration 7.5 μg/200 μl.

Our results show that the fully activated binding is insensitive to large increases in the protein content of incubation mixtures. Failure to use fully activated binding proteins could contribute markedly to the interference noted in other assays [5—7,10] by proteins present in assay samples.

(4) Choice of optimum method of separation of bound and free cyclic AMP

An ideal method should give a clean separation and be free from interference by materials likely to be present in extracts. It should also be simple, reproducible, rapid and cheap. Because of the difficulty of accurately counting tritium-labelled compounds in heterogeneous solution, it is desirable to use a technique which gives a soluble fraction for counting to avoid the need for tedious or expensive solubilization procedures.

These requirements ruled out many separation methods: membrane filtration methods are tedious and expensive, require solubilization before counting and are reported to be subject to interference by EDTA [7,12] and by proteins [16]. Salt precipitation methods are greatly influenced by protein concentration, and the presence of salts causes counting problems. Organic solvent precipitation methods suffer similar defects and, in our hands, caused excessive dissociation of the protein—cyclic AMP complex. Ion-exchange methods are affected by salts, give incomplete uptake of cyclic AMP, and suffer interference from plasma extracts [7].

Only charcoal (centrifugation) and Sephadex (column) separation methods satisfied most requirements. A charcoal separation method was chosen because it allowed the simpler centrifugation separation step.

Charcoal separation methods are very effective for the separation of low molecular weight ligands such as cyclic AMP. It is, however, important to use the correct amount and grade of charcoal [1,17]; failure to do so will result in poor performance [7,8]. Optimal conditions must be determined for maximal adsorption of free ligand and minimal adsorption of bound ligand by the adsorbent.

With charcoal adsorbents it is relatively easy to get complete adsorption of low molecular weight ligands but it is less easy to get 100% exclusion of bound ligand. Adsorption of bound ligand can be reduced, but not eliminated by adding dextran or protein to the separation mixture [18]. Our results have shown that the optimum amounts of charcoal and bovine serum albumin used in the assay (see Methods) resulted in the adsorption of more than 99% of the free cyclic AMP and the exclusion of 90% of the bound cyclic AMP. The comparative freedom of the assay from non-specific interference by a wide range of substances (see section B) indicates that the efficiency of separation of bound and free cyclic AMP is constant over a wide range of environmental conditions.

(B) Specificity of the assay

Specificity has been defined [3] as the extent of freedom from interference by substances other than the one intended to be measured. Interference in cyclic AMP assays can be expected from: other nucleotides which cross-react with the binding protein; other proteins present in the biological extract; phosphodiesterase inhibitors; bivalent metal ions added in some extraction procedures and to activate adenylate cyclase.

The effects on the cyclic AMP assay of large variations in the concentrations of these potentially interfering factors have been determined:

(1) Interference by other nucleotides

The naturally occurring nucleotides most likely to interfere in cyclic AMP assays are cyclic GMP and ATP. Fig. 4 shows the cross-reactivity of cyclic GMP and ATP in the assay.

Approximately 200 times as much cyclic GMP as cyclic AMP is needed to give an equivalent reduction in the binding of tritiated cyclic AMP. Consequently cyclic GMP is unlikely to interfere in assays at concentrations reported in normal tissues.

ATP does not interfere in this assay at concentrations up to 5 mM which represents an approximately million-fold excess over the tritiated cyclic AMP. This freedom from interference from ATP is significantly greater than that reported in other assays [1,9—11]. Gilman [1,11], for example, reported 50% inhibition of binding by 1 mM ATP.

(2) Interference by other proteins

Interference by proteins and unidentified non-dialysable substances present in biological extracts is a common source of trouble in cyclic AMP assays.

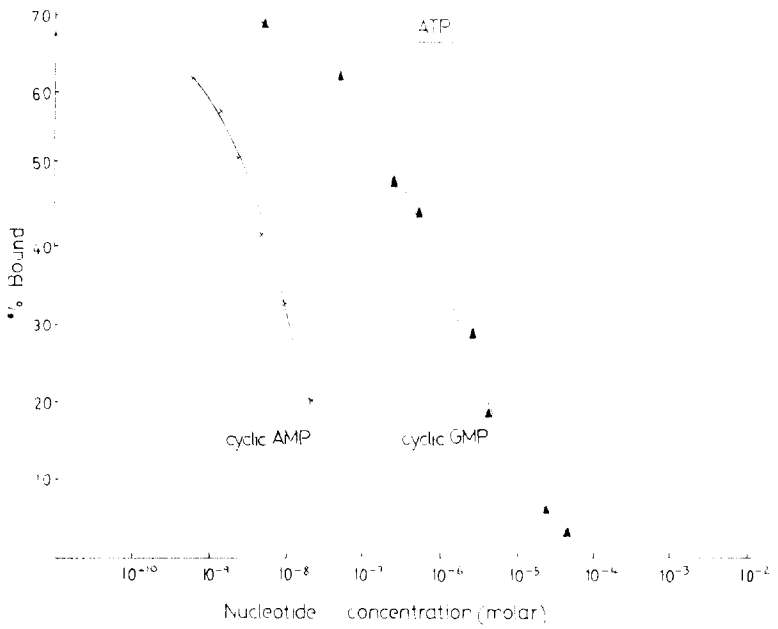

Fig. 4. Cross-reactivity of various nucleotides with cyclic AMP binding protein in the presence of $4.5 \cdot 10^{-9}$ M tritiated cyclic AMP.

The results discussed previously (section A, part 3) indicate that provided sufficient BSA is added to fully activate the binding protein, neither the binding of cyclic AMP nor the separation procedure are affected by large increases in BSA concentration. Further evidence of the freedom from interference by proteins and other non-dialysable substances is given in Tables III and IV.

TABLE III

THE VALIDITY OF DIRECT MEASUREMENTS OF CYCLIC AMP IN PLASMA

The results are means ± S.D. (n = 3)

Assay sample	Volume	pmoles cyclic AMP		
		Added	Measured in assay sample	Per ml plasma
Plasma*	50 µl		1.06 (± 0.12)	21.2
Plasma**	50 µl	—	1.00 (± 0.09)	20.0
Plasma**	25 µl	—	0.45 (± 0.05)	18.0
Plasma**	25 µl	0.50	1.00 (± 0.12)	20.0
Cyclic AMP-free plasma***	50 µl	—	< 0.03	< 0.6

* Prepared from blood containing 5 units of heparin/ml as anti-coagulant and 2 mg theophylline/ml as phosphodiesterase inhibitor.

** Prepared from blood containing EDTA as anti-coagulant and phosphodiesterase inhibitor.

*** Prepared by incubating plasma, prepared from blood containing 5 units of heparin/ml, for 20 h at 30°.

TABLE IV

THE VALIDITY OF DETERMINATIONS OF CYCLIC AMP IN OX-BRAIN EXTRACT

The results are means ± S.D. (n = 8).

Assay sample	Volume	pmoles cyclic AMP			
		Added	Measured	Calculated	Per ml original extract
5.6-fold diluted extract	50 µl	—	9.25 ± 0.93	—	1036 ± 104
11.2-fold diluted extract	50 µl	—	4.57 ± 0.25	—	1024 ± 56
11.2-fold diluted extract	25 µl	4.0	6.20 ± 0.60	6.29	—
Cyclic AMP-free extract* diluted 5,6-fold	50 µl	—	0.066 ± 0.002	—	7.4 ± 0.2

* Prepared by treating 1 ml of brain extract with 10 mg of Norit GSX charcoal.

(3) Interference from phosphodiesterase inhibitors

EDTA and theophylline are commonly used as phosphodiesterase inhibitors. Fig. 2 shows that there is no interference in assays from EDTA at concentrations up to 40 mM. Other experiments have shown that there is no interference from theophylline at concentrations up to 32 mM in the assay samples. These concentrations are much larger than those which might be expected in normal assay samples.

(4) Interference from bivalent metal ions

Bivalent metal ions, such as Ca^{2+}, Zn^{2+} and Mg^{2+}, cause interference in some cyclic AMP assays [14]. The effects of these cations on the binding of cyclic AMP are shown in Fig. 5.

Concentrations of up to 80 mM Ca^{2+} in the 50 µl assay sample (added to a 200 µl final volume incubation mixture) caused no decrease in binding. Similarly concentrations of up to 20 mM Zn^{2+} caused no decrease in binding. Concentrations of Zn^{2+} greater than 20 mM precipitated in the assay buffer and their effect could not be studied. Interference from Mg^{2+} became significant (> 2 standard deviations) at 10 mM and increased steadily with increasing concentration causing 25% inhibition at 80 mM.

Magnesium ions are most likely to be present when the assay is used to measure adenylate cyclase activity [10,19] and it is unlikely that Mg^{2+} concentrations as high as 10 mM will be present in such samples which are normally diluted considerably before cyclic AMP assay.

Interference by bivalent cations in other assays has been shown to occur in the separation step [14]. The marked freedom from interference in our assay results in part from the presence of EDTA in the reaction mixture and in part from the use of optimal proportions of charcoal and BSA in the separation procedure. In addition we noted that calcium and zinc ions form sparingly soluble complexes with the assay buffer and this might help to minimise metal ion interference in the assay.

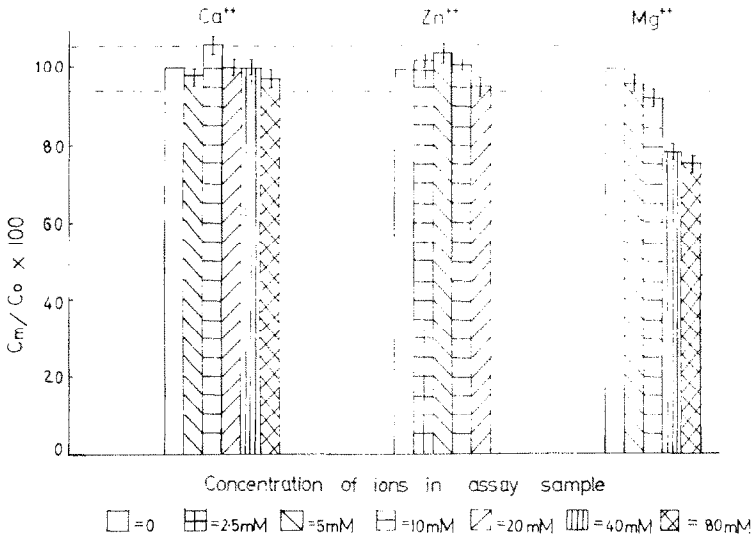

Fig. 5. The effect of bivalent metal ions on the binding of tritiated cyclic AMP at zero-dose was determined in reaction mixtures containing 100 µl binding protein, 50 µl tritiated cyclic AMP and 50 µl of solutions of the various metal ions, at the concentrations indicated, in assay buffer. C_m, cpm bound in the presence of metal ions; C_0, cpm bound in the absence of metal ions. Broken lines indicate the 95% confidence limits for the binding in the absence of metal ions.

(5) Interference from high salt and high buffer concentrations

High salt and buffer concentrations usually result when samples are concentrated before assay. Fig. 6 shows that the assay is insensitive to very large increases in salt and buffer concentrations.

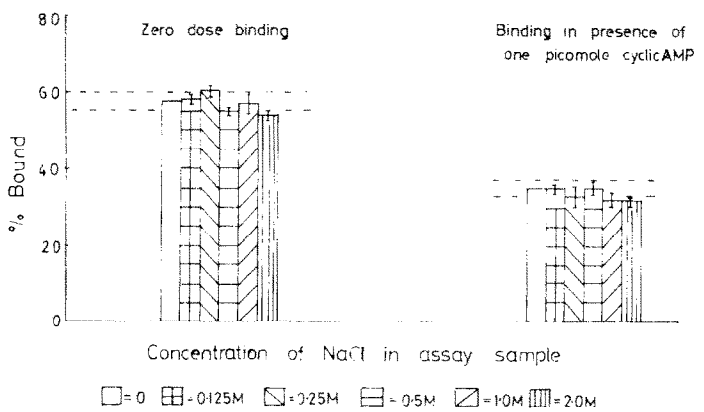

Fig. 6. The effect of NaCl concentration on cyclic AMP binding; measured at zero-dose and in the presence of 1 pmole cyclic AMP. The effect of increasing the NaCl concentration at zero-dose cyclic AMP was determined by measuring the percentage of cyclic AMP bound in reaction mixtures containing 100 µl binding protein, 50 µl tritiated cyclic AMP, and 50 µl of NaCl at the concentration indicated. For experiments in the presence of 1 pmole cyclic AMP the 50 µl aliquots of NaCl solutions were supplemented with 1 pmole unlabelled cyclic AMP. Broken lines indicate 95% confidence limits for binding in the absence of NaCl.

(6) Interference by fluoride ion

Many extracts contain fluoride ions. Potassium fluoride in assay samples at concentrations up to 80 mM causes no significant (> 2 standard deviations) change in zero-dose binding in the cyclic AMP assay.

(C) the assay of cyclic AMP in urine, plasma and tissue extracts

(1) Cyclic AMP in urine

Table II shows that, in assays of cyclic AMP in urine carried out on the same sample on six separate occasions over a period of seven weeks, a 1 : 50 dilution gave a value of 7.00 (± 0.52) μmoles/l and a 1 : 100 dilution gave a value of 6.80 (± 0.28) μmoles/l. Treatment of an undiluted urine sample with charcoal to remove cyclic AMP reduced the cyclic AMP content of 50 μl of the undiluted sample to zero (within the standard deviation of the zero-dose binding).

(2) Cyclic AMP in plasma

No previously published assay has permitted the direct assay of plasma cyclic AMP levels. The high specificity of the present assay has permitted a simple and rapid direct assay of plasma cyclic AMP.

Plasma contains a high level of phosphodiesterase activity which must be completely inhibited immediately on sampling to prevent significant degradation of cyclic AMP. Plasma is usually collected in heparinized tubes to which the poorly soluble phosphodiesterase inhibitor theophylline is added. This procedure has been simplified by using EDTA which acts both as an anti-coagulant and as a phosphodiesterase inhibitor. Freshly collected blood is added to a tube containing 1% of its volume of 0.5 M EDTA pH 7.5, mixed and centrifuged. Table III shows that cyclic AMP levels in plasma treated in this way are identical to those in heparinized plasma containing theophylline. The high activity of phosphodiesterase in plasma was demonstrated by incubation of heparinized plasma containing no inhibitor for 20 h at 30°. This resulted in a decrease in measured cyclic AMP from 20 pmoles/ml to < 0.6 pmoles/ml. Incubation of the EDTA/plasma for 20 h at 30° showed no decrease in cyclic AMP content indicating that the added EDTA was sufficient to cause complete inhibition of phosphodiesterase.

The validity of the direct assay of plasma cyclic AMP levels was demonstrated by showing that the result was independent of sample size; by using internal standards; and by showing that the addition of cyclic AMP-free plasma had no effect on the assay. The results are given in Table III.

It is of interest to note that this simple procedure for the preparation of plasma cannot be used in many cyclic AMP assays because EDTA interferes both with membrane filtration techniques [7,12] and with the binding of antibody to cyclic AMP in radioimmunoassays of cyclic AMP [13].

(3) Cyclic AMP in extracts of ox brain

Many tissue extracts have been reported to contain unknown materials which interfere with saturation analyses. Weller et al. [10] have reported that extracts of ox brain contained material which enhanced cyclic AMP binding at

low concentrations of extract and which caused inhibition at higher concentrations. We have used such an extract (a kind gift from Dr M. Weller, Institute of Psychiatry, London) and shown that a simple direct assay is possible with the present assay (see Table IV). The increase in binding at low concentrations of extract noted by Weller et al. [10] may be explained as being due to activation of the binding protein by non-dialysable material in the crude extract; the apparent inhibition of binding at high concentrations of extract may be explained as being due to interference by high molecular weight impurities in the membrane filtration step [16].

Failure of these extracts to cause interference in the present assay would then be explained by the use of a fully activated binding protein and a carefully designed charcoal separation procedure which, unlike membrane filtration methods, is insensitive to large changes in protein concentration.

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