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Adenosine 3′:5′-Cyclic Monophosphate-Binding Proteins in Bovine and Rat Tissues

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1. At least two classes of high-affinity cyclic AMP-binding proteins have been identified: those derived from cyclic AMP-dependent protein kinases (regulatory subunits) and those that bind a wide range of adenine analogues (adenine analogue-binding proteins). 2. In fresh-tissue extracts, regulatory subunits could be further subdivided into 'type I' or 'type II' depending on whether they were derived from 'type I' or 'type II' protein kinase [see Corbin *et al.* (1975) *J. Biol. Chem.* **250**, 218–225]. 3. The adenine analogue-binding protein was detected in crude tissue supernatant fractions of bovine and rat liver. It differed from the regulatory subunit of cyclic AMP-dependent protein kinase in many of its properties. Under the conditions of assay used, the protein accounted for about 45% of the binding of cyclic AMP to bovine liver supernatants. 4. The adenine analogue-binding protein from bovine liver was partially purified by DEAE-cellulose and Sepharose 6B chromatography. It had mol.wt. 185000 and was trypsin-sensitive. As shown by competition and direct binding experiments, it bound adenosine and AMP in addition to cyclic AMP. At intracellular concentrations of adenine nucleotides, binding of cyclic AMP was essentially completely inhibited *in vitro*. Adenosine binding was inhibited by only 30% under similar conditions. 5. Rat tissues were examined for the presence of the adenine analogue-binding protein, and, of those examined (adipose tissue, heart, brain, testis, kidney and liver), significant amounts were only found in the liver. The possible physiological role of the adenine analogue-binding protein is discussed. 6. Because the adenine analogue-binding protein or other cyclic AMP-binding proteins in tissues may be products of partial proteolysis of the regulatory subunit of cyclic AMP-dependent protein kinase, the effects of trypsin and aging on partially purified protein kinase and its regulatory subunit from bovine liver were investigated. In all studies, the effects of trypsin and aging were similar. 7. In fresh preparations, the cyclic AMP-dependent protein kinase had mol.wt. 150000. Trypsin treatment converted it into a form of mol.wt. 79500. 8. The regulatory subunit of the protein kinase had mol.wt. 87000. It would reassociate with and inhibit the catalytic subunit of the enzyme. Trypsin treatment of the regulatory subunit produced a species of mol.wt. 35500 which bound cyclic AMP but did not reassociate with the catalytic subunit. Trypsin treatment of the protein kinase and dissociation of the product by cyclic AMP produced a regulatory subunit of mol.wt. 46500 which reassociated with the catalytic subunit. 9. These results may be explained by at least two trypsin-sensitive sites on the regulatory subunit. A model for the effects of trypsin is described.

In mammalian tissues, cyclic AMP-dependent protein kinases‡ (EC 2.7.1.37) are the only well characterized receptor proteins for cyclic AMP (see Krebs, 1972; Rubin & Rosen, 1975, for reviews). There are at least two protein kinase isoenzymes (types I and II) in tissues. These can be separated by DEAE-cellulose chromatography and differ in several properties (Corbin *et al.*, 1975, 1976). However, both isoenzymes are similar in that they contain two types of subunit, a regulatory (R) subunit and a catalytic (C) subunit (Brostrom *et al.*, 1971; Corbin *et al.*, 1976). The regulatory subunit inhibits the catalytic subunit in the absence of cyclic AMP. Certain hormones increase the intracellular concentration of cyclic AMP (Soderling *et al.*, 1973; Keely *et al.*, 1975) and dissociate the protein kinase

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‡ Abbreviations: In this paper protein kinase refers to cyclic AMP-dependent protein kinase holoenzyme which consists of regulatory (R) and catalytic (C) subunits. PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene; QAE-Sephadex, quaternary aminoethyl-Sephadex.

into its subunits (Brostrom *et al.*, 1971; Rubin *et al.*, 1972; Rosen *et al.*, 1973; Bechtel & Beavo, 1974) according to the equation:

$$R_2C_2 + 2\,\text{cyclic AMP} \rightleftharpoons R_2(\text{cyclic AMP})_2 + 2C \quad (1)$$
(inactive) (active)

When the catalytic subunit is dissociated from the regulatory subunit, it can catalyse ATP-dependent phosphorylation of certain enzymes and possibly other proteins (see Krebs, 1972; Rubin & Rosen, 1975; for reviews), causing activity changes. The regulatory subunit is thus an important receptor for cyclic AMP.

However, there have been consistent reports of cyclic AMP-binding proteins that are not regulatory subunits occurring in prokaryotes (Zubay *et al.*, 1970; de Crombrugghe *et al.*, 1971) and eukaryotes (Chambaut *et al.*, 1971; Donovan & Oliver, 1972; Sy & Richter, 1972; Yuh & Tao, 1974; Døskeland & Ueland, 1975; Hsu, 1975; Tsuzuki & Kiger, 1975). It has been proposed that the latter class of proteins may be important in the mediation of cyclic AMP action in eukaryotes. It therefore seemed important to investigate the existence and properties of cyclic AMP-binding proteins in tissue homogenates and to attempt to ascertain the physiological significance of such binding proteins. Further, since some cyclic AMP-binding proteins may be products of protein kinase degradation (Corbin *et al.*, 1972), the effects of proteolysis and aging on protein kinase and regulatory subunit were studied in detail. The results are reported in the present paper.

Materials and Methods

Materials

All materials were from sources described by Sugden *et al.* (1976) with the addition of the following: ^{3}H-labelled chemicals were obtained from New England Nuclear, Boston, MA 02118, U.S.A., and were of the highest specific radioactivity available; catalase was from Worthington Biochemicals, Freehold, NJ 07728, U.S.A.; apoferritin was from Calbiochem, La Jolla, CA 92037, U.S.A. Male Sprague–Dawley rats were used and had free access to food and water.

Buffer solutions. Potassium phosphate buffers, Tris/HCl buffers, CO_3^{2-}/HCO_3^- buffers and acetate buffers were prepared by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4, Tris base and Tris/HCl, Na_2CO_3 and $NaHCO_3$ or sodium acetate and acetic acid respectively, to give the desired pH. EDTA was neutralized to pH6.8 with 6M-NaOH before use.

Methods

Assay of binding of nucleosides and nucleotides to proteins. Binding of nucleosides and nucleotides to proteins was assayed as a routine by a method similar to that of Gilman (1970). A sample [10–20μl, suitably diluted in homogenization buffer (10mM-potassium phosphate/1mM-EDTA/0.1mM-dithiothreitol, pH6.8)] was added to 110μl of a solution of 50mM-potassium phosphate/1mM-EDTA/0.5mg of histone (Sigma, type II-A) per ml/2M-NaCl, pH6.8, containing 1μM-cyclic [^{3}H]AMP (6330c.p.m./pmol) or 1μM-[^{3}H]adenosine (2490c.p.m./pmol) or 1μM-[^{3}H]-AMP (2060c.p.m./pmol). The mixture was incubated for 60–90min at 30°C and then 1ml of ice-cold 10mM-potassium phosphate/1mM-EDTA, pH6.8, was added. The mixture was filtered through a Millipore filter (HA 0.45μm) previously moistened with the same buffer. The reaction tube was rinsed out with a further 1ml of the same buffer. The filter was rinsed with 8ml of buffer and dried in an oven at 150°C. Radioactivity retained by the filter was measured by counting in 10ml of toluene containing 4g of PPO/litre and 0.1g of dimethyl POPOP/litre in a Beckman LS-230 liquid-scintillation system. Suitable blanks were included concurrently.

Enzyme assays. Protein kinase was assayed as described previously (Sugden *et al.*, 1976). One unit of activity catalysed the incorporation of 1pmol of ^{32}P from [γ-^{32}P]ATP into histone per min at 30°C. In some cases, kinase activity is expressed as an activity ratio, i.e. the ratio of the activity in the absence of cyclic AMP to that in the presence of 2μM-cyclic AMP.

Phosphodiesterase (EC 3.1.4.17) was assayed essentially as described by Beavo *et al.* (1970) at a concentration of 1μM-cyclic [^{3}H]AMP.

Xanthine oxidase (EC 1.2.3.2) was assayed spectrophotometrically. Cuvettes contained, in a final volume of 1ml, 25mM-potassium phosphate (pH6.8), 1mM-EDTA, 0.3mM-xanthine and a suitable volume (100–200μl) of enzyme (with which the reaction was initiated). The increase in E_{292} at 37°C was followed in a Beckman model 24 spectrophotometer with a recorder attachment.

Adenosine deaminase (EC 3.5.4.4) was also assayed spectrophotometrically. Cuvettes contained, in 1ml, 25mM-potassium phosphate (pH6.8), 1mM-EDTA, 0.05mM-adenosine and a sample (50–200μl) of enzyme (with which the reaction was initiated). The decrease in E_{265} was measured at 30°C as described above.

Preparation of supernatant fractions for DEAE-cellulose chromatography. Bovine liver or rat tissue was homogenized in 10mM-potassium phosphate/1mM-EDTA/0.1mM-dithiothreitol, pH6.8 (1g of tissue/2ml of buffer for bovine and rat liver, rat adipose tissue and rat testis; 1g of tissue/4ml of buffer for other tissues including perfused rat liver), in a Servall Omni-Mixer (Ivan Sorvall, Norwalk, CT, U.S.A.) for two intervals of 15s at 0°C. Homogenates were centrifuged at 30000g (at $r_{av.} = 7.0$cm) for 45min, and supernatant fractions were retained.

Partial purification of cyclic AMP-binding proteins from bovine liver. Supernatant (80ml) was incubated with 0.25mM-cyclic AMP at 0°C for 1h and applied to a column (2.6cm×32cm) of DEAE-cellulose (Whatman DE-11). Protein kinase was dissociated by this procedure into its regulatory and catalytic subunits; the latter was eluted in the flow through. The column was washed with homogenization buffer (2.5litres). A linear NaCl gradient (500ml, 0–0.5M) in the same buffer was started, and fractions (10ml) were collected. Fractions containing cyclic AMP-binding activities were pooled separately, dialysed against several changes of homogenization buffer (3litres) at 4°C for 24h and concentrated by surrounding the dialysis tubing with dry Sephadex G-200. The cyclic AMP-binding proteins were further purified by Sepharose 6B chromatography (see below). Such preparations were purified approximately 80-fold.

Preparation of protein kinase holoenzyme. Bovine liver supernatant (22ml) was applied to a column (0.9cm×10cm) of DEAE-cellulose equilibrated with homogenization buffer. The column was washed with homogenization buffer (50ml), and a linear NaCl gradient (100ml; 0–0.4M) in the same buffer was started. Fractions (5ml) were collected and assayed for protein kinase activity in the presence of cyclic AMP (2μM).

Tryptic hydrolysis. Trypsin solutions were freshly prepared in 50mM-sodium acetate buffer, pH4.0. Protein solutions were incubated for 10min at 30°C with crystallized bovine pancreatic trypsin (trypsin/protein, 1:100, w/w). Tryptic hydrolysis was terminated by the addition of a fivefold excess (w/w) of soya-bean trypsin inhibitor to the reaction mixture and subsequent storage at 0°C.

Preparation of fresh R subunit–cyclic [^{3}H]AMP complex. Bovine liver supernatant (22ml) was applied to a column (0.9cm×10cm) of DEAE-cellulose equilibrated with homogenization buffer. The column was washed with homogenization buffer (50ml) and protein kinase was dissociated by subsequently washing with 50ml of 50mM-potassium phosphate/1mM-EDTA/0.1mM-dithiothreitol/1μM-cyclic [^{3}H]AMP (1900–2000c.p.m./pmol), pH6.8. Excess of cyclic [^{3}H]AMP was removed by washing the column with homogenization buffer (100ml), and the R subunit–cyclic [^{3}H]AMP complex was eluted with a linear NaCl gradient (100ml; 0–0.4M) in the same buffer. Fractions (5ml) were collected, and ^{3}H in samples (100μl) was determined in 0.9ml of water and 10ml of a fluor containing 4g of PPO/litre, 0.1g of dimethyl POPOP/litre in 2vol. of toluene and 1vol. of Triton X-100. Such preparations of R subunit–cyclic [^{3}H]AMP complex contained only very low amounts of protein kinase activity (assayed in the presence of 2μM-cyclic AMP).

Preparation of R subunit–cyclic [^{3}H]AMP complex from trypsin-treated fresh protein kinase. Protein kinase holoenzyme was prepared from bovine liver supernatant (as described above), treated with trypsin and chromatographed on a Sepharose 6B column as described. Sepharose 6B fractions (3ml) containing protein kinase activity (assayed in the presence of 2μM-cyclic AMP) were pooled and incubated with cyclic [^{3}H]AMP (2μM; 5680c.p.m./pmol) for 1h at room temperature (21°C). This mixture was applied to a column (0.9cm×3cm) of DEAE-cellulose equilibrated with homogenization buffer. The column was washed with the same buffer (100ml) and R subunit–cyclic [^{3}H]AMP complex was eluted batchwise by the same buffer (10ml) containing 0.5M-NaCl. Fractions (1ml) were collected and R subunit–cyclic [^{3}H]AMP complex was determined as described above.

Determination of Stokes radii by Sepharose 6B chromatography. Stokes radii were measured on a column (1.5cm×90cm) of Sepharose 6B equilibrated with 50mM-Tris/HCl/1mM-EDTA, pH7.5. Samples (1ml) containing 10% (w/v) sucrose were placed on the column, and fractions (2–3ml) were collected by downward flow. The flow rate was about 4ml/h. The column was standardized with proteins of Stokes radii calculated (Siegel & Monty, 1966) from values of physical parameters given in the literature, namely bovine serum albumin (Stokes radius 3.5nm; Creeth, 1952), bovine serum albumin dimer (4.75nm; Hughes, 1950; Creeth, 1952), soya-bean trypsin inhibitor (2.45nm; Rackis *et al.*, 1962), ovalbumin (2.95nm; Kegeles & Gulter, 1951; Castellino & Barker, 1968), bovine liver catalase (5.2nm; Sumner & Gralen, 1938) and horse spleen apoferritin (5.94nm; Rothen, 1944). Inclusion (144ml) and exclusion (42ml) volumes were determined with cyclic [^{3}H]AMP and Blue Dextran respectively. All protein standards were applied to the column separately at a concentration of 10mg/ml and were determined spectrophotometrically. Cyclic [^{3}H]AMP was determined by liquid-scintillation counting. Data were plotted by the method of Laurent & Killander (1964); see also Siegel & Monty (1966).

Separation and identification of purine derivatives. Adenine, adenosine, cyclic AMP, AMP, ADP and ATP were separated by chromatography on columns (0.9cm×12cm) of QAE-Sephadex A-25 equilibrated with 20mM-Na_2CO_3/$NaHCO_3$ buffer, pH9.7. Extracts were adjusted to pH9.7 with 20mM-Na_2CO_3 and applied in 10% (w/v) sucrose to columns which were then washed with 50ml of 20mM-Na_2CO_3/$NaHCO_3$ buffer, pH9.7, and eluted with linear NaCl gradients (100ml; 0–0.4M-NaCl) in the same buffer. Fractions (5ml) were collected.

Adenine, adenosine, inosine, hypoxanthine, xanthine, uric acid, cyclic AMP, AMP, ADP and ATP were also separated by chromatography on a column

(0.9cm×55cm) of Sephadex G-25 (superfine grade) equilibrated with 5mM-Tris/HCl/1mM-EDTA/100mM-NaCl, pH7.5. Extracts were applied to the column in 10% (w/v) sucrose, and fractions (1–2ml) were collected.

Other methods. Protein was determined by measurement of E_{280}. Sucrose-density-gradient centrifugation was performed as described by Sugden *et al.* (1976). Rat liver was perfused for 10min with 0.9% (w/v) NaCl as described by Exton (1975).

Results

DEAE-cellulose chromatography of bovine liver and rat tissue supernatant

Chromatography of bovine liver supernatant is shown in Fig. 1. There were two peaks of cyclic AMP-binding activity eluted at 0.02 and 0.17M-NaCl respectively. The latter peak was co-eluted with a single peak of protein kinase. The chromatographic behaviour (and other properties, see below) of this protein kinase was consistent with that of a 'type II' isoenzyme (see Corbin *et al.*, 1975). In this tissue, cyclic AMP phosphodiesterase was eluted in a single peak at 0.21M-NaCl (results not shown).

Chromatography of tissue supernatants after incubation with cyclic AMP (to dissociate protein kinase) is shown in Fig. 2. Catalytic subunit was eluted before the start of the NaCl gradient (results not shown). For bovine liver supernatant, there were two peaks of cyclic AMP-binding activity eluted at 0.03 and 0.26M-NaCl (Fig. 2*a*). The former peak of material was eluted in a similar position to that observed in Fig. 1. The latter peak of cyclic AMP-binding activity, as will become apparent later, was probably the regulatory subunit of the dissociated protein kinase. It was eluted at a higher NaCl concentration than protein kinase holoenzyme (Fig. 1) because of its lower pI (see Corbin *et al.*, 1976). For rat liver supernatant (Fig. 2*b*), there were two clear peaks of cyclic AMP-binding activity, eluted at 0.19 and 0.27M-NaCl, and a shoulder, eluted at 0.05M-NaCl.

Adenosine- and AMP-binding activities in these fractions were also assayed (Fig. 2). For bovine or rat liver, binding of these compounds coincided only with the peak of cyclic AMP-binding activity eluted at 0.03–0.05M-NaCl. Thus this binding protein differs from regulatory subunit (which did not bind adenosine or AMP, see Fig. 2) and is known hereafter as the adenine analogue-binding protein. The binding of cyclic [^{3}H]AMP, [^{3}H]adenosine or [^{3}H]AMP to fractions from sucrose-density-gradient centrifugation or Sepharose 6B chromatography of bovine liver adenine analogue-binding protein was superimposable. Thus it is probable that these compounds were binding to the same protein species. Binding of adenosine to adenine analogue-binding protein (in terms of pmol/ml) was always greater than that of cyclic AMP or AMP, under the conditions of assay. This may be caused by a greater affinity for adenosine, or more binding sites on adenine analogue-binding protein for adenosine.

Competition studies

Many purine compounds (e.g. adenosine, ADP, adenine, NAD^+, inosine) inhibited the binding of cyclic [^{3}H]AMP or [^{3}H]AMP to adenine analogue-binding protein (Table 1). However, only adenosine itself and adenine inhibited the binding of [^{3}H]-adenosine to adenine analogue-binding protein. In contrast, only certain cyclic nucleotides inhibited the binding of cyclic AMP to regulatory subunit. Because the adenine analogue-binding protein may be a 'receptor' for cyclic AMP and/or adenosine *in vivo*, binding of cyclic [^{3}H]AMP (1μM) or [^{3}H]adenosine (1μM) to adenine analogue-binding protein was investigated at approximately intracellular concentrations of adenine nucleotides (5mM-ATP, 0.5mM-ADP and 0.1mM-AMP). Binding of cyclic [^{3}H]AMP or [^{3}H]adenosine decreased to 1 or 70% of their control values respectively under such conditions. It is therefore unlikely that the adenine analogue-binding protein binds cyclic AMP in the cell but possible that adenosine would be bound.

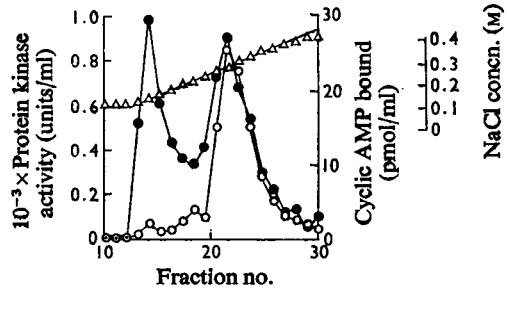


Fig. 1. *DEAE-cellulose chromatography of bovine liver supernatant*

Bovine liver supernatant (1ml) was applied to a column (0.9cm×10cm) of DEAE-cellulose equilibrated with homogenization buffer. The column was washed with 50ml of homogenization buffer and a linear NaCl gradient (100ml; 0–0.4M) in the same buffer was started. Fractions (5ml) were collected and assayed for protein kinase activity in the presence of 2μM-cyclic AMP (○), cyclic [^{3}H]AMP-binding protein (●) and NaCl concn. (△), which was determined by using a Beckman cationic electrode. Fractions (1ml) were diluted with 200mM-Tris/HCl, pH8.0 (4ml), for measurement of NaCl concn.

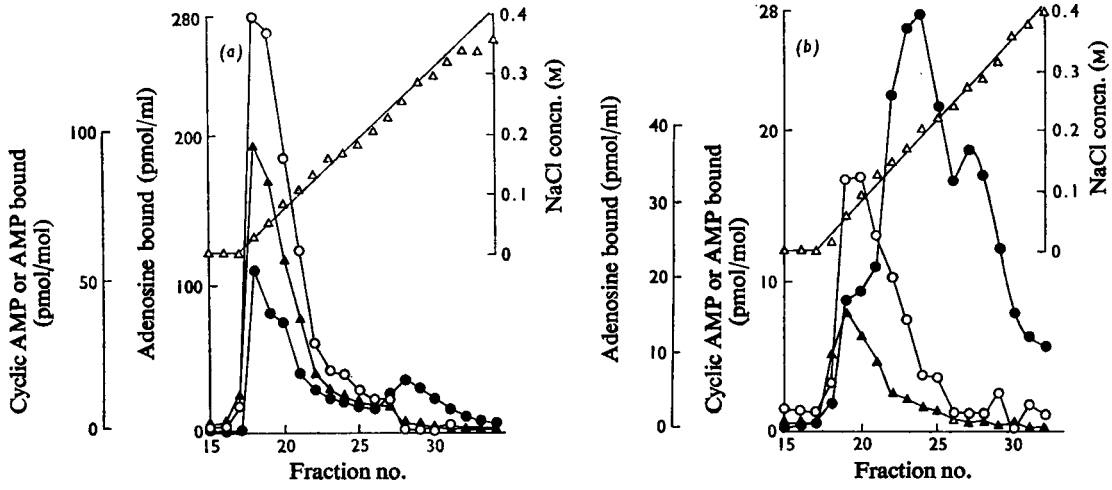


Fig. 2. *DEAE-cellulose chromatography of cyclic AMP-treated bovine (a) and rat (b) liver supernatants*

Liver supernatants (4ml) were incubated with cyclic AMP (0.25mM) for 1h at 0°C and applied to columns (0.9cm×10cm) of DEAE-cellulose equilibrated with homogenization buffer. Otherwise, methodology was as described in Fig. 1. Fractions were assayed for the binding of cyclic [^{3}H]AMP (●), [^{3}H]adenosine (○) and [^{3}H]AMP (▲), and for NaCl concn. (△) as described in the Materials and Methods section and in Fig. 1.

Table 1. *Competition studies for adenine analogue-binding protein and regulatory subunit*

Binding assays were as described in the Materials and Methods section. The binding reaction was initiated by the addition of adenine analogue-binding protein or regulatory subunit to the binding mixture containing ^{3}H-labelled compound (1 μM) and competitor and incubated for 90min. Results are expressed as (c.p.m. bound in the presence of competitor/c.p.m. bound in the absence of competitor)×100. —, Not determined.

		Activity [(c.p.m. bound in the presence of competitor/c.p.m. bound in the absence of competitor)×100]										
		Adenine analogue-binding protein									Regulatory subunit	
	^{3}H-labelled compound ...	Cyclic [^{3}H]AMP			[^{3}H]Adenosine			[^{3}H]AMP			Cyclic [^{3}H]AMP	
Unlabelled competitor	Concn. unlabelled competitor (μM) ...	1	10	100	1	10	100	1	10	100	10	100
No addition			100			100			100		100	
Cyclic AMP		64	12	1	99	90	88	91	35	33	15	2
Cyclic GMP		—	102	95	—	—	—	—	—	—	93	76
Cyclic IMP		—	90	97	—	—	—	—	—	—	101	89
Cyclic UMP		—	105	94	—	—	—	—	—	—	72	31
8-Bromo cyclic AMP		—	18	3	—	—	—	—	—	—	50	11
Dibutyryl cyclic AMP		—	99	78	—	—	—	—	—	—	41	21
AMP		68	17	3	101	94	88	82	39	23	91	94
GMP		—	95	95	—	—	—	—	—	—	106	106
UMP		—	106	106	—	—	—	—	—	—	105	104
IMP		—	100	92	—	—	—	—	—	—	101	105
ADP		82	48	10	102	98	94	108	78	34	99	94
ATP		93	93	53	97	102	95	106	99	84	105	104
Adenosine		24	3	1	97	50	11	45	16	3	109	97
Adenine		59	37	29	99	87	67	83	50	40	96	104
NAD^+		49	8	1	98	92	88	68	32	24	—	—
Inosine		113	91	31	98	100	88	91	83	42	—	—
Xanthine		98	109	107	109	106	107	98	91	105	—	—
Hypoxanthine		135	139	128	91	100	98	95	96	91	—	—
Uric acid		98	111	123	92	88	92	100	101	99	—	—

Cyclic AMP-binding proteins in rat tissues

A survey of binding of cyclic [^{3}H]AMP by DEAE-cellulose fractions of cyclic AMP-treated rat tissue supernatants is shown in Fig. 3. Of six tissues tested (testis, adipose tissue, brain, heart, perfused liver and kidney), only perfused liver and brain showed clear peaks of cyclic AMP-binding activity eluted at less than 0.1M-NaCl. Several tissues (testis, adipose tissue and kidney) showed small peaks or shoulders of binding activity. Since adenosine inhibited binding of cyclic AMP to adenine analogue-binding protein (see Table 1), fractions from adipose tissue (fractions 17 and 21), brain (fractions 13, 18 and 23), heart (fractions 18 and 23), kidney (fractions 15, 18, and 21) and perfused rat liver (fractions 14, 16 and 20) were tested for the inhibition of binding of 1μM-cyclic [^{3}H]AMP by 100μM-adenosine. Only perfused rat liver fraction 14 showed significant inhibition (20%). Therefore, of the tissues examined, the adenine analogue-binding protein occurred in significant quantities only in bovine and rat liver and, at least in rat liver, the presence of adenine analogue-binding protein was not caused by the presence of blood in the tissue. The former conclusion was confirmed by examination of the binding of 1μM-cyclic [^{3}H]AMP to whole-tissue supernatants in the absence or the presence of 100μM-adenosine (Table 2). Under such conditions, adenine analogue-binding protein contributed 21 and 44% of the total cyclic AMP-binding protein in rat and bovine liver supernatants respectively. Other tissues tested showed no significant inhibition of cyclic [^{3}H]AMP-binding activity by adenosine.

A comparison of some properties of adenine analogue-binding protein and bovine liver regulatory subunit

In addition to competition studies (see Table 1), the adenine analogue-binding protein differed from regulatory subunit in other properties. It did not inhibit homogeneous bovine liver catalytic subunit, whereas regulatory subunit inhibited by 80% (results not shown). The proteins differed in their physical properties (Table 3). The adenine analogue-binding protein was larger than protein kinase and much larger than any form of regulatory subunit. In contrast with bovine liver, rat liver adenine analogue-binding protein showed two peaks of binding activity on sucrose-gradient centrifugation or Sepharose 6B chromatography: a smaller species (30% of total) of mol.wt. 180500 and a larger species (70% of total) of mol.wt. 370500. The larger species was possibly a dimer of the smaller species. The adenine analogue-binding protein from bovine liver was relatively stable (26% loss of activity) to high concentrations of trypsin (0.2mg/ml final concentration; 2h incubation; pH6.8; 30°C) when assayed by cyclic AMP binding, but regulatory subunit from bovine liver was more labile (99% loss of activity). Adenine analogue-binding protein from bovine liver was more stable to heat, losing 50% of cyclic AMP-binding activity (assayed at 30°C) at 49°C (5min incubation) as compared with 45°C for regulatory subunit from bovine liver. The pH profiles of cyclic AMP binding to the two proteins differed under a variety of conditions (Fig. 4). Their affinities for cyclic AMP also differed. Half-maximal binding was at 1μM for adenine analogue-binding protein compared with 0.05μM for regulatory subunit. On the basis of these differences, adenine analogue-binding protein was not a regulatory subunit.

Rates of binding of cyclic AMP, adenosine and AMP to adenine analogue-binding protein

The rates of binding or of release of [^{3}H]adenosine, [^{3}H]AMP or cyclic [^{3}H]AMP (each at 1μM) to or from adenine analogue-binding protein differed (Fig. 5). Such kinetic experiments should be interpreted with caution, since the rate of binding of ^{3}H-labelled compounds may be concentration-dependent under such conditions and may represent, at least in part, an exchange between the ^{3}H-labelled compound and unlabelled compounds bound to the adenine analogue-binding protein. However, under the conditions used, [^{3}H]adenosine bound to adenine analogue-binding protein more quickly (Fig. 5*a*) and was released more slowly than the two ^{3}H-labelled nucleotides tested (Fig. 5*b*).

Metabolism of adenine nucleotides, adenosine and adenine by extracts containing adenine analogue-binding protein

Since experiments on adenine analogue-binding protein used crude tissue preparations, it was important to establish that metabolism of cyclic AMP, AMP, adenosine or adenine did not occur during binding experiments. Samples (50μl) of adenine analogue-binding protein from bovine liver (purified by DEAE-cellulose and Sepharose 6B chromatography) were incubated for 90min with samples (0.55ml) of binding assay mixtures (see the Materials and Methods section) containing 1μM-cyclic [^{3}H]AMP or 1μM-[^{3}H]adenosine or 1μM-[^{3}H]AMP or 40μM-[^{14}C]adenine (15c.p.m./pmol). Incubations were terminated by immersion in a boiling-water bath for 30s.

Radioactivity present as cyclic [^{3}H]AMP, [^{3}H]-AMP or [^{14}C]adenine was recovered almost completely as such (>97%) by chromatography after incubations with adenine analogue-binding protein in the presence or the absence of 5mM-magnesium acetate. Cyclic [^{3}H]AMP was slightly (2%) contaminated by ^3H_2O, and heating at 100°C produced

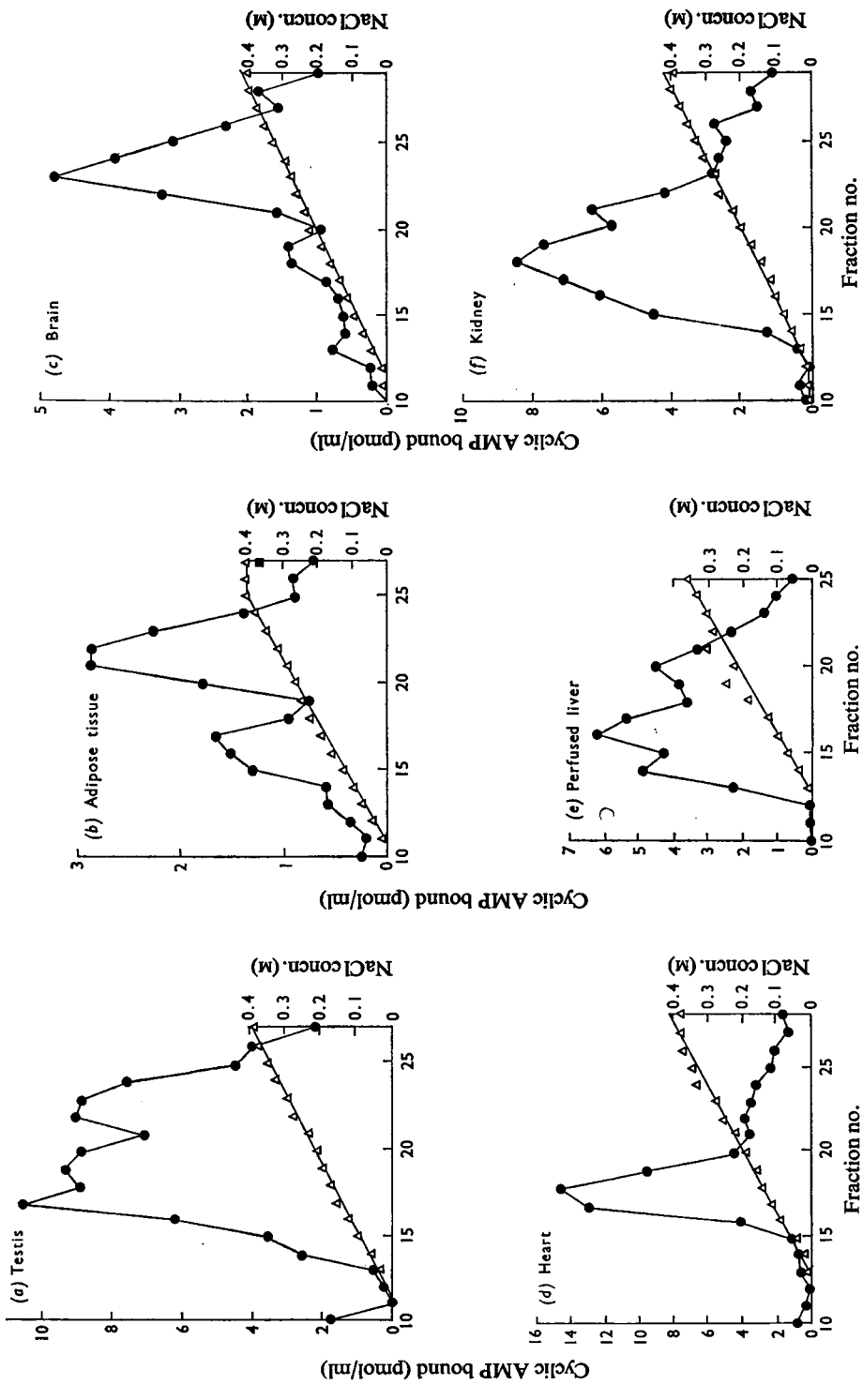


Fig. 3. *DEAE-cellulose chromatography of cyclic AMP-treated rat tissue supernatants*

Rat tissue supernatants were incubated with cyclic AMP for 1 h at 0°C and applied to columns (0.9 cm × 5 cm) of DEAE-cellulose equilibrated with homogenization buffer. For adipose tissue, 4 ml of supernatant was applied to the column; for all other tissues, 2 ml of supernatant was applied. Otherwise, methodology was as described in Fig. 1. Fractions were assayed for the binding of cyclic [^{3}H]AMP (●) and NaCl concn. (△) as described.

Table 2. *Inhibition of binding of cyclic AMP to tissue supernatants by adenosine*

Tissue supernatants were prepared as described in the Materials and Methods section. Binding of cyclic [^{3}H]AMP (1 μM) to supernatants was assayed as described in the absence and in the presence of adenosine (100 μM). The incubation was initiated by the addition of 20 μl of tissue supernatant suitably diluted with homogenization buffer.

Tissue	Cyclic [^{3}H]AMP bound (pmol/ml of undiluted supernatant)	Cyclic [^{3}H]AMP bound in the presence of adenosine (pmol/ml of undiluted supernatant)	Inhibition of cyclic [^{3}H]AMP-binding activity by adenosine (%)
Rat adipose tissue	15.25	15.25	0
Rat liver	149.1	118.3	21
Rat heart	64.91	66.98	0
Rat brain	38.80	40.87	0
Rat kidney	66.98	63.83	5
Bovine liver	207.0	116.3	44

Table 3. *Physical properties of adenine analogue-binding protein from bovine and rat liver, and protein kinase and its regulatory subunits from bovine liver*

Adenine analogue-binding proteins and regulatory subunits were partially purified by DEAE-cellulose chromatography. Physical parameters were calculated from data obtained by sucrose-density-gradient centrifugation and Sepharose 6B gel filtration as described by Siegel & Monty (1966). A value of 0.725 $cm^3 \cdot g^{-1}$ was assumed for $\bar{v}$. At least three separate determinations of $s_{20,w}$ and Stokes radius were performed for each protein. The terms peak I and peak II refer to the peaks of protein kinase or binding activity observed on sucrose density gradients or gel filtration. It was assumed that the protein with the larger $s_{20,w}$ corresponded to the larger protein observed on gel filtration.

	$s_{20,w}$ (S)	Stokes radius (nm)	f/f_0	Mol.wt.
Adenine analogue-binding protein				
Bovine liver	9.8	4.60	1.22	185000
Rat liver peak I	10.1	4.35	1.17	180500
peak II	14.2	6.35	1.34	370500
Regulatory subunit				
Fresh	4.4	4.80	1.64	87000
Trypsin-treated fresh	3.1	2.80	1.29	35500
Dissociation of trypsin-treated fresh protein kinase	3.1	3.65	1.54	46500
Protein kinase				
Fresh	7.3	5.00	1.43	150000
Trypsin-treated	5.3	3.65	1.29	79500
Reassociation of fresh regulatory subunit and pure catalytic subunit				
peak I	7.2	5.00	1.43	148000
peak II	5.4	3.55	1.25	79000

a small amount (1%) of [^{3}H]AMP. [^{3}H]AMP was also slightly (2%) contaminated by ^3H_2O. Such experiments exclude contamination of adenine analogue-binding protein by cyclic nucleotide phosphodiesterase (EC 3.1.4.17) or 5'-nucleotidase (EC 3.1.3.5). Thus cyclic AMP, AMP and adenine probably bound by adenine analogue-binding protein themselves and were not metabolized under the conditions of assay.

In contrast, [^{3}H]adenosine was metabolized (approx. 20–30%) by preparations of adenine analogue-binding protein. Products isolated from the incubation mixtures co-chromatographed with water, inosine, uric acid and adenine after Sephadex G-25 chromatography. Of these products, only adenine inhibited binding of [^{3}H]adenosine to adenine analogue-binding protein (Table 1) and then inhibition was only 33%, even at concentrations 100-fold greater than those of adenosine. Were a prior conversion of [^{3}H]adenosine into [^{3}H]adenine required before binding to adenine analogue-binding protein occurred, unlabelled adenine would inhibit binding of [^{3}H]adenosine much more strongly than was observed (Table 1). [^{3}H]Adenosine itself was therefore bound to adenine analogue-binding protein. Because of these findings, it was considered probable that preparations of adenine analogue-binding protein were contaminated by

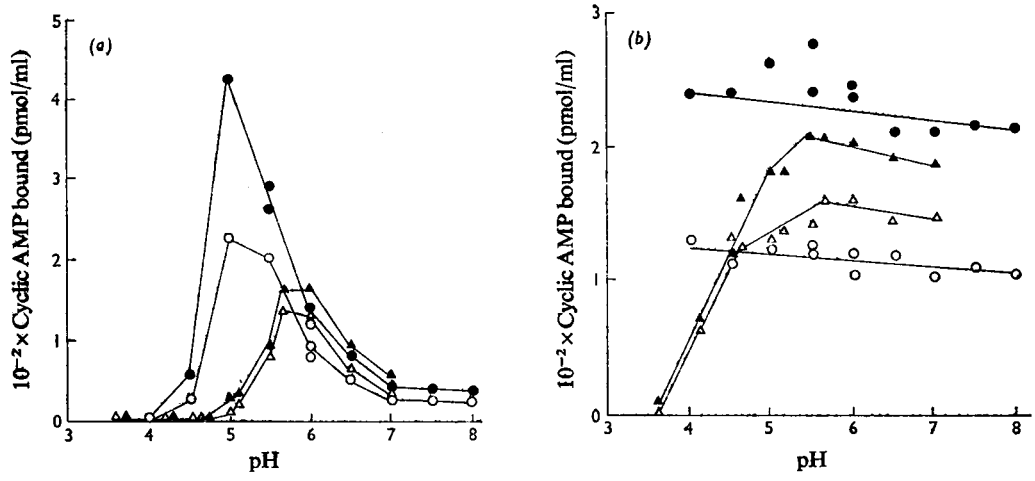


Fig. 4. *pH-dependence of cyclic AMP-binding to adenosine analogue-binding protein and regulatory subunit from bovine liver*

Adenine analogue-binding protein (*a*) or regulatory subunit (*b*) was partially purified by DEAE-cellulose chromatography as described in the Materials and Methods section. Sodium acetate buffers were used at pH4.0–6.0 and potassium phosphate buffers were used at pH5.5–8.0. Proteins were incubated for 90min in the presence of 50mM buffer, 1mM-EDTA and 1μM-cyclic [^{3}H]AMP (○). The following additions were made to the basic incubation medium: 0.5mg of histone/ml (Sigma, type II-A) (●); 2M-NaCl (△); or 0.5mg of histone/ml (Sigma, type II-A) and 2M-NaCl (▲).

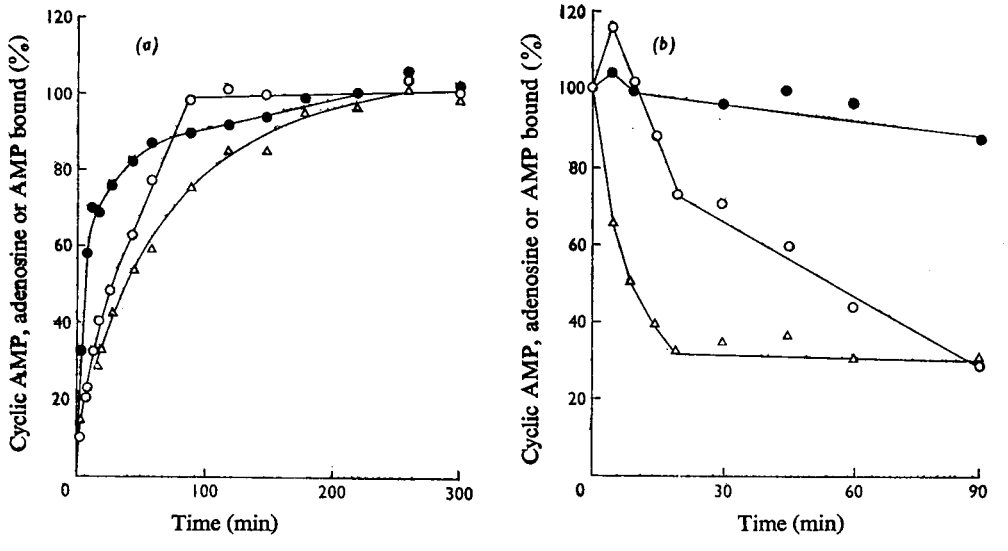


Fig. 5. *Time-course of adenine analogues binding to or released from adenine analogue-binding protein from bovine liver*

The time-course of binding of cyclic [^{3}H]AMP (○), [^{3}H]adenosine (●) or [^{3}H]AMP (△) was estimated as described in the Materials and Methods section. The concentration of adenine analogues was 1μM in each case. The time-course of release (*b*) of adenine analogues was estimated by incubating adenine analogue-binding protein with [^{3}H]adenine analogue (1μM) for 90min and then, at zero time, a 100-fold molar excess of unlabelled cyclic AMP, adenosine or AMP was added to adenine analogue-binding protein incubated with cyclic [^{3}H]AMP (○), [^{3}H]adenosine (●) or [^{3}H]AMP (△) respectively. Results are expressed as a percentage of maximum radioactivity (c.p.m.) bound (*a*) or as a percentage of radioactivity (c.p.m.) bound at zero time (*b*).

adenosine deaminase (EC 3.5.4.4) and xanthine oxidase (EC 1.2.3.2). Both these enzyme activities were detected in the preparations.

Sepharose 6B chromatography of regulatory subunits and protein kinase holoenzymes from bovine liver

Because of the possibility that some cyclic AMP-binding proteins are derived from protein kinase and regulatory subunit by partial proteolysis (Corbin *et al.*, 1972), the effects of proteolysis and aging were investigated in detail. Methods of purification were such that there was no contamination of regulatory subunit preparations by adenine analogue-binding protein (see the Materials and Methods section). At least three species of regulatory subunit were resolved (Fig. 6). Fresh R subunit–cyclic [^{3}H]-AMP complex (Fig. 6*a*) was eluted mainly at 72ml, but showed a smaller peak at 94ml. R subunit–cyclic [^{3}H]AMP complex aged for 24 days at 4°C (Fig. 6*b*) was eluted mainly at 94ml (but showed a smaller more diffuse peak at 76–82ml). Similarly, trypsin-treated fresh R subunit–cyclic [^{3}H]AMP complex was eluted at 92ml (Fig. 6*c*). In contrast, R subunit–cyclic [^{3}H]AMP complex derived from trypsin-treated fresh protein kinase holoenzyme was eluted at 82ml (Fig. 6*d*).

At least two species of protein kinase holoenzyme could be resolved. Fresh protein kinase was eluted mainly at 70ml but showed a smaller peak of activity at 84ml (Fig. 6*e*). With protein kinase aged for 9 days at 4°C, an increased proportion was eluted at 85ml (Fig. 6*f*). Trypsin-treated fresh protein kinase was eluted in a single peak at 82.5ml (Fig. 6*g*). Protein kinase produced by reassociation of fresh regulatory subunit purified by DEAE-cellulose chromatography and homogeneous bovine liver catalytic subunit was eluted mainly at 70ml (Fig. 6*h*), as did fresh native protein kinase (Fig. 6*e*), but showed a shoulder of material at 80–85ml which was presumed to be identical with protein kinase shown in Fig. 6(*g*).

Physical properties of bovine liver protein kinase and its regulatory subunit

Sedimentation coefficients, Stokes radii, frictional ratios and molecular weights of protein kinase and its regulatory subunits are shown in Table 3. Fresh regulatory subunit had a large frictional ratio characteristic of a highly asymmetric ellipsoid. The regulatory subunit obtained by trypsin treatment of fresh protein kinase and its subsequent dissociation by cyclic AMP had a molecular weight of approximately one-half that of fresh regulatory subunit. It was also highly asymmetric, having a frictional ratio of 1.54. Trypsin-treated fresh regulatory subunit was much less asymmetric. These results indicate that trypsin treatment of fresh regulatory subunit probably removed a peptide fragment(s). However, such treatment did not inhibit cyclic AMP binding with saturating concentrations of cyclic AMP. The behaviour of the aged regulatory subunit on Sepharose 6B chromatography (Fig. 6*b*) indicated that it was also of this type, i.e. it has been attacked proteolytically.

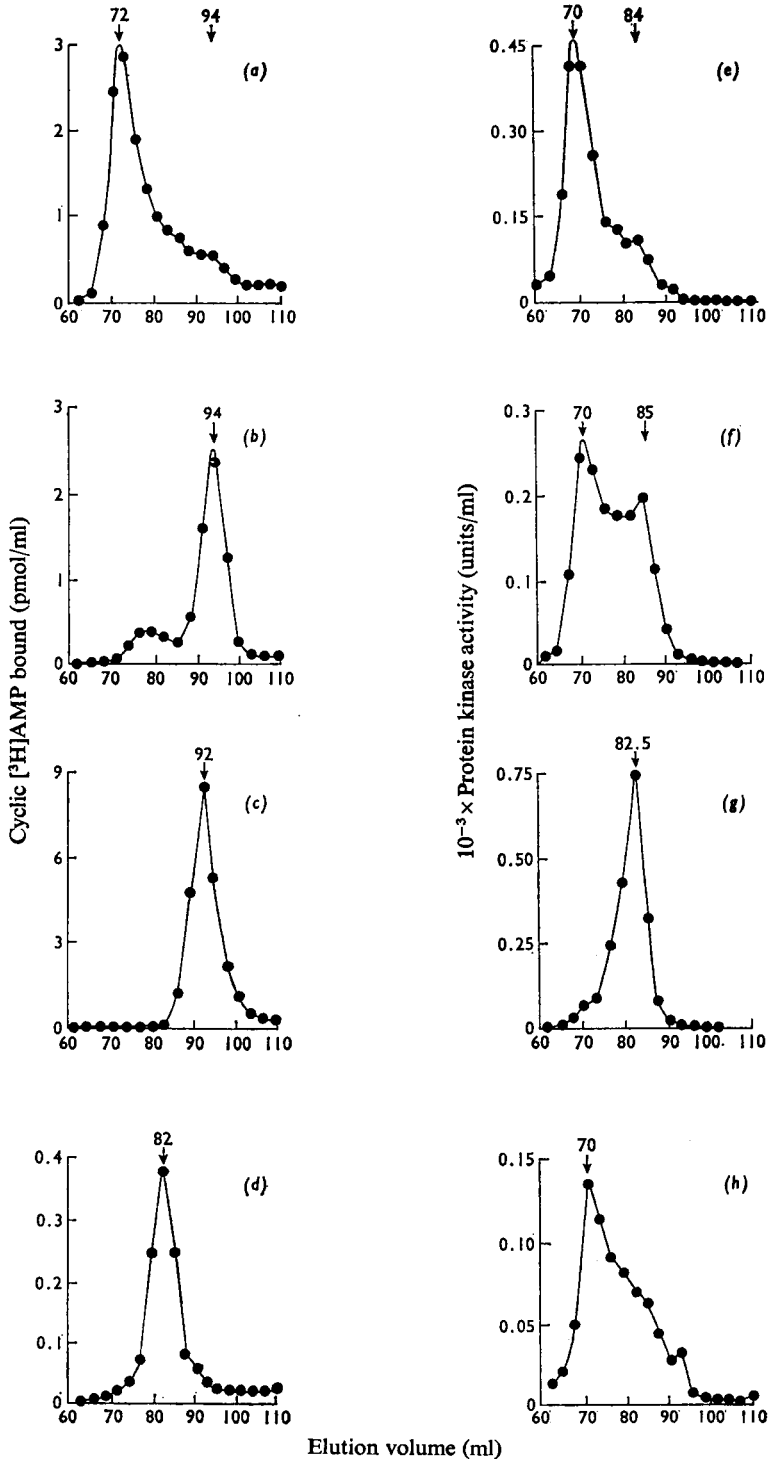
Fresh protein kinase had mol.wt. 150000 (Table 3); aging at 4°C or trypsin treatment produced a species with a molecular weight approximately one-half of that of the native enzyme. Trypsin treatment thus possibly produced RC from native R_2C_2. Reassociation of fresh regulatory subunit and pure catalytic subunit thus probably resulted mainly in the formation of R_2C_2 (mol.wt. 148000).

Reassociation of regulatory and catalytic subunits

Because their ability to inhibit catalytic subunit has been used to identify regulatory subunits, experiments were undertaken to test the validity of this criterion for the various bovine liver regulatory subunits isolated (see Table 3 and Fig. 6). Reassociation was investigated by the release of cyclic [^{3}H]AMP from the R subunit–cyclic [^{3}H]AMP complex on incubation with catalytic subunit via reversal of eqn. (1) (see the introduction) and, in some cases, by measurement of activity ratios. Fresh R subunit–cyclic [^{3}H]AMP complex (mol.wt. 87000) readily released cyclic [^{3}H]AMP on incubation with catalytic subunit (Fig. 7). Loss of cyclic [^{3}H]AMP was maximum at a C/R subunit molar ratio of 1.0, when the activity ratio was 0.26. Thereafter, the activity ratio increased as C/R subunit ratio increased. The increase was not as great as expected, probably because of the instability of catalytic subunit under the conditions of incubation. Trypsin-treated,

Fig. 6. *Sepharose 6B chromatography of regulatory subunits and protein kinases from bovine liver*

A volume (1ml) of R subunit–cyclic [^{3}H]AMP complex or protein kinase was applied to a column of Sepharose 6B, and fractions were collected and assayed for ^{3}H or protein kinase activity as described in the Materials and Methods section. The following species of R subunit–cyclic [^{3}H]AMP complexes were used: fresh (*a*), aged 24 days at 4°C (*b*), trypsin-treated (*c*) and that derived from trypsin-treated fresh protein kinase (*d*). The following species of protein kinases were used: fresh (*e*), aged 9 days at 4°C (*f*), trypsin-treated fresh (*g*) and that from the reassociation of fresh regulatory and pure catalytic subunits (*h*).


72
94
(a)
70
84
(e)
94
(b)
70
85
(f)
92
(c)
82.5
(g)
82
(d)
70
(h)
Cyclic [³H]AMP bound (pmol/ml)
10⁻³ × Protein kinase activity (units/ml)
Elution volume (ml)

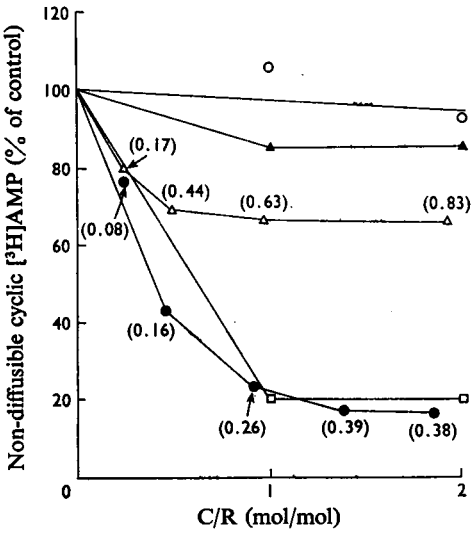


Fig. 7. *Reassociation of partially purified regulatory subunit with homogeneous catalytic subunit from bovine liver*

Bovine liver R subunit–cyclic [^{3}H]AMP complexes (5–25 pmol, prepared as described in the Materials and Methods section) were incubated with homogeneous bovine liver catalytic subunit (see Sugden *et al.*, 1976). The following species of R subunit–cyclic [^{3}H]AMP complexes were used in the experiments: aged 60 days at 4°C (○), aged 20 days at 4°C (△), R subunit–cyclic [^{3}H]-AMP complex produced by the dissociation of trypsin-treated fresh protein kinase by cyclic [^{3}H]AMP (□), fresh (●) and trypsin-treated fresh (▲) R subunit–cyclic [^{3}H]AMP complexes. Molar ratios of C/R were calculated from the protein concentrations and molecular weight of the homogeneous catalytic subunit, and from the known specific radioactivity of cyclic [^{3}H]AMP in the R subunit–cyclic [^{3}H]AMP complexes. The incubation mixtures contained, in a final volume of 1 ml, 10 mM-potassium phosphate/1 mM-EDTA/0.1 mM-dithiothreitol/0.5 mg of bovine serum albumin/ml, pH 6.8, and various ratios of catalytic/regulatory subunits. The mixtures were dialysed at room temperature for 6 h against 500 vol. of 10 mM-potassium phosphate/1 mM-EDTA/0.1 mM-dithiothreitol, pH 6.8. The dialysis buffer was changed every hour. After 6 h the contents of the dialysis tubes were collected, weighed and the cyclic [^{3}H]AMP retained in each tube was estimated by making the volume up to 1 ml with water and counting for radioactivity in a Triton X-100/toluene-based fluor. In some experiments the protein kinase activity ratio (values in parentheses) was measured on the contents of the dialysis tube.

fresh R subunit–cyclic [^{3}H]AMP complex (mol.wt. 35500) lost only about 15% of its bound cyclic [^{3}H]AMP on incubation with catalytic subunit. At a high C/R subunit ratio, loss of cyclic [^{3}H]AMP from fresh R subunit–cyclic [^{3}H]AMP complex was only 85% complete possibly because the fresh R subunit–cyclic [^{3}H]AMP complex was contaminated with the smaller species of R subunit–cyclic [^{3}H]AMP complex (mol.wt. 35500). R subunit–cyclic [^{3}H]AMP complex aged for 20 days at 4°C showed a 35% release of cyclic [^{3}H]AMP on incubation with catalytic subunit. At a C/R subunit molar ratio of 0.23, the activity ratio fell to 0.17 and increased thereafter with increasing addition of catalytic subunit. After aging for 60 days, the R subunit–cyclic [^{3}H]AMP complex no longer released cyclic [^{3}H]AMP on incubation with catalytic subunit, i.e. it behaved similarly to trypsin-treated fresh R subunit–cyclic [^{3}H]AMP complex, presumably because the R subunit–cyclic [^{3}H]AMP complex (mol.wt. 87000) was essentially completely degraded to its mol.wt.-35500 form. However, R subunit–cyclic [^{3}H]AMP complex produced by dissociation of trypsin-treated fresh protein kinase readily released cyclic [^{3}H]AMP on incubation with catalytic subunit and resembled the behaviour of fresh R subunit–cyclic [^{3}H]AMP complex in this respect.

Dissociation of fresh and trypsin-treated bovine liver protein kinase holoenzyme by histone

Types I and II protein kinase holoenzymes differ in their ability to be dissociated into their subunits by histone (Corbin *et al.*, 1975); the former is more easily dissociated by histone, as shown by the increase in the activity ratio. Fresh bovine liver protein kinase was prepared as described in the Materials and Methods section, and dialysed overnight against 2000 vol. of homogenization buffer at 4°C. A sample was partially hydrolysed by trypsin as described, and the trypsin-treated protein kinase was further purified by Sepharose 6B chromatography. Samples (200 μl) of fresh or trypsin-treated protein kinase were incubated with water (10 μl) or histone (Sigma, type II-A; 10 μl of a 15 mg/ml solution) at 30°C for 5 min, after which time the activity of protein kinase was measured in the absence or the presence of cyclic AMP (2 μM) in a 10 min incubation. The activity ratio of fresh protein kinase was 0.02 in the control (+water) incubation and was only increased to 0.05 on incubation with histone. In contrast, the activity ratio of trypsin-treated protein kinase in the control incubation was 0.09 and was increased to 0.50 on incubation with histone. Thus the behaviour of fresh bovine liver protein kinase was typical of a 'type II' enzyme. The trypsin-treated enzyme was much more readily dissociated by histone than the fresh enzyme, suggesting that the removal of a small peptide(s) from the regulatory subunit decreased the affinity of the regulatory subunit for the catalytic subunit.

Discussion

Tissue distribution and function of cyclic AMP-binding proteins

At least two distinct classes of cyclic AMP-binding proteins were distinguished in this study: those derived from protein kinase(s) (regulatory subunits) and those of the adenine analogue-binding protein type. Studies of fresh extracts of tissues showed that regulatory subunits can be divided into 'type I' or 'type II', depending presumably on whether they are derived from 'type I' or 'II' protein kinase (Fig. 3). 'Type I' regulatory subunit was eluted from DEAE-cellulose at a NaCl concentration lower than 0.2M, whereas 'type II' regulatory subunit was eluted at a NaCl concentration higher than 0.2M under the experimental conditions used. Such behaviour indicates a difference in net charge. There are considerable differences in regulatory subunit isoenzymes between tissues in the same animal and also between the same tissue in different animals. For example, the rat heart and kidney have mainly 'type I' regulatory subunit, brain and adipose tissue have mainly 'type II' regulatory subunit, and testis and liver have both 'type I' and 'type II' regulatory subunit in about equal quantities (Fig. 3). Although rat liver has both 'type I' and 'type II' regulatory subunits, bovine liver has predominantly 'type II' regulatory subunit (Figs. 1 and 2). The reasons for and the function of the differences in regulatory subunit composition between tissues are not known though they may be important in the regulation of protein kinase activity by cyclic AMP.

In bovine or rat liver, another species of cyclic AMP-binding protein was detected (adenine analogue-binding protein). By several criteria, it was not a regulatory subunit of protein kinase. Adenine analogue-binding protein accounted for about 45 and 20% of the cyclic AMP-binding activity of bovine liver and rat liver supernatants respectively. Binding of cyclic AMP to crude tissue extracts has been used to determine the concentration of regulatory subunit in tissues (Hofmann & Krebs, 1974) and the state of activation of protein kinase (Do Khac *et al.*, 1973; Walaas *et al.*, 1973). Our data indicate that such studies should be performed at low pH, or in the presence of adenosine, to inhibit binding of cyclic AMP to adenine analogue-binding protein.

Cyclic AMP-binding proteins, which are not protein kinase regulatory subunits, have been described in eukaryotic organisms. A protein(s) with similar properties to the adenine analogue-binding proteins described here occurs in rabbit erythrocytes and in embryonic supernatants of *Drosophila melanogaster* (Yuh & Tao, 1974; Tsuziki & Kiger, 1975). These proteins have mol.wts. of about 200000–240000 and show certain similarities to adenine analogue-binding protein in their specificity of binding. There have been other reports of less well characterized binding proteins (Chambaut *et al.*, 1971; Døskeland & Ueland, 1975; Hsu, 1975) from rat and mouse livers. It is conjecture that adenine analogue-binding protein is another receptor for cyclic AMP or any other type of receptor in the complete absence of physiological evidence. Under physiological adenine nucleotide concentrations, cyclic AMP binding by adenine analogue-binding protein was completely inhibited, although adenosine was still bound. Adenine analogue-binding protein is possibly an enzyme that binds a wide variety of purine derivatives. Other proteins, such as xanthine oxidase (EC 1.2.3.2) and arginase (EC 3.5.3.1), for example, are inhibited by a wide variety of purines (Bray, 1963; Rosenfield *et al.*, 1975) and presumably such compounds must bind to these enzymes. Adenine analogues could therefore be binding to either an active or regulatory site of an enzyme.

Species of protein kinase and regulatory subunit in bovine liver

Although multiple forms of protein kinases and regulatory subunits have been observed previously (Corbin *et al.*, 1972; Rubin *et al.*, 1972; Garbers *et al.*, 1973; Tao & Hackett, 1973), there have been no detailed studies on the effects of trypsin or aging on protein kinase or regulatory subunit. A model for these effects on bovine liver protein kinase is shown

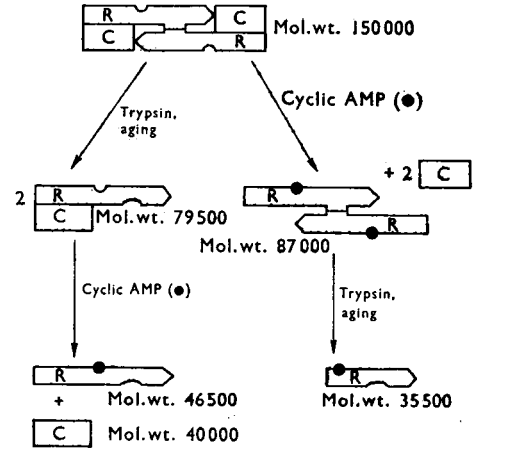


Fig. 8. *A model for the effects of cyclic AMP, aging and trypsin on protein kinase and regulatory subunit from bovine liver*

in Fig. 8. In this model, the effects of aging and proteolysis are considered to be similar. Protein kinase was an asymmetrical molecule of mol.wt. 150000. Partial tryptic hydrolysis produced an asymmetrical kinase of mol.wt. 79500. This kinase was still activated by cyclic AMP, but it was more easily dissociated by histone than the native enzyme. It was possibly a monomer (RC) of the fresh enzyme produced by the loss of a small peptide. Dissociation of this species produced a regulatory subunit (mol.wt. 46500) which reassociated with the catalytic subunit (Fig. 7).

When fresh protein kinase was dissociated by cyclic AMP, an asymmetrical regulatory subunit (mol.wt. 87000) was produced (Table 3). This reassociated with the catalytic subunit (Fig. 7) to produce the native form of protein kinase (Fig. 6). However, trypsin treatment or aging of the regulatory subunit (mol.wt. 87000) produced a regulatory subunit of mol.wt. 35500 which did not reassociate with the catalytic subunit (Fig. 7). It is proposed that there are at least two sites on the regulatory subunit that are sensitive to low concentrations of trypsin: one which is important in R–R interactions and hence in RC–RC interactions in the holoenzyme, and a second which is important in R–C interactions. The latter site is protected against tryptic attack by binding of the catalytic subunit. Although we have not observed an alteration of the catalytic subunit by low concentrations of trypsin (see Corbin *et al.*, 1972; Sugden *et al.*, 1976), a small change in molecular size of this protein could not be completely ruled out. Only a single species of mol.wt. 40000 could be observed in crude extracts (Corbin *et al.*, 1976). It is not known whether proteolytic attack on protein kinase or regulatory subunit has any significance, other than possibly a degradative role, in the regulation of protein kinase *in vivo*. From the results reported here, it is clear that partial tryptic hydrolysis is a valuable tool in studying the mechanism of protein kinase action. It is also technically interesting that forms of the regulatory subunit can be isolated that will not inhibit the catalytic subunit. This criterion is frequently used to demonstrate whether or not certain cyclic AMP-binding proteins are derived from protein kinase, and as such may be in error.

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