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Purification and Properties of a Microsomal Cyclic Adenosine Monophosphate Binding Protein Required for the Release of Tyrosine Aminotransferase from Polysomes†

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ABSTRACT: The isolation and partial purification of a microsomal protein which binds adenosine 3′,5′-cyclic monophosphate are described. The protein, together with cAMP, is required for the release of tyrosine aminotransferase from neonatal rat liver polysomes. Experiments on heat stability and degradation by pronase, ribonuclease and deoxyribonuclease confirm the protein nature of the binding factor. The protein has a molecular weight of 40,000 by zone sedimentation and binds cAMP with an intrinsic association constant of 3.6×10^{-9} M. cAMP remains unchanged as a result of binding. The most powerful competitors of the binding of [^{3}H]cAMP to the protein are: unlabeled cAMP, monobutyryl-cAMP, dibutyryl-cAMP, cGMP, and cIMP. None of the cyclic pyrimidine nucleotides which were tested are competitors and deoxy-cAMP is only a very weak competitor. 2′,3′-cAMP and a range of other nucleotides are without effect on binding of cAMP.

In previous work from this laboratory a cytoplasmic translational control mechanism for enzyme synthesis has been characterized (Chuah and Oliver, 1971, 1972). The system was found during investigations on the mechanism of induction of tyrosine aminotransferase in neonatal rat liver, and by experiments *in vitro* it was demonstrated to operate at the release step in the synthesis of subunits of the enzyme on microsomal-bound polysomes (Chuah and Oliver, 1971, 1972). Adenosine 3′,5′-cyclic monophosphate[1] was shown to be a specific effector involved in the mechanism, and by fractionation of the system *in vitro* a further component was demonstrated. This component, a release factor, is normally found in microsomes of fetal, neonatal, and adult rat liver (Chuah and Oliver, 1971) and it binds cAMP with high specificity and high affinity. The factor has no activity in the release function in the absence of cAMP and is not active with other cyclic nucleotides. In the present paper, the purification and characterization of the release factor are described.

Methods

Animals. Adult male or female rats of the Wistar strain of *Rattus norwegicus* were used without special preparation. Animals were killed by a blow to the head and cervical fracture. Livers were excised immediately and chilled in ice-cold 0.25 M sucrose in TKM buffer.

Preparation of the Crude Microsomal Extract. All steps in the preparation of the crude microsomal extract were at 0–4°. Approximately 40 g of rat liver was homogenized in a coaxial homogenizer in two volumes of 0.25 M sucrose in TKM buffer. The homogenate was centrifuged at 600*g* for 10 min in the PR-2 International centrifuge and the resultant supernatant was further centrifuged at 6000*g* for 15 min. The 6000*g* supernatant was centrifuged at 105,000*g* (1 hr) in the type 50

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[1] Abbreviations used for nucleoside 3′,5′-cyclic monophosphates have the word cyclic (or the symbol c) preceding the conventional abbreviation for 5′-nucleoside monophosphates: adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP); N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dibutyryl-cAMP); N^6-monobutyryladenosine 3′,5′-cyclic monophosphate (monobutyryl-cAMP); deoxyadenosine 3′,5′-cyclic monophosphate (deoxy-cAMP). Other nucleotides are abbreviated conventionally. The Tris–EDTA–boric acid buffer of Aronsson and Grönwall (1957) is abbreviated as TEB. Double strength TEB is written as 2 × TEB. Other abbreviations used are: TTM buffer, 50 mM Tris-HCl–8 mM theophylline–6 mM mercaptoethanol (pH 7.4); TM buffer, 50 mM Tris-HCl–6 mM mercaptoethanol (pH 7.4); TKM buffer, 50 mM Tris-HCl–25 mM KCl–5 mM $MgCl_2$ (pH7.4).

rotor of the Spinco L2-65 ultracentrifuge. The pellet was washed twice by resuspension in the homogenizing solution and centrifugation at 105,000*g* (1 hr). The pellet was resuspended in 0.5 M NH_4Cl in TM buffer, and the suspension centrifuged at 105,000*g* (1 hr). The supernatant was carefully removed and brought to 55% saturation with ammonium sulfate. The precipitate was harvested by centrifugation, resuspended in 5 mM Tris-HCl–6 mM β-mercaptoethanol (pH 7.4), and dialyzed against 200 volumes of the same buffer for 16 hr on a rotating dialyzer. The dialyzed fraction was centrifuged at 25,000*g* for 30 min in the PR-2 centrifuge, and the supernatant was collected and stored on ice.

Further Purification of the Crude Microsomal Extract by Preparative Electrophoresis in Acrylamide Gel. The procedure was based on the method of Shuster and Schrier (1967) utilizing a U-tube apparatus. A sucrose gradient of 20–60% with an inverse buffer gradient of 2 × TEB (pH 9.2) to 1.5 × TEB (pH 9.2) was used. The left arm of the U-tube contained 10 ml of acrylamide solution (7.5% acrylamide, 3% bis(acrylamide) as a percentage of the acrylamide concentration, 10% sucrose, 0.25 × TEB, pH 9.2) which was layered over the 20% sucrose. The right arm contained 5.5 ml of acrylamide–bis(acrylamide) solution as above, 10% sucrose, 3 × TEB layered over the sucrose. Polymerization was effected using *N,N,N′,N′*-tetramethylethylenediamine as catalyst and ammonium persulfate as accelerator. The left arm was connected to the cathode and the right arm to the anode. The apparatus was prerun for 30 min at an applied voltage of 500 V and a current of 14 mA. The left-hand arm was flushed with fresh electrode buffer and 2 ml of crude microsomal extract made to 10% sucrose was layered beneath the buffer in the left-hand arm. A small amount of Bromophenol Blue in 20% sucrose was layered beneath the microsomal extract. The apparatus was run at 500 V and 14 mA for approximately 3 times the length of time required for the dye to run through the acrylamide gel. About 20 fractions of 1 ml were collected by displacement with 60% sucrose applied in the right-hand outlet hole. The fractions were assayed for cAMP binding, and the active fractions were pooled and dialyzed against 200 volumes of TKM buffer.

Assay of cAMP Binding. Equilibrium dialysis was used for the determination of cAMP binding. Protein solution (0.5–1.0 ml) was dialyzed for 16 hr at 4° against 40 ml of TTM buffer containing [^{3}H]cAMP (10^{-8} M, 1.4 Ci/mmole). Binding was expressed as the difference in radioactive counts between equal volumes of solution from inside and outside the dialysis sac. Radioactive counting was carried out in a Nuclear Chicago Mark I scintillation counter using Diotol (Herberg, 1960) as the counting solvent. Results were corrected for dilution by estimation of the protein concentration before and after dialysis.

Zone Ultracentrifugation. This technique was carried out in the SW 56 rotor of the Spinco L2-65 ultracentrifuge. Linear sucrose concentration gradients from 5 to 20% sucrose in TM buffer were prepared in a multiple gradient maker (Hoefer Scientific Instruments, San Francisco, Calif.). Tubes were fractionated by bottom puncture and drop counting from the bottom of the tube using a fractionater made by Hoefer Scientific Instruments.

Analytical Disc Acrylamide Gel Electrophoresis. Acrylamide gel (7.5% acrylamide–3% bis(acrylamide)–0.25 × TEB, pH 9.2) was prepared in 6-mm diameter quartz tubes. Gels containing 8 M urea were also prepared. Electrophoresis was carried out in a disc gel electrophoresis apparatus made by Hoefer Scientific Instruments. TEB (pH 9.2) was used as the running buffer. Samples were applied in 20% sucrose beneath the buffer. A current of 3 mA/gel was maintained throughout electrophoresis. After electrophoresis, gels were either stained with 1% Napthalene Black in 5% acetic acid, fractionated into counting vials using an auto gel divider (Savant Instruments, Hickwelle, New York), or scanned at 280 mμ using a disc gel scanner fitted to a Gilford 2400 spectrophotometer. After staining, gels were destained with 5% acetic acid.

Assay for Tyrosine Aminotransferase Release Activity. Release of tyrosine aminotransferase from rat liver microsomes was assayed according to the procedure described by Chuah and Oliver (1971). Rat liver microsomes from 1- to 2-day-old rats were prepared and washed with 50 mM Tris-HCl–27 mM KCl–5 mM $MgCl_2$ (pH 8.0) to remove the release activity. Release activity was reconstituted by addition of extracts prepared from adult rat liver microsomes to a 0.5-ml suspension of microsomes in a final volume of 1 ml of 50 mM Tris-HCl–275 mM KCl–5 mM $MgCl_2$ (pH 8.0)–1 mM cAMP–5 mM theophylline. Reaction was initiated by the addition of the microsomal suspension. The suspension was incubated at 37° for 1 hr, the reaction stopped by dilution, and the mixture assayed for tyrosine aminotransferase activity. Controls were incubated under the same conditions in the absence of the microsomal extract.

Protein Determination. The method of Lowry *et al.* (1951) was used to determine protein in suitably diluted aliquots. Lyophylized crystalline bovine serum albumin was used as a standard.

Chemicals. cAMP, 5′-AMP, 2′,3′-cAMP were obtained from Sigma Chemical Co. (St. Louis, Mo.). cIMP, cGMP, cUMP, cTMP, 2′deoxy-cAMP, dibutyryl-cAMP, cCMP, ADP, ATP, 3′AMP, 2′-deoxy-AMP, 2′-deoxy-GMP, 5′-GMP were obtained from Boehringer und Sohne (Mannheim, GmbH) or from Sigma. Monobutyryl-cAMP was prepared from dibutyryl-cAMP by the method described by Falbriard *et al.* (1967). Theophylline was obtained from Prosana Laboratories (Sydney, Australia). Pronase was obtained from K & K Laboratories (Plain View, N. Y.). Ribonuclease (100 Kunitz units/mg) and deoxyribonuclease (1950 Kunitz units/mg) were obtained from Sigma. Lyophylized bovine serum albumin was obtained from Sigma. [^{3}H]cAMP was obtained from either Schwartz (Orangeburg, N. Y.) or from the Radiochemical Centre (Amersham, Buckinghamshire, England) at various specific activities. The tritium label was in position 8 of the adenine ring.

Results

The crude microsomal extract was prelabeled with [^{3}H]-cAMP and then dialyzed to remove free nucleotide. Zone centrifugation then demonstrated the existence of two cAMP binding species of which a slower sedimenting species was predominant. Disc acrylamide gel electrophoresis of the same crude microsomal extract and subsequent scanning at 280 mμ showed a fast-moving peak of absorbance in addition to several slower moving bands. Fractionation of the gel and scintillation counting showed a large, predominant, fast-moving band of radioactivity which coincided exactly with the fast-moving peak of absorbance at 280 mμ. Figure 1 shows the 280-mμ profile of the crude fraction after disc polyacrylamide electrophoresis and the radioactive counts after fractionation of the same gel. Subsequent characterization of the cAMP binding species, to be described later in the text, showed that the fast-moving cAMP binding band in electrophoresis corresponded to the slower sedimenting species

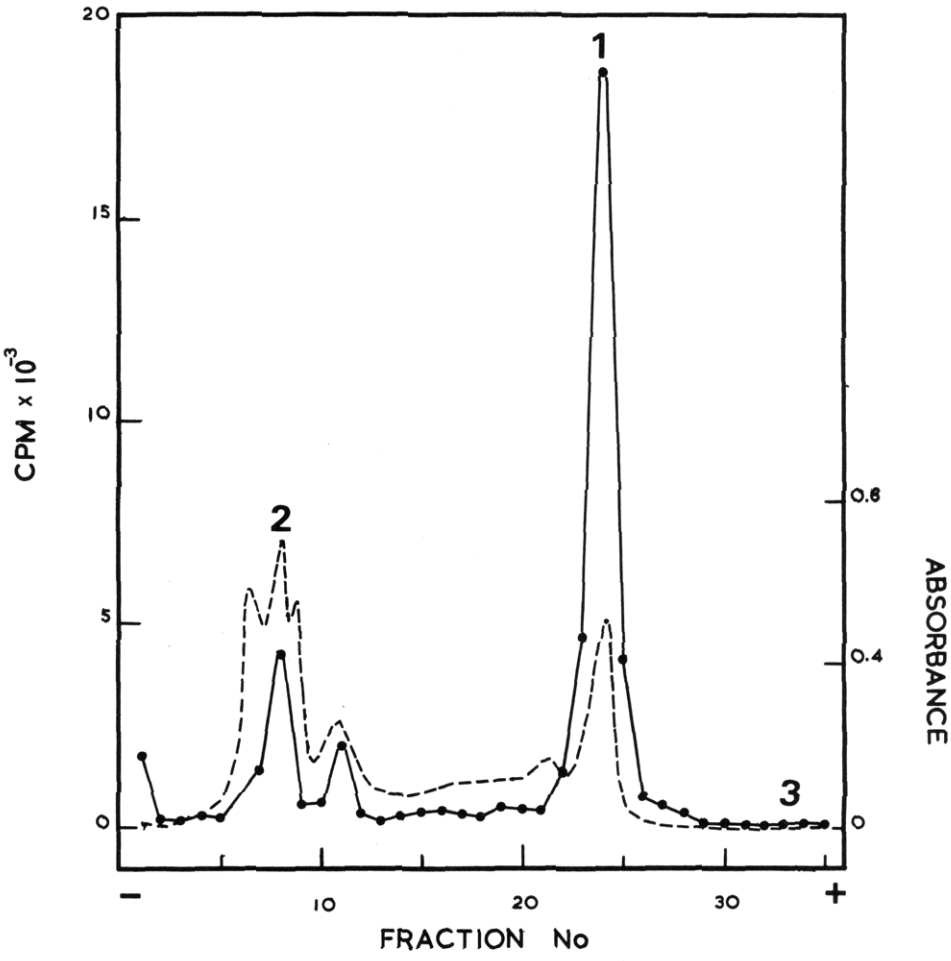

FIGURE 1: Disc polyacrylamide gel electrophoresis of the protein–[^{3}H]cAMP complex prepared from the crude microsomal fraction. A highly concentrated protein sample was treated with a saturating level of [^{3}H]cAMP and dialyzed for 16 hr against two changes of 200 volumes of TM buffer to remove free radioactivity. After electrophoresis, the gel was scanned at 280 mμ (- - - - - -), then fractionated and each fraction counted in 10 ml of Diotol (●). Sections of gel (3 mm) numbered 1, 2, and 3 were taken from other polyacrylamide gels using the 280-mμ absorbance profile as a guide. These sections were macerated and tested for tyrosine aminotransferase release activity.

found in zone ultracentrifugation. Only the lower molecular weight species from zone ultracentrifugation showed release activity. Fractionation of a disc gel into three sections of 3 mm each showed only the fast-moving peak of absorbance (area 1) to have release activity (see Figure 1). The subsequent experiments deal solely with the characterization and purification of the microsomal tyrosine aminotransferase release factor.

The cAMP binding factor was characterized as protein by heat treatment at 50° and digestion studies with pronase, ribonuclease, and deoxyribonuclease at 37°. Heat treatment and pronase digestion rendered the material incapable of binding cAMP. Ribonuclease and deoxyribonuclease digestion had no effect on binding.

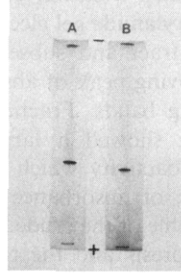

FIGURE 2: Analytical disc polyacrylamide gel electrophoresis of the purified release protein; 40 μg of protein was applied to the gel. Stained gels of the release protein are shown in the absence (A) and presence (B) of 8 M urea.

The use of electrophoretic purification was based upon the rapid migration of the cAMP-binding protein. A 66-fold purification of the cAMP-binding protein relative to microsomes was obtained using polyacrylamide gel–sucrose density preparative electrophoresis. Table I gives the total units, specific radioactivity, and percentage yield throughout the course of purification. Analytical acrylamide gel electrophoresis or the product showed a single strongly stained band even in the presence of 8 M urea. Disc gels of the purified fraction are shown in Figure 2.

Affinity chromatography using Sepharose 4B (Cuatrecasas, 1970) with cAMP covalently attached either to an acetaminoethyl Sepharose derivative through the nitrogen of position 6 of the adenine residue or directly to cyanogen bromide activated Sepharose through the same adenine position was attempted, but was found unsuitable. Although up to 80% of the binding activity could be attached to the Sepharose when cAMP was attached through an acetaminoethyl side chain, it was impossible to elute more than a minute fraction using 10^{-3} M cAMP. No attachment of the protein was obtained

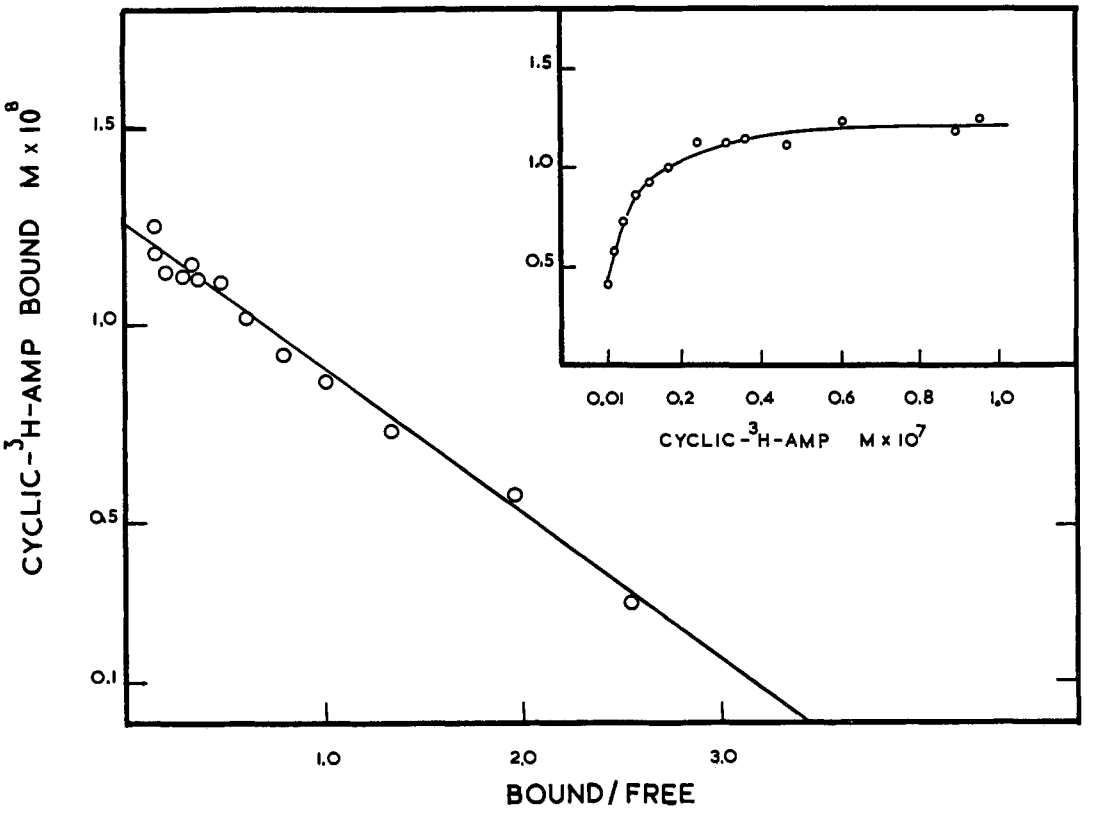

FIGURE 3: Inset: The interaction of [^{3}H]cAMP with the release protein as a function of the equilibrium concentration of the nucleotide. Concentrations of bound nucleotides were standardized to equal protein concentrations (180 mg/l). A Scatchard plot of the same data using a linear regression yielded an intrinsic association constant of 3.6×10^{-9} M.

when cAMP was linked through the 6-amino position directly to the Sepharose.

The purified protein bound [^{3}H]cAMP with a high affinity. A measure of the amount of cAMP bound as a function of ligand concentration is shown in Figure 3. A Scatchard plot (Scatchard, 1949) of these data was performed to determine the intrinsic association constant of the protein complex. The value of k was found to be 3.6×10^{-9} M for the electrophoretically purified protein. The properties of the crude microsomal fraction showed good agreement with those of the purified fraction. The value of the mean and standard error from determinations of the intrinsic association constant using the crude microsomal fraction was $(3.50 \pm 0.20) \times 10^{-9}$ M. Only 25% of bound [^{3}H]cAMP could be removed after 96 hr dialysis with several buffer changes. Assay of the crude microsomal extract for cAMP by the method of Brown *et al.* (1970) showed that considerable endogenous cAMP was bound to protein prior to assay by equilibrium dialysis.

Acid extraction of the protein-[^{3}H]cAMP complex and subsequent paper chromatography of these extracts showed cAMP was not degraded after binding. Table II gives the results of this experiment.

Zone ultracentrifugation of the electrophoretically purified protein bound with tritiated cAMP gave only a single peak of radioactivity having an $s_{20,w}$ of 3.13 calculated by the method of McEwen (1967). Binding of each fraction with [^{3}H]cAMP *after* zone centrifugation gave an $s_{20,w}$ identical with the sedimentation coefficient obtained using the protein-cAMP complex. The crude microsomal fraction prelabeled with [^{3}H]-cAMP contained a minor peak having an $s_{20,w}$ of 4.66. This heavy component amounted to less than 10% of the 3S peak and was inactive in the enzyme-release function. An approximate estimation of molecular weight was obtained using the formula $s_1/s_2 = (M_1/M_2)^{2/3}$ (Martin and Ames, 1961) and the value of $s_{20,w}$ obtained for lyophylized crystalline bovine serum albumin. A molecular weight of approximately 40,000 was obtained for the 3S protein and 72,000 for the 4.6S protein. The sedimentation profile of the purified labeled 3S protein is shown in Figure 4.

Competition studies using other nucleotides showed that only purine 3′,5′-cyclic monophosphates were effective com-

TABLE I: Purification of the cAMP-Binding Release Protein from Rat Liver Microsomes.[a]

Fraction	Total (pmoles) cAMP Bound	Total Protein (mg)	Binding Activity (pmoles of cAMP/mg of Protein)	% Yield
Microsomes	422	715	0.5	
Crude microsomal fraction	273	46.2	5.9	67
Acrylamide gel sucrose density fraction	107	3.2	29.5	23

[a] [^{3}H]cAMP at a concentration of 10^{-8} M (1.4 Ci/mmole) was used to assay binding. The microsomal protein was first fractionated and then assayed for cAMP binding by equilibrium dialysis.

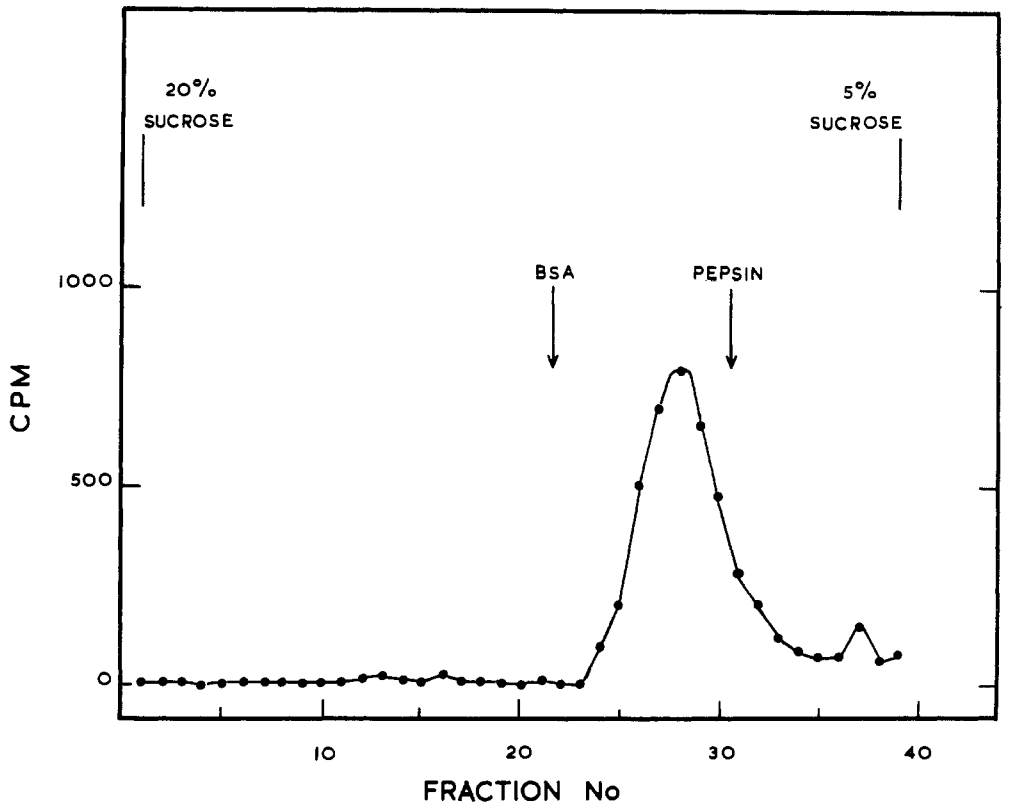

FIGURE 4: Zone ultracentrifugation of the protein–[^{3}H]cAMP complex. The electrophoretically purified protein was exposed to a saturating concentration of [^{3}H]AMP and dialyzed for 16 hr against 2 changes of 200 volumes of TM buffer to remove free radioactivity. Centrifugation was for 12 hr at 56,000 rpm. After fractionation into counting vials the fractions were counted in 10 ml of Diotol. Six-drop fractions were collected. Bovine serum albumin and pepsin were used as markers.

petitors. Table III shows the effect of a wide range of cyclic and other nucleotides on the binding of [^{3}H]cAMP to the crude microsomal fraction. Similar studies restricted to the purine 3′,5′-cyclic monophosphates using the electrophoretically purified protein were essentially identical with those using the crude microsomal extract. Cyclic pyrimidine nucleotides show inhibition of cAMP binding only when they are present at an excess concentration ratio of 10,000. Noteworthy is the apparent obligatory requirement of the 2′-oxygen atom since 2′-deoxy-cAMP is a poor competitor.

The purified protein was also tested for protein kinase activity by the method of Chambaut *et al.* (1971). Using histone and bovine serum albumin as substrates for phosphorylation, protein kinase activity which was stimulated threefold by 5×10^{-6} M cAMP was found at pH 6.6, but no activity dependent on cAMP was found at pH 8.0, which is the pH optimum for the release activity.

TABLE II: Characterization of the Bound Radioactivity of the Protein–Ligand Complex as cAMP.[a]

Nucleotide	Recovered Counts (cpm)
5′-AMP	27
Adenosine	0
cAMP	1793

[a] The protein was prelabeled with [^{3}H]cAMP and exhaustively dialyzed to remove free radioactivity. The preparation was extracted with 6% trichloroacetic acid. The acid supernatant was extracted three times with 10 volumes of ether. The aqueous phase after ether extraction was concentrated by freeze-drying and 10 μl was applied to EDTA-washed Whatman No. 1 chromatography paper. cAMP, 5′-AMP, and adenosine were applied to the labeled spot as markers. The paper was chromatographed in 2-propanol–ammonia–water (7:1:2) for 12 hr in descending chromatography. Spots were identified under ultraviolet light, cut out, and counted together with 0.5 ml of water in 10 ml of Diotol.

Discussion

Disc polyacrylamide gel electrophoresis and zone ultracentrifugation show the tyrosine aminotransferase release activity of rat liver microsomes to be associated with a cAMP-binding factor having a high mobility in electrophoresis and a molecular weight of approximately 40,000. Studies using heat treatment at 50° and digestion at 37° using pronase, ribonuclease, and deoxyribonuclease demonstrated the release factor to have a protein moiety. The high mobility in disc polyacrylamide electrophoresis was used to (partially) purify the release protein.

The intrinsic association constant of 3.6×10^{-9} M shows the very high affinity of the release factor for cAMP. In addition, the tight binding is indicated by the fact that only about 25% of bound [^{3}H]cAMP can be removed by dialysis even after 96 hr with several buffer changes. The protein as prepared from liver microsomes was shown by direct assay to contain bound cAMP, presumably derived from endogeneous sources. However, it is obvious from the data of the binding

assay that such cAMP is exchangeable, even though it is not removable by dialysis. Experiments are in progress to attempt the removal of endogeneous cAMP.

Competitive studies with cyclic nucleotides and other nucleotides gave further correlation between the properties of the cAMP-binding protein and the tyrosine aminotransferase release activity. It is clear from these studies that the purine 3′,5′-cyclic monophosphates are effective inhibitors of both binding of cAMP and tyrosine aminotransferase release (Chuah and Oliver, 1971). Both these studies show the obligatory requirement of the 3′,5′-phosphodiester linkage and the 2′-O of the ribose moiety for both binding and release activity (Chuah and Oliver, 1972). From the competitive studies it can be seen that the effectiveness of competitors for binding of labeled cAMP decreases in the following order: cAMP, monobutyryl-cAMP, cIMP, dibutyryl-cAMP, cGMP, deoxy-cAMP, pyrimidine 3′,5′-cyclic monophosphates. The tyrosine aminotransferase system may differ in that dibutyryl-cAMP appears to be as effective as cAMP in eliciting enzyme release at a concentration of 1 mM. However, the study did not include an analysis of the effect of concentration of the esterified derivative.

cAMP-binding proteins have been found in a variety of tissues and biochemical activity which is dependent on cAMP, *e.g.*, protein kinase activity has been defined for some of them (Walsh *et al.*, 1968; Corbin and Krebs, 1969; Gill and Garren, 1969, 1970, 1971; Eil and Wool, 1971). Emmer *et al.* (1970) have reviewed evidence for the function of a cAMP-binding protein in the induced synthesis of enzymes in bacteria.

The mechanism by which the cAMP-binding protein brings about release of nascent tyrosine aminotransferase from polysomes is currently unknown. Chuah and Oliver (1971) have shown that the release phenomenon is specific to cAMP which is the nucleotide most strongly bound to the microsomal protein. A reasonable theory would be that cAMP binds to the release protein and activates the protein by some conformation change, into an active species. It is important to note that it is the protein–AMP complex which is active, and not a protein from which a cAMP-binding protein has dissociated, such as the protein phosphokinase from adrenal cortical tissue (Gill and Garren, 1971) and beef heart (Erlichman *et al.*, 1971). The phosphorylation of microsomal protein *in vivo* (Blat and Harel, 1964) and ribsomal protein *in vivo* and *in vitro* (Loeb and Blat, 1970; Eil and Wool, 1971) suggests a possible mechanism for activation of release. Release protein as purified by gel electrophoresis contains a protein kinase activity, but the fact that tyrosine aminotransferase release occurs in the absence of ATP (Chuah and Oliver, 1971) would appear to eliminate phosphorylation reaction as part of the release mechanism. In any case, the stimulation of protein kinase activity by cAMP occurs with the release protein at pH 6.6 but not at the optimum pH for release of tyrosine aminotransferase from polysomes. A recent paper presents data on the complete absence of protein phosphokinase activity from the microsome fraction of rat liver (Chen and Walsh, 1971), but Eil and Wool (1971) have published contrary evidence for the occurence of protein phosphorylation in rat liver ribosomes which is slightly stimulated by cAMP and effectively removed by washing at high ionic strength.

Other possible mechanisms of release could be the enzymatic hydrolysis of the polypeptide–tRNA ester linkage by an esterase which might be activated by cAMP or perhaps the protein–cAMP complex is a necessary structural protein whose replacement to washed microsomes restores the integrity of the release mechanism.

TABLE III: Competition for cAMP Binding Sites Using a Range of Nucleotides.[a]

Competitor	% Inhibition
cAMP	99
Monobutyryl-cAMP	82
Dibutyryl-cAMP	39
cGMP	19
cIMP	46
cTMP	0
cUMP	0
cCMP	0
Deoxy-cAMP	9
ADP	1
ATP	0
2′,3′-cAMP	2
3′-AMP	0
Deoxy-5′AMP	0
Deoxy-ATP	2
Deoxy-GMP	2
5′-AMP	3
5′-GMP	0

[a] 1 ml of crude microsomal fraction was dialyzed in the presence of the competitor nucleotide and [^{3}H]cAMP (10^{-8} M, 1.4 Ci/mmole). The concentration ratio of competitor nucleotide to [^{3}H]cAMP was 100:1.

Satre *et al.* (1971) have concluded that adenylyl cyclase activity of bovine adrenal cortex is mainly associated with the microsomes and that the microsomal fraction displays a high affinity for cAMP. The total distribution of adenylyl cyclase in rat liver is not clear, but Pohl *et al.* (1969) have demonstrated a glucagon-sensitive enzyme, which is insensitive to epinephrine associated with plasma membrane. A close association of adenylyl cyclase to the release mechanism would seem a favorable situation and Chuah and Oliver (1972) have presented indirect evidence for the occurrence of adenylyl cyclase in the rough-surfaced endoplasmic reticulum of liver cells. This structure also contains the nascent tyrosine aminotransferase.

The experiments in this paper show that a cAMP-binding protein is involved in the release of a specific enzyme from polysomes *in vitro*. The close association of cAMP binding activity and polysomes in the microsomal fraction suggest a further possible control point in protein synthesis.

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N-[(9-β-D-Ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine, a Modified Nucleoside Isolated from *Escherichia coli* Threonine Transfer Ribonucleic Acid†

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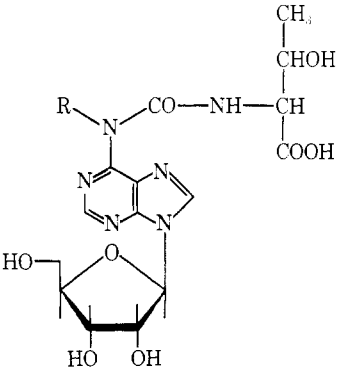
ABSTRACT: A hitherto undiscovered modified nucleoside was isolated from *Escherichia coli* $tRNA^{Thr}$ and its structure determined to be *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine (mt^6A). Its chemical and physical properties, including ultraviolet spectra, thin-layer chromatographic and electrophoretic mobilities, and the liberation of N^6-methyladenosine and threonine upon treatment with alkali, are similar to those of *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine, previously found in unfractionated yeast tRNA. Trimethylsilylation produced a mixture of the pentasilyl derivative of mt^6A (molecular ion, *m/e* 786) and the trisilyl derivative of N^6-methyladenosine (molecular ion, *m/e* 386), which confirmed both the molecular weight and the presence of methyl at the N^6 position of the base in the underivatized nucleoside.

N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]threonine(I) was first isolated from unfractionated yeast tRNA and characterized by Hall and his coworkers (Chheda *et al.*, 1969; Schweizer *et al.*, 1969). A survey of its distribution in individual *Escherichia coli* tRNAs indicated that it is present in $tRNA_3^{Ser}$ (AGU, AGC), $tRNA_1^{Met}$, $tRNA^{Lys}$, $tRNA^{Ile}$, $tRNA^{Thr}$, and $tRNA^{Asn}$, presumably in the position next to the 3′-hydroxyl end of the anticodon in all cases, but not in other tRNAs (Ishikura *et al.*, 1969; Kimura-Harada *et al.*, 1972). There is a striking similarity among tRNAs that contain t^6A,[1] since they always recognize codons starting with A.



I, R = H
II, R = CH_3

It is also known that several other modified nucleosides with structures similar to that of t^6A are present in these *E. coli* tRNAs (Ishikura *et al.*, 1969; Cory and Marcker, 1970;

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[1] Abbreviations used are: t^6A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-carbamoyl]threonine; mt^6A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine; m^7G, 7-methylguanosine; s^4U, 4-thiouridine; A_{260} or A_{280} unit, an amount of material with an absorbance of 1.0 at 260 mμ or 280 mμ when dissolved in 1 ml of water and measured with a 1-cm light path.