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Factors Affecting the Binding of [^{3}H]Adenosine 3′:5′-Cyclic Monophosphate to Protein Kinase from Bovine Adrenal Cortex

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Inorganic salts, several proteins and traces of protein precipitants were tested to find out by what mechanisms they modulate the binding of cyclic [^{3}H]AMP to protein kinase (ATP–protein phosphotransferase; EC 2.7.1.37). The separation of free and bound cyclic AMP by $(NH_4)_2SO_4$ precipitation was unaffected by the above agents and was more reliable than the Millipore filtration technique. Several binding sites for cyclic AMP were revealed in adrenal-cortex extract. When this extract was used as binding reagent in an assay for cyclic AMP, the standard curve was distorted in the presence of KCl because the salt affected the different binding sites to a varying extent. At high ionic strength the protein kinase isoenzyme I dissociated and showed an extraordinarily high affinity for cyclic AMP. Trichloroacetate and perchlorate at very low concentrations were able to dissociate the protein kinase and modulate its binding characteristics as well. A progressive decrease in the cyclic AMP-binding capacity occurred on prolonged incubations. The binding protein was protected against inactivation by 2-mercaptoethanol, EDTA and several proteins. It was more resistant to denaturation when complexed to cyclic AMP. The enhancement of cyclic AMP binding by bovine serum albumin was investigated in some detail and appeared to be a pure stabilizing effect. It is proposed that the competitive-binding assays for cyclic AMP based on protein kinase be conducted at high ionic strength and in the presence of stabilizers (protein, EDTA, 2-mercaptoethanol). The interference from agents that may dissociate the protein kinase or influence its stability will thus be decreased.

The cyclic AMP-dependent protein kinases have subunits whose specificity and high affinity for cyclic AMP make these enzymes useful as binding reagents in competitive-binding assays for this nucleotide. The binding reagent is minimally purified tissue extracts (Brown *et al.*, 1971; Ichii, 1972; Tsang *et al.*, 1972; Rabinowitz & Katz, 1973; Cooper *et al.*, 1972), the isoenzyme form of protein kinase eluted from DEAE-cellulose at low ionic strength (Brostrom & Kon, 1974; Tovey *et al.*, 1974) or the protein kinase isoenzyme eluted at higher ionic strength (Gilman, 1970; Walton & Garren, 1970; Sanborn *et al.*, 1973). Bound and free isotope is most often separated by membrane filtration (Gilman, 1970; Walton & Garren, 1970; Ichii, 1972; Sanborn *et al.*, 1973; Gilman & Murad, 1974; Gill & Walton, 1974) or dextran-coated charcoal (Brown *et al.*, 1971; Tovey *et al.*, 1974).

The usefulness of these assays is limited mainly because of the interference by inorganic salts (Brostrom & Kon, 1974; Albano *et al.*, 1974; Sheppard & Tsien, 1975), proteins (Murad & Gilman, 1971; Tsang *et al.*, 1972; Weller *et al.*, 1972; Brostrom & Kon, 1974; Barling *et al.*, 1974) or traces of protein precipitants (Albano *et al.*, 1974; Wright & Price, 1975).

In the present study the mechanisms by which added proteins, salt and protein precipitants may influence the cyclic AMP binding were investigated. Some methods for the separation of bound and free nucleotide were compared. Crude adrenal-cortex extract and protein kinase isoenzyme I from the same tissue were compared as binding reagents. Optimum incubation conditions for the latter binding protein will be described.

Experimental

Reagents

Crystallized bovine serum albumin, bovine haemoglobin (type I), calf thymus histone (type II), EGTA, ATP (disodium salt), cyclic AMP (free acid) and other nucleoside derivatives were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Human γ-globulin was from KABI AB, Stockholm 30, Sweden. Cyclic [^{3}H]AMP (27 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. [γ-^{32}P]ATP (approx. 10 Ci/mmol) was prepared by the method of Glynn & Chappell (1964). Sucrose and 2-mercaptoethanol were from the British Drug House, Poole, Dorset, U.K. Sodium dodecyl sulphate

and Unisolve were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Other chemicals, all of analytical grade, were obtained from Merck, Darmstadt, Germany. DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 (medium grade) was from Pharmacia, Uppsala, Sweden. Polyethyleneimine–cellulose thin-layer sheets (Polygram Cel 300, PEI) were purchased from Macherey, Nagel and Co., Düren, Germany. Millipore filters (HAWP 0.45 μm) of 25mm diameter were obtained from Millipore Corp., Bedford, MA, U.S.A.

Protein kinase activity

This was measured essentially as described by Ueland & Døskeland (1976). Incubations were carried out for 10min at 30°C in 15mM-Hepes*/NaOH, pH7.0, containing 0.3mM-EGTA, 0.1mM-EDTA, 10mM-magnesium acetate, 30μM-[γ-^{32}P]ATP (1μCi/ml) and 0.67mg of histone/ml. The protein kinase activity ratio is defined as the activity obtained in the absence of cyclic AMP divided by the activity in the presence of 2μM-cyclic AMP.

Measurement of cyclic [^{3}H]AMP binding

Except where otherwise indicated in the text, the binding protein was incubated at 0°C with cyclic [^{3}H]AMP in 50mM-Hepes/NaOH, pH7.2 (adjusted at 0°C) containing 800mM-NaCl, 30mM-EDTA, 20mM-2-mercaptoethanol and 1mg of bovine serum albumin/ml. The incubation was left for 2½h, and was stopped by mixing 400μl of incubation mixture with 3.6ml of ice-cold 80%-satd. $(NH_4)_2SO_4$ (adjusted to pH7.2 with NH_3). The resulting precipitate was collected on Millipore filters and rinsed with 3×3ml of ice-cold 65%-satd. $(NH_4)_2SO_4$. The filters were transferred to scintillation vials containing 1ml of aq. 2% (w/v) sodium dodecyl sulphate, the vials well shaken, and 7ml of scintillation fluid (Unisolve) was added.

Assay for cyclic AMP phosphodiesterase (EC 3.1.4.17) activities

Samples to be tested were incubated at 30°C in the presence of 10mM-magnesium acetate and 0.5μM- or 50μM-cyclic [^{3}H]AMP. After 1h the reaction was stopped by heating. A portion of the supernatant, obtained by centrifuging the heated reaction mixture, was spotted on to a polyethyleneimine–cellulose sheet, together with unlabelled AMP, cyclic AMP and adenosine. Separation was by ascending chromatography with 0.5M-ammonium acetate/96% ethanol (5:2, v/v) as the solvent. The positions of radioactive substances were determined by liquid-scintillation counting of the powder scraped off from different areas on the plate.

Preparation of the heat-stable inhibitor protein

This was as described by Walsh *et al.* (1971), except that the protein was fractionated with 20–40%-satd. $(NH_4)_2SO_4$ after the precipitation with trichloroacetic acid.

Preparation of adrenal-cortex extract

This was exactly as described by Brown *et al.* (1971). To obtain cytosol the homogenization (1:5, w/v) was in 10mM-Tris/HCl, pH7.5, containing 4mM-EDTA, 10mM-2-mercaptoethanol and 0.25M-sucrose. The high-speed (20000 $g_{av.}$, 20min) supernatant of this homogenate was centrifuged for 1h at 100000 $g_{av.}$, and is referred to as bovine adrenal-cortex cytosol.

Preparation of protein kinase

The cytosol was passed through a column (2.6cm×40cm) that had been packed with Sephadex G-25 and equilibrated with 10mM-Tris/HCl, pH7.5, with 4mM-EDTA and 10mM-2-mercaptoethanol. The desalted eluate was then applied to a DEAE-cellulose column (2.4cm×30cm) equilibrated with the same buffer. After washing with 2litres of starting buffer, an 800ml gradient of NaCl (0–0.08M) was started. A single peak of concomitant protein kinase and cyclic AMP-binding activities was eluted between 0.01M- and 0.025M-NaCl. The peak fractions were pooled and designated protein kinase isoenzyme I. The other major peak of protein kinase could be eluted (between 0.15M- and 0.25M-NaCl) with a second NaCl gradient (0.08–0.3M). Only the protein kinase I was used in the following experiments.

The pooled fractions of protein kinase were precipitated with 55%-satd. $(NH_4)_2SO_4$ and kept as the precipitate in liquid N_2. Before use, the precipitate was dissolved in a minimal volume of 15mM-Hepes/NaOH, pH7.2, and desalted on a Sephadex G-25 column equilibrated with the same buffer. The preparation did not contain phosphodiesterase activity.

Results

Separation of bound and free cyclic [^{3}H]AMP by $(NH_4)_2SO_4$ precipitation

The bulk of the cyclic AMP-binding activity associated with protein kinase I precipitated between 35%- and 55%-satd. $(NH_4)_2SO_4$ (Fig. 1*a*). In a similarly designed experiment all the cyclic AMP-binding activity of crude adrenal-cortex extract was

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

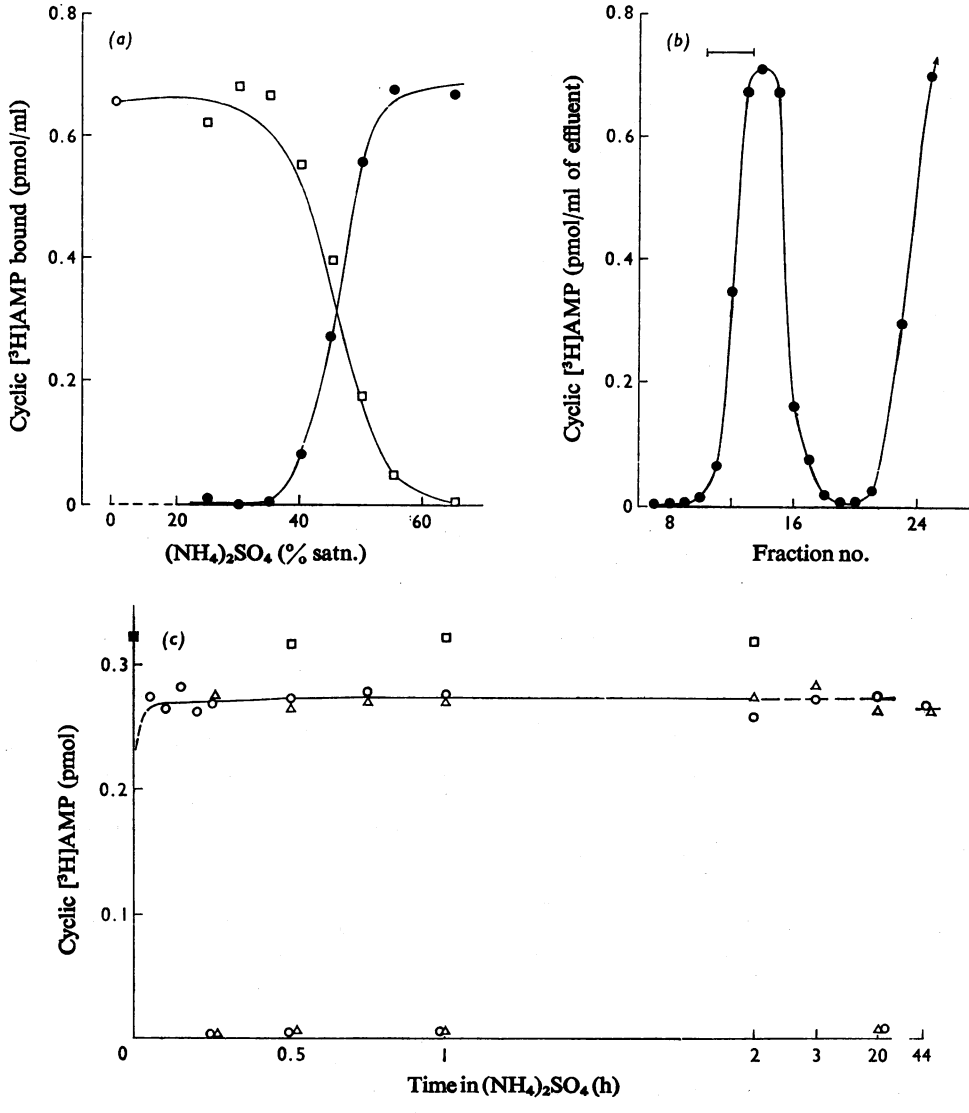

Fig. 1. *Separation of free and protein-bound cyclic AMP by $(NH_4)_2SO_4$ precipitation*

(*a*) To 5ml portions of the pooled DEAE-cellulose fractions containing protein kinase I was added enough 95%-satd. $(NH_4)_2SO_4$, pH7.2, to give the final concentrations of $(NH_4)_2SO_4$ indicated on the abscissa. After 1h at 0°C the precipitates were pelleted by spinning for 20min at $15000g_{av.}$. Portions (100μl) from the pellets (redissolved in 5ml of the original buffer) and from the supernatants were assayed for binding activity in the presence of 20nM-cyclic [^{3}H]AMP. The numbers of binding sites in the pellets (●) and the supernatants (□) are given per ml of the original (5ml portions) protein kinase preparation. (*b*) Protein kinase I (500μl) incubated with 50mM-Hepes/NaOH, pH7.2, 800mM-NaCl, 30mM-EDTA, 20mM-2-mercaptoethanol, 1mg of albumin/ml and 20nM-cyclic [^{3}H]AMP was applied to a column (0.9cm×30cm) of Sephadex G-25. Fractions (500μl) were collected, and the radioactivity was determined in 100μl portions from each fraction. (*c*) The same column of Sephadex G-25 was run exactly as above; the eluate corresponding to fractions 11–14 in (*b*) was pooled, and 400μl portions were either taken directly into scintillation vials (■) or mixed with 80%-satd. $(NH_4)_2SO_4$ and filtered after various periods of time (□). In another experiment protein kinase I was incubated in the buffer described in (*b*) with 1.5nM-cyclic [^{3}H]AMP, and 400μl portions were mixed with 3.6ml of 80%-satd. $(NH_4)_2SO_4$ supplemented with unlabelled 0.2mM-cyclic AMP (△) or without nucleotide (○). After the time-periods indicated, the mixtures were filtered with suction. The lower unconnected symbols show the radioactivity measured in the absence of binding protein.

Table 1. *Binding of cyclic [^{3}H]AMP to protein kinase I during $(NH_4)_2SO_4$ precipitation*

The preparation of protein kinase I was diluted with buffer (15mM-Hepes/NaOH, pH7.2, 30mM-EDTA, 20mM-2-mercaptoethanol and 1mg of albumin/ml) to give a binding capacity (measured at saturation) of 0.8pmol of cyclic [^{3}H]AMP/200μl. Samples (200μl) of this preparation (or buffer alone to obtain backgrounds) were pipetted into tubes with 1.8ml of ice-cold 80%-satd. $(NH_4)_2SO_4$ containing the concentrations of cyclic [^{3}H]AMP indicated in the left column. The tubes were then quickly capped and their contents mixed by repeated inversions. After 2h at 0°C the precipitates were filtered and their radioactivity was measured.

Concentration of cyclic [^{3}H]AMP in the $(NH_4)_2SO_4$ solution (nM)	Cyclic [^{3}H]AMP retained without binding protein (background) (fmol)	Amount of cyclic [^{3}H]AMP retained in the presence of binding protein		Cyclic [^{3}H]AMP retained in the presence of binding protein and unlabelled 0.2mM-cyclic AMP (fmol)
		(fmol)	(% of total binding capacity)	
0.05	0.1	0.2	—	0.1
0.1	0.2	0.5	—	0.1
0.2	0.6	0.9	0.005	0.2
0.4	0.6	1.9	0.2	0.3
0.8	0.8	4.2	0.4	0.6
1.6	1.5	8.7	0.9	1.4

precipitated with 65%-satd. $(NH_4)_2SO_4$. The final concentration of $(NH_4)_2SO_4$ was therefore kept above 65% saturation when the cyclic [^{3}H]AMP bound to receptor protein was precipitated with $(NH_4)_2SO_4$.

A few minutes after the addition of $(NH_4)_2SO_4$, the complex between cyclic [^{3}H]AMP and binding protein had formed a precipitate, which was completely retained by the membrane filters (Fig. 1*c*). The amount of cyclic [^{3}H]AMP retained was not decreased after 48h in $(NH_4)_2SO_4$ with a 10000-fold excess of unlabelled nucleotide (Fig. 1*c*).

Free cyclic [^{3}H]AMP was separated from that bound to protein kinase by Sephadex G-25 chromatography (Fig. 1*b*). The samples from the excluded fraction which had been precipitated in $(NH_4)_2SO_4$ contained as much radioactivity as those taken directly into scintillation vials (Fig. 1*c*). The $(NH_4)_2SO_4$ precipitation method thus allowed a complete recovery of the cyclic [^{3}H]AMP bound to protein kinase I.

The amount of cyclic [^{3}H]AMP retained by the filters in the absence of binding protein was about 0.3% of that applied to the filters. This proportionality between background and amount of added radioactivity was observed over a wide range of isotope concentrations and was unaffected by the presence of a large excess of unlabelled cyclic AMP.

As shown in Table 1, binding of cyclic [^{3}H]AMP can occur to a small extent during $(NH_4)_2SO_4$ precipitation. This was completely avoided by adding excess unlabelled cyclic AMP. Precipitation was therefore carried out in the presence of unlabelled 100μM-cyclic AMP if the concentration of labelled nucleotide was expected to be above 1nM during the precipitation step.

Filtration on Sephadex G-25 and the present $(NH_4)_2SO_4$ precipitation method gave similar recoveries of bound cyclic [^{3}H]AMP and were highly reproducible under all the conditions tested. Less of the bound isotope was recovered on the membrane filters when the binding protein was in solution before filtration (Table 2).

The adsorption of protein to the membrane filters commonly used is facilitated by Mg^{2+} (Kihara & Kuno, 1968; Gill & Walton, 1974; Nakamura & Pisano, 1976), and only a limited amount of protein can be adsorbed (Gilman, 1970; Walton & Garren, 1970; Gill & Walton, 1974). The inclusion of EDTA and relatively high concentrations of protein, found to be optimal for preservation of the binding capacity in the present study, is therefore not readily compatible with the use of membrane filters to recover bound nucleotide.

The separation of bound and free cyclic [^{3}H]AMP by $(NH_4)_2SO_4$ precipitation was not affected by the presence of proteins (provided the amount of precipitate was not so high that the membrane filters were obstructed), traces of neutralized trichloroacetate or perchlorate, EDTA, 2-mercaptoethanol, Mg^{2+}, Ca^{2+}, several heavy metals in mM concentrations, and up to 200mM-NaCl or -KCl. When the protein concentration of the samples was known to be low, carrier protein (serum albumin or γ-globulin) was added to either the $(NH_4)_2SO_4$ solution or the sample immediately before the mixing of the two.

Characteristics of cyclic [^{3}H]AMP binding by adrenal-cortex extract

The cyclic AMP assay of Brown *et al.* (1971) has been reported to be useful also when bound and free radioactivity is separated with membrane filters (Tsang *et al.*, 1972; Ichii, 1972; Albano *et al.*, 1974) or by $(NH_4)_2SO_4$ precipitation (Rabinowitz & Katz, 1973). The standard curve for this assay by $(NH_4)_2SO_4$ precipitation was plotted in the conventional manner (Fig. 2*a*) and as

Table 2. *Influence of separation method on the estimation of cyclic [^{3}H]AMP bound to protein kinase I*

Samples (500μl) of incubation mixture I (15mM-Hepes/NaOH, pH7.2, 800mM-NaCl, 20mM-2-mercaptoethanol, 0.1mg of albumin were applied directly to the Millipore filters with suction (A), or diluted with 4.5ml of ice-cold incubation buffer (without albumin and isotope) (B) or 15mM-Hepes/NaOH, pH7.2, with 30mM-$MgCl_2$ (C), left for 1min, and then applied to the filters. Filters A and B were washed with 3×3ml of incubation buffer (without albumin and isotope), and filter C with 15mM-Hepes/NaOH, 30mM-$MgCl_2$. Samples were also precipitated in 10 times their own volume (F) of 80%-satd. $(NH_4)_2SO_4$, filtered 30min later, and washed with 3×3ml of 65%-satd. $(NH_4)_2SO_4$. Samples (100μl) of incubation mixture II (50mM-Hepes/NaOH, pH7.2, 800mM-NaCl, 30mM-EDTA, 20mM-2-mercaptoethanol, 1mg of albumin/ml, 2mg of bovine haemoglobin/ml, 3nM-cyclic [^{3}H]AMP) were either applied directly to the filters (A) and washed with incubation buffer (without proteins and isotope) or diluted with 1.9ml of ice-cold 20mM-potassium phosphate, pH6.0, (D) and the filters washed with the same buffer. Portions were also precipitated in 10 times (F) or 50 times (G) their own volumes of 80%-satd. $(NH_4)_2SO_4$, and the filters washed with 65%-satd. $(NH_4)_2SO_4$ or with 20mM-potassium phosphate, pH6.0, (E). Samples (100μl) of the same incubation mixture were also chromatographed on Sephadex G-25 columns (0.8cm×4cm) (H) equilibrated with incubation buffer (without proteins and isotope). The excluded fractions were detected by their content of haemoglobin, and could be collected quantitatively in 500μl of eluate. The eluates were transferred to scintillation vials containing 10ml of Unisolve, and counted in a Nuclear–Chicago Isocap-300 liquid-scintillation counter. The results given are the means for duplicate determinations, except in two cases where the means for 14 determinations are given together with the standard deviations. The counting efficiency for the samples precipitated with $(NH_4)_2SO_4$ and those eluted from Sephadex G-25 was determined by adding 50μl of 0.16μM-cyclic [^{3}H]AMP to the respective vials. The data are given as pmol of cyclic AMP bound/ml of incubation mixture and are corrected for the slight difference in counting efficiency between the two groups. The counting efficiency for cyclic [^{3}H]AMP with the $(NH_4)_2SO_4$ precipitation method was about 33%.

Incubation medium	Membrane filtration of non-precipitated protein				Membrane filtration of $(NH_4)_2SO_4$ precipitate			Sephadex G-25 chromatography
	A	B	C	D	E	F	G	H
I	0.12	0.09	0.12	—	—	0.74	—	—
II	0.27	—	—	0.22	0.24	0.763±0.04 (14)	0.78	0.765±0.08 (14)

described by Wunderwald *et al.* (1974) (Fig. 2*a*, inset). The inclusion of 150mM-KCl during the incubation increased the amount of cyclic [^{3}H]AMP bound, especially at low concentrations of nucleotide (Fig. 2*a*). The C_0/C_x plot (Fig. 2*a*, inset) was not linear in either the absence or presence of added salt, suggesting that more than one binding site was present (Wunderwald *et al.*, 1974). The Scatchard plot (Scatchard, 1949) also suggested the existence of several binding sites with different affinities for cyclic AMP (Fig. 2*b*). As non-linear Scatchard plots may be expected because of interaction between the protein kinase subunits (Swillens *et al.*, 1974; MacKenzie & Stellwagen, 1974), the plot proposed by Swillens *et al.* (1974) was also constructed (Fig. 2*b*, inset). The shape of this curve was different from that expected from subunit interaction (straight line according to Swillens *et al.*, 1974) or incomplete recovery of bound nucleotide (downward curvature according to Swillens & Dumont, 1975). The results were essentially the same for adrenal-cortex cytosol as for the high-speed supernatant, and when NaCl was added instead of KCl.

The existence of different binding sites for cyclic AMP in bovine adrenal-cortex cytosol was confirmed by the separation by DEAE-cellulose chromatography of several receptors which differed in their affinity for cyclic AMP (not shown). The highest affinity for cyclic AMP was in the DEAE-cellulose fractions containing protein kinase isoenzyme I (PKI). The characteristics of the cyclic AMP binding to this isoenzyme were therefore studied in more detail.

Effect of incubation conditions on the cyclic [^{3}H]AMP binding to protein kinase I

The results of preliminary experiments, where the composition of the reaction mixture and the time of incubation were varied, are summarized in Table 3. At low concentrations of cyclic [^{3}H]AMP the preparation bound considerably more nucleotide in the presence of salt. On prolonged incubations at high ionic strength there was a decrease in bound ligand. The enhancement of the binding brought about by several proteins seemed not to be specific, because bovine serum albumin as well as the heat-stable inhibitor protein (known to interact with the catalytic subunit of the protein kinase: Ashby & Walsh, 1973) and histone (thought to interact with the regulatory subunit: Krebs, 1972; Ueland & Døskeland, 1976) were efficient in this respect. Furthermore, the degree of enhancement of binding by different inhibitor preparations did not always parallel their inhibitory potency (S. O. Døskeland & P. M. Ueland, unpublished work).

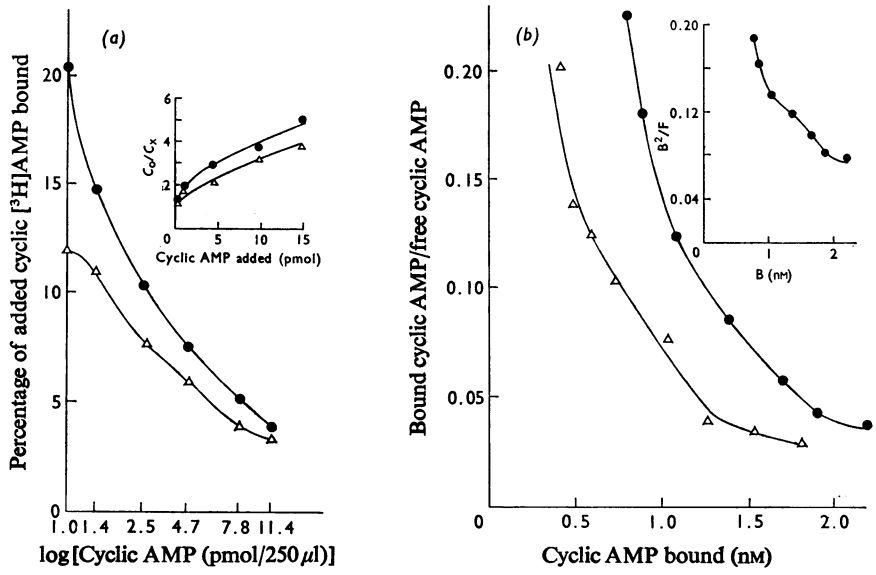

Fig. 2. *Binding of cyclic AMP to adrenal-cortex extract*

(*a*) Bovine adrenal-cortex extract was incubated at 4°C for 2h in 250µl of 50mM-Tris/HCl (pH7.4)/80mM-theophylline/6mM-2-mercaptoethanol (△) or in the same buffer supplemented with 150mM-KCl (●). The concentration of cyclic [^{3}H]AMP was 1pmol/tube (4nM), whereas the concentrations of the standards of unlabelled nucleotide ranged from 0.4 to 15pmol/tube. The standard curve for cyclic AMP was plotted as percentage of bound cyclic [^{3}H]AMP (determined as described by Brown *et al.*, 1971) against log (total cyclic AMP content per tube). The inset shows a plot of the ratio between cyclic [^{3}H]AMP bound in the absence of labelled nucleotide (C_0) and that in the presence of the different standards (C_x) versus the amount of standard added. (*b*) Bovine adrenal-cortex cytosol was desalted by passage through a Sephadex G-25 column equilibrated with 15mM-Hepes/NaOH (pH7.2)/4mM-EDTA/20mM-2-mercaptoethanol. It was then incubated for 2h at 0°C in the same buffer and various concentrations of cyclic [^{3}H]AMP either in the absence (△) or presence (●) of 150mM-KCl. The amount of bound cyclic [^{3}H]AMP (B in inset) was determined for each concentration of nucleotide. The amount of free nucleotide (F in inset) was calculated by subtraction of bound from total nucleotide. The results were plotted as described by Scatchard (1949) and (in the inset) as described by Swillens *et al.* (1974).

The results of Table 3 suggested that the binding protein was unstable at high ionic strengths and low concentrations of cyclic AMP. The first row of Table 4 showed that less nucleotide was bound if the preparation had been preincubated with salt in the absence of cyclic AMP. Vigorous mixing of the reaction mixture resulted in a loss of binding ability whether the preparation had been preincubated or not.

The inclusion of 2-mercaptoethanol, EDTA or albumin protected against inactivation of binding sites during preincubation, but only albumin protected against the loss on vortexing. The highest recovery of bound cyclic [^{3}H]AMP was observed when the protein kinase had been preincubated in the presence of 2-mercaptoethanol, EDTA and albumin (Table 4).

Effect of protein precipitants on the binding of cyclic AMP to protein kinase I

When protein kinase I was incubated at high ionic strength, the presence of neutralized trichloroacetate or perchlorate decreased the cyclic [^{3}H]AMP binding in a dose-dependent manner (Table 5, second column). The relation between amount of bound nucleotide and concentration of protein precipitant was biphasic when the incubations were carried out at low ionic strength. Increasing amounts of cyclic [^{3}H]AMP were bound up to about 10mM-trichloroacetate and 40mM-perchlorate (Table 5, first column). Above these concentrations the cyclic AMP binding declined, but to a lesser extent than in the presence of added salt (Table 5).

The ratio between cyclic AMP binding in the absence and presence of added salt increased in parallel with the protein kinase activity ratio (Fig. 3). Since much higher molar concentrations of NaCl, KCl, NaF, $MgCl_2$ and magnesium acetate are required than of trichloroacetate to activate the protein kinase (P. M. Ueland & S. O. Døskeland, unpublished work), trichloroacetate must be especially efficient in perturbing protein kinase I (Huang & Huang, 1975).

Table 3. *Effect of incubation time, KCl, inhibitor protein, histone and albumin on the amount of cyclic [^{3}H]AMP bound to protein kinase I*

The incubations were performed for 2 or 12h in 15mM-Hepes/NaOH, pH7.2, in the presence of the agents indicated in the first column. In some experiments the binding protein was preincubated for 10h in the presence of the same agents, and then enough concentrated cyclic [^{3}H]AMP was added to give 1nM- or 20nM-nucleotide during the following 2h of incubation. The results are means of duplicate determinations. Essentially the same results were obtained when NaCl was substituted for KCl.

Concentrations (final) of agents present during preincubation and incubation	Time of preincubation (h)	Time of incubation (h)	Concentration of cyclic [^{3}H]AMP present during the incubation (nM)	Cyclic [^{3}H]AMP bound (pmol/ml)
—	—	2	1	0.016
			20	0.12
—	10	2	1	0.017
			20	0.12
—	—	12	1	0.049
			20	0.31
1mg of albumin/ml	—	2	1	0.018
			20	0.13
	10	2	1	0.019
			20	0.14
	—	12	1	0.050
			20	0.34
800mM-KCl	—	2	1	0.12
			20	0.18
	10	2	1	0.074
			20	0.12
	—	12	1	0.11
			20	0.16
800mM-KCl+1mg of albumin/ml	—	2	1	0.20
			20	0.30
	10	2	1	0.13
			20	0.22
	—	12	1	0.17
			20	0.25
800mM-KCl+0.05mg of albumin/ml	—	2	1	0.18
800mM-KCl+5mg of albumin/ml	—	2	1	0.20
800mM-KCl+0.25mg of inhibitor/ml	—	2	1	0.19
800mM-KCl+0.5mg of inhibitor/ml	—	2	1	0.20
800mM-KCl+1mg of inhibitor/ml	—	2	1	0.21
800mM-KCl+2mg of inhibitor/ml	—	2	1	0.22
800mM-KCl+0.05mg of histone/ml	—	2	1	0.20
800mM-KCl+0.25mg of histone/ml	—	2	1	0.20
800mM-KCl+0.75mg of histone/ml	—	2	1	0.21

An aqueous solution of trichloroacetate (5%, w/v) and HCl (0.1M), which had been extracted with 5×3vol. of water-saturated diethyl ether and then neutralized, did not affect the binding of cyclic [^{3}H]AMP at high ionic strength, even when it occupied half of the incubation volume. Such an extraction procedure was therefore adequate to avoid interference by trichloroacetate in the presence of added salt. It should be noted, however, that very low concentrations of trichloroacetate augmented the binding of cyclic [^{3}H]AMP in the absence of added salt (Table 5, first column).

Nature of the effect of albumin on the binding of cyclic AMP to protein kinase I

The presence of albumin enhanced the high-affinity binding of cyclic AMP to protein kinase I at high ionic strength (Table 3). A similar observation has been made by Tovey *et al.* (1974), who concluded that albumin increased the affinity of protein kinase for cyclic AMP. As the free regulatory subunit of protein kinase I is known to have a very high affinity for cyclic AMP (Brostrom *et al.*, 1971; Døskeland & Ueland, 1975), albumin might possibly act by dissociating the protein kinase. The findings that

Table 4. *Test of stability of the cyclic AMP-binding capacity of isoenzyme I*

In one series of experiments (A) the binding protein was preincubated for 6h in 15mM-Hepes/NaOH, pH7.2, 800mM-NaCl and the agents indicated in the first column, and then incubated for another hour in the presence of 20nM-cyclic [^{3}H]AMP. In another series (B) the preincubation was omitted. The tube contents were either mixed by careful rocking or by vortexing for 6s on a rotamixer at maximum setting.

Concentrations (final) of agent(s) present during preincubation and incubation	Cyclic [^{3}H]AMP bound to enzyme (pmol/ml)			
	Gentle mixing		Vigorous mixing	
	A	B	A	B
—	0.17	0.37	0.12	0.22
5mM-2-Mercaptoethanol	0.31	0.41	0.20	0.22
50mM-2-Mercaptoethanol	0.30	0.42	0.19	0.22
30mM-EDTA	0.30	0.41	0.22	0.25
0.5mg of albumin/ml	0.34	0.47	0.33	0.43
0.5mg of albumin/ml+50mM-2-mercaptoethanol	0.37	0.46	0.39	0.42
0.5mg of albumin/ml+50mM-2-mercaptoethanol+30mM-EDTA	0.39	0.48	0.43	0.40
5mM-$BaCl_2$	0.21	0.37	0.14	0.16
16.5% Glycerol	0.16	0.32	0.11	0.20

Table 5. *Effect of neutralized protein precipitants on the binding of cyclic [^{3}H]AMP to protein kinase I at low or high ionic strength*

Solutions of 600mM-trichloroacetic acid or -perchloric acid were made 10mM in Tris and brought to pH7.2 (0°C) with 10M-NaOH. The binding protein was incubated in the presence of increasing concentrations of neutralized trichloroacetate or perchlorate in either 15mM-Hepes/NaOH (pH7.2)/0.1mM-EDTA/0.3mM-EGTA/20mM-2-mercaptoethanol/1mg of albumin/ml (left-hand column) or 50mM-Hepes/NaOH (pH7.2)/800mM-NaCl/30mM-EDTA/20mM-2-mercaptoethanol/1mg/ml of albumin (right-hand column).

Concentrations (final) of agents present during the incubation		Cyclic [^{3}H]AMP bound (pmol/ml of incubation mixture)	
		Absence of NaCl	800mM-NaCl and 30mM-EDTA present
—		0.071	0.51
Trichloracetate,	0.24mM	0.085	0.50
	0.48mM	0.10	0.48
	1.2 mM	0.11	0.48
	2.4 mM	0.12	0.44
	4.8 mM	0.13	0.43
	12 mM	0.13	0.37
	24 mM	0.12	0.28
	48 mM	0.12	0.21
	120 mM	0.078	0.077
Perchlorate,	2.4 mM	0.12	0.52
	7.5 mM	0.15	0.45
	24 mM	0.22	0.41
	45 mM	0.29	0.31
	112 mM	0.20	0.23

protein kinase I was not dissociated by albumin alone (Fig. 4*b*) argues against this possibility. Furthermore, serum albumin did not increase appreciably the binding of cyclic AMP in the absence of salt (Table 3).

In Fig. 5 the number of binding sites for cyclic AMP remaining in samples which had been incubated in the presence of various concentrations of cyclic [^{3}H]AMP was estimated by reincubating a portion from each sample under optimal binding conditions (in the presence of 2-mercaptoethanol, EDTA, albumin and a high concentration of labelled nucleotide). The loss of binding capacity during incubation at low concentrations of cyclic AMP appeared to be most pronounced for the samples without albumin (Fig. 5*a*, upper part). When the loss of binding sites during incubation was corrected for, the affinity of protein kinase for cyclic AMP turned out to be the same whether albumin was present or not (Fig. 5*b*). The presence of albumin

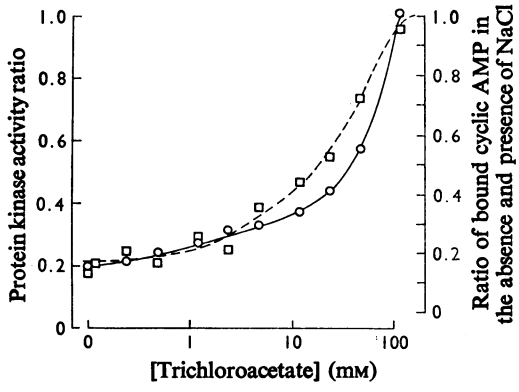

Fig. 3. *Activation of protein kinase by trichloroacetate* From the same incubation mixtures as in the first column of Table 5, 25 μl portions were mixed with 125 μl of protein kinase reaction mixture, either without added cyclic AMP or with 2 μM-cyclic AMP. The ratio between protein kinase activity in the absence and presence of 2 μM-cyclic AMP (□) is given as a function of the concentration of trichloroacetate during the first incubation. The ratio between the amount of cyclic [^{3}H]AMP bound in the absence of added salt (first column, Table 5) and in the presence of added salt (second column, Table 5) is also given (○).

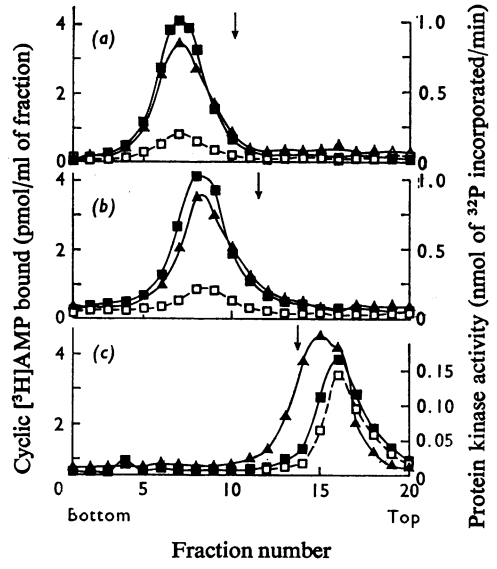

Fig. 4. *Sucrose-density-gradient centrifugation of protein kinase I in the absence and presence of salt or albumin* Samples (250 μl) containing bovine haemoglobin as a marker were layered on the top of 10ml linear gradients of 10–20% (w/v) sucrose in 15mM-Hepes/NaOH (pH7.2)/0.1mM-EDTA/0.3mM-EGTA/20mM-2-mercaptoethanol. The gradients were spun at 40000rev./min at 2°C in the 488 rotor of an International B 60 ultracentrifuge. Fractions (500 μl) were collected, and protein kinase activity was determined in the absence (□) or presence (■) of 2 μM-cyclic AMP. Samples (100 μl) were tested for cyclic [^{3}H]AMP binding activity (▲). The position of bovine haemoglobin is indicated by an arrow. (*a*) Protein kinase I (in 15mM-Hepes/NaOH, pH7.2) was layered on the gradient without preincubation. (*b*) The protein kinase had been preincubated for 12h at 0°C in the presence of 10mg of albumin/ml before centrifugation. The gradient contained 10mg of albumin/ml. (*c*) The protein kinase had been preincubated for 12h in the presence of 800mM-NaCl+30mM-EDTA. The gradient contained 800mM-NaCl.

thus did not affect the affinity of protein kinase for cyclic AMP, but rather the total number of binding sites for the nucleotide.

The question remained, however, whether albumin only protected the regulatory subunit of the protein kinase from inactivation or whether new sites were made available for cyclic AMP. In the experiment shown in Fig. 6, the amount of bound cyclic [^{3}H]AMP was monitored after addition of several agents known to enhance the binding of nucleotide. No new binding sites for cyclic AMP appeared after the addition of bovine serum albumin, human γ-globulin, 2-mercaptoethanol or EDTA. On the other hand all of these agents, especially albumin, were able to protect the binding protein against inactivation.

Discussion

The cyclic AMP-dependent protein kinase holoenzyme is assumed to be activated by dissociation into an active catalytic subunit and a regulatory subunit with binding sites for cyclic AMP (Gill & Garren, 1971; Krebs, 1972).

Substrate phosphorylation and cyclic [^{3}H]AMP binding have been measured simultaneously under various incubation conditions for protein kinase, and a close correlation was found between the degree of activation of the protein kinase and the amount of cyclic [^{3}H]AMP bound (Ueland & Døskeland, 1976). Several results obtained in the present study indicate a correlation between the activation of the protein kinase and an increased affinity for cyclic AMP. Thus high concentrations of salt dissociated the protein kinase isoenzyme I (Fig. 4*c*) and also increased its binding of cyclic [^{3}H]AMP at low nucleotide concentrations (Table 3). Small amounts of neutralized trichloroacetate or perchlorate enhanced the binding of cyclic [^{3}H]AMP (Table 5). This enhancement could be related to activation of protein kinase (Fig. 3). No enhancement was

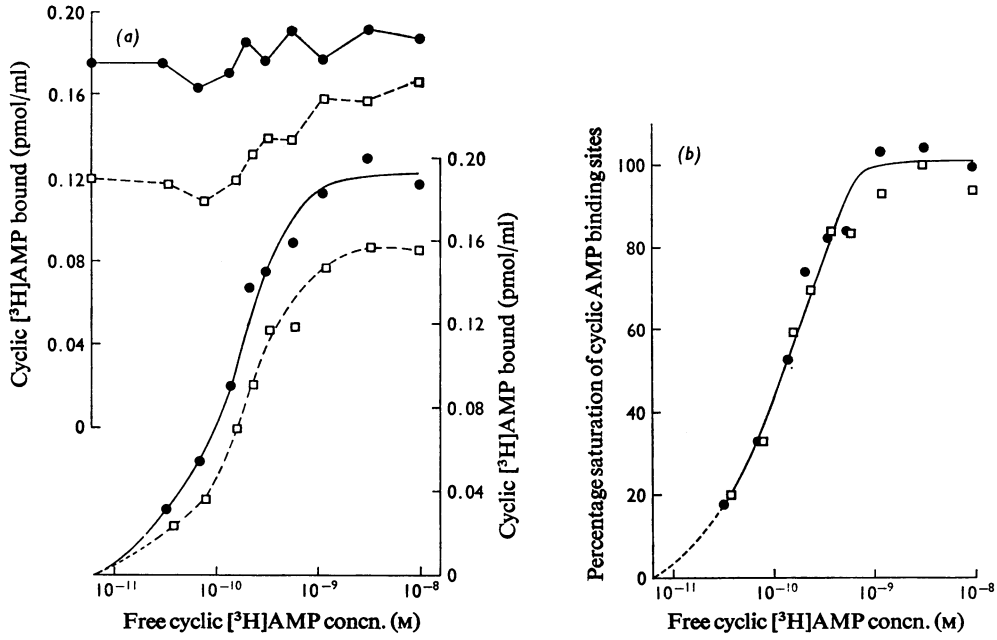

Fig. 5. *Binding of cyclic [^{3}H]AMP and stability of the binding sites as a function of cyclic AMP concentration*
(*a*) Parallel incubations of 2.4ml were set up for each concentration of nucleotide. After 6h of incubation, 1ml was taken directly into 80%-satd. $(NH_4)_2SO_4$ and 1ml incubated for another hour in the presence of 10nM-cyclic [^{3}H]AMP, 30mM-EDTA, 20mM-2-mercaptoethanol and 1mg of albumin/ml. The lower two curves show the amount of cyclic [^{3}H]AMP bound when the incubation was performed in the presence of 800mM-NaCl (□) or 800mM-NaCl+1mg of albumin/ml (●). The upper two curves show the number of binding sites that could be occupied in the presence of a saturating concentration of cyclic [^{3}H]AMP after preincubation with NaCl (□) or NaCl+albumin (●). (*b*) The amount of cyclic [^{3}H]AMP bound at various concentrations of the nucleotide was plotted as a percentage of the amount bound at saturation. The data were taken from the two sets of curves in (*a*).

observed when the kinase was already dissociated by salt (Table 5). Bovine serum albumin neither dissociated protein kinase I (Fig. 4*b*) nor increased its affinity for cyclic AMP (Fig. 5*b*). From the above it may be expected that inorganic salts and traces of protein precipitants as well as other agents known to affect the degree of activation of protein kinases (Beavo *et al.*, 1974) may influence the measurement of cyclic AMP by methods where the protein kinase is not saturated with nucleotide (Brown *et al.*, 1971, 1972, 1974; Cooper *et al.*, 1972). NaCl or KCl did influence one such assay (Fig. 2*a*).

The activation of protein kinase isoenzymes is affected differently by several agents (Corbin *et al.*, 1975; Hofmann *et al.*, 1975; Ueland & Døskeland, 1976). The degree of interference with cyclic AMP assays caused by such agents will therefore probably depend on the type of isoenzyme used in the assay. Bovine adrenal-cortex cytosol contains more than one protein kinase isoenzyme (cf. the Experimental section; Gill & Garren, 1971). The pattern of interference by, e.g. salt, would therefore be expected to be complex. The presence of KCl or NaCl preferentially increased the high-affinity binding in the extract, but also had some effect at relatively high concentrations of cyclic AMP (Figs. 2*a* and 2*b*). The shape of the standard curve was thus changed in the presence of salt (Fig. 2*a*). Similar complex patterns of interference have been reported by others (Albano *et al.*, 1974).

Prolonged incubation of protein kinase I at high ionic strength decreased the binding capacity for cyclic AMP (Tables 3 and 4; Fig. 6). This may be due to a lower stability of the free regulatory subunit than of the holoenzyme (Kumon *et al.*, 1972; Miyamoto *et al.*, 1971), as the binding capacity was stable in the absence of added salt. It is also possible that NaCl and KCl have a direct denaturing effect on the protein kinase (Brostrøm & Kon, 1974). The enhancement of high-affinity binding by salt described in the preceding paragraph would result in underestimation of the cyclic AMP in samples containing salt, whereas the instability of the binding preparation in the presence of salt, referred to above,

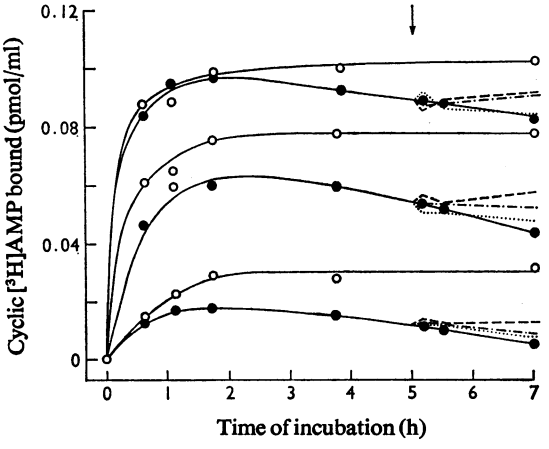

Fig. 6. *Time-course of cyclic* [^{3}H]*AMP binding to and stability of protein kinase I*

Protein kinase I was incubated in the presence of 800mM-NaCl (●) or 800mM-NaCl/30mM-EDTA/20mM-2-mercaptoethanol/1mg of albumin/ml (○), and 400μl portions were withdrawn at the times indicated. The concentration of cyclic [^{3}H]AMP was 0.1nM (lower set of curves), 0.5nM (middle set of curves) or 5nM (upper set of curves). After 5h of incubation (indicated by the arrow) 1.5ml portions were withdrawn from each of the tubes in which the protein kinase was incubated with 800mM-NaCl and then mixed with 300μl of concentrated solutions of substances known to enhance the binding of cyclic [^{3}H]AMP, or a similar volume of distilled water (●). The final concentrations of presumed stabilizers were 30mM-EDTA, 20mM-2-mercaptoethanol (····) alone or together with either 1mg of human γ-globulin/ml (–·–·) or 1mg of bovine serum albumin/ml (––––). Portions (48μl) were removed 10min, 30min and 2h after the addition of stabilizers.

would lead to an overestimation of cyclic AMP in the sample.

The presence of 2-mercaptoethanol, EDTA (Table 4, Fig. 6) and several proteins which themselves do not bind cyclic AMP (Table 3, Fig. 6) enhanced the binding of cyclic [^{3}H]AMP to protein kinase. Enhancement of binding to protein kinase by addition of proteins is widely recognized (Murad & Gilman, 1971; Weller *et al.*, 1972; Brostrøm & Kon, 1974; Tovey *et al.*, 1974) and was observed also for the binding of cyclic [^{3}H]AMP to diluted crude adrenal-cortex extract and to immunoglobulin raised against conjugated succinyl (3-carboxypropionyl)-cyclic AMP (S. O. Døskeland, H. J. Haga & P. M. Ueland, unpublished work). The stabilizing effect of bovine serum albumin was most pronounced for receptor incubated at low concentrations of cyclic AMP (Fig. 5*a*), and the affinity of the receptor was in fact unaffected by bovine serum albumin (Fig. 5*b*).

EDTA has been found to enhance the binding of cyclic [^{3}H]AMP in crude tissue extracts, which has been ascribed to inhibition of cyclic AMP phosphodiesterase (Sanborn *et al.*, 1973; Reimann & Rapino, 1974). Since our protein kinase preparation was devoid of phosphodiesterase activity (see the Experimental section), EDTA must also be able to act by other mechanisms. One possibility, which is especially attractive as 2-mercaptoethanol also stabilized the receptor, is that EDTA chelates traces of metal ions that may catalyse an oxidative process (Kono *et al.*, 1975), making the binding protein more prone to inactivation.

The binding protein was less subject to inactivation when incubated in the presence of a high concentration of cyclic AMP (Fig. 6). A similar finding has been reported for rat liver extracts (MacKenzie & Stellwagen, 1974). The extent of protection afforded by cyclic AMP was related to the degree of occupancy of receptor sites by cyclic AMP (Fig. 5*a*), indicating that the complex of protein kinase regulatory subunit and cyclic AMP is more stable than the regulatory subunit alone.

The results discussed in the preceding paragraphs suggest that competitive cyclic AMP assays based on protein kinase and conducted at neutral pH should be performed at high ionic strength and in the presence of stabilizers (albumin, 2-mercaptoethanol, EDTA).

The inclusion of salt (e.g. 800mM-NaCl, 30mM-EDTA) in order to dissociate fully the protein kinase (Fig. 4*c*) would decrease the interference by agents (e.g. traces of protein precipitants) which may dissociate the kinase at low ionic strength (Table 5, Fig. 3). In addition, the affinity for cyclic AMP of the protein kinase I preparation at high ionic strength is 10–100 times higher than that reported for any other naturally occurring binding protein used for cyclic AMP assays (Gilman, 1970; Walton & Garren, 1970; Tsang *et al.*, 1972; Sanborn *et al.*, 1973; Brostrøm & Kon, 1974; Tovey *et al.*, 1974). Half-maximal saturation was achieved at 0.1–0.2nM-cyclic [^{3}H]AMP, and the binding protein was nearly saturated at 1–2nM (Fig. 5*b*).

In the 'disequilibrium method' for cyclic AMP measurement (Brown *et al.*, 1974), originally described by Cooper *et al.* (1972), the binding protein is first incubated with the sample, and the number of unoccupied binding sites then measured after addition of cyclic [^{3}H]AMP. The inclusion of salt in such an assay, by increasing the affinity of the binding protein for the cyclic AMP in the sample (Table 3, Fig. 5), would enhance the binding of cyclic AMP during the first incubation step, and thereby make the assay more sensitive.

Interaction between protein kinase subunits seems to facilitate the dissociation of bound cyclic AMP

(Brostrom *et al.*, 1971). We have recently found that addition of the catalytic subunit did not affect the binding of cyclic [^{3}H]AMP to the isolated regulatory subunit of protein kinase I, provided a high concentration of NaCl was present (S. O. Døskeland, P. M. Ueland & H. J. Haga, unpublished work). One could thus expect that the presence of salt would also be of advantage during the second incubation step in the 'disequilibrium method', by retarding the exchange between cyclic [^{3}H]AMP and the unlabelled nucleotide bound during the first incubation step.

The $(NH_4)_2SO_4$ precipitation method used in the present study was unaffected by inorganic salts, EDTA, several bivalent ions and proteins. The observed modulation by those agents of the amount of bound ligand recovered must therefore be assumed to be during the incubation step, that is, before the separation of bound and free nucleotide by $(NH_4)_2SO_4$ precipitation. Marked differences in recoveries of bound cyclic [^{3}H]AMP have been described when Sephadex G-25 chromatography, membrane filtration and dextran-coated charcoal were used to separate free and bound ligand. There were also discrepancies between those methods with respect to the effect of ionic strength, pH and source of binding protein on the apparent amount of cyclic [^{3}H]AMP bound (Sheppard & Tsien, 1975). Whereas bivalent ions increased the amount of bound cyclic [^{3}H]AMP as detected by the membrane filtration method, less bound isotope was detected with the charcoal method (Albano *et al.*, 1974). Like Illiano *et al.* (1973), we found $(NH_4)_2SO_4$ precipitation to be superior to membrane filtration of the non-precipitated protein (Table 2). We have not tested dextran-coated charcoal, but that method is not able to give more than 90% recovery of bound isotope, even when source of charcoal, concentration of charcoal and concentration of bovine serum albumin are optimum (Tovey *et al.*, 1974). With that method a volatile substance, presumably ^3H_2O formed on prolonged storage of cyclic [^{3}H]AMP in aqueous solution (Tsang *et al.*, 1972), may increase the background counts (Tsang *et al.*, 1972; Albano *et al.*, 1974). With the $(NH_4)_2SO_4$ precipitation method we found no increase in background radioactivity for aqueous solutions of cyclic [^{3}H]AMP kept for more than 1 month at 4°C (S. O. Døskeland & P. M. Ueland, unpublished work).

We hope that application of the modifications suggested in this paper will extend the usefulness of the cyclic AMP assays based on naturally occurring binding proteins. Preliminary results obtained with protein kinase I from bovine adrenal cortex as binding reagent indicate that higher sensitivity and less interference is achieved in the presence of salt and stabilizers.

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