

# An Adenosine 3':5'-Monophosphate-Adenosine Binding Protein from Mouse Liver

PURIFICATION AND PARTIAL CHARACTERIZATION\*

(Received for publication, July 12, 1976)

PER MAGNE UELAND† AND STEIN OVE DØSKELAND

From the Cell Biology Research Group, Preclinical Institutes, University of Bergen, Bergen, Norway

A cyclic AMP-adenosine binding protein from mouse liver has been purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate and by analytical ultracentrifugation. The binding protein had a Stokes radius of 48 Å based on gel chromatography. Both the purified binding protein and the binding activity in fresh cytosol sedimented as 9 S on sucrose gradient centrifugation. The homogeneous protein had a sedimentation coefficient ( $s_{20,w}$ ) of  $8.8 \times 10^{-13}$  s, as calculated from sedimentation velocity experiments. By use of the Stokes radius and  $s_{20,w}$ , the molecular weight was calculated to be 180,000. The protein was composed of polypeptides having the same molecular weight of 45,000 as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and thus appeared to consist of four subunits of equal size. The isoelectric point,  $pI = 5.7$ .

The binding capacity for cyclic AMP increased by preincubating the receptor protein in the presence of  $Mg^{2+}$  ATP. This process, tentatively termed activation, was studied in some detail and was shown not to be accompanied by dissociation, aggregation, or phosphorylation of the binding protein.

Cyclic AMP was bound to the protein with an apparent dissociation constant ( $K_d$ ) of  $1.5 \times 10^{-7}$  M. The binding of cyclic AMP was competitively inhibited by adenosine, AMP, ADP, and ATP whose inhibition constants were  $8 \times 10^{-7}$  M,  $1.2 \times 10^{-6}$  M,  $1.5 \times 10^{-6}$  M, and higher than  $5 \times 10^{-6}$  M respectively.

A hyperbolic Scatchard plot was obtained for the binding of adenosine to the activated binding protein, indicating more than one site for adenosine. The binding of adenosine to the site with the highest affinity ( $K_d = 2 \times 10^{-7}$  M) for this nucleoside was not suppressed by excess cyclic AMP and was thus different from the aforementioned cyclic AMP binding site.

Cyclic GMP, GMP, guanosine, cyclic IMP, IMP, and inosine did not inhibit the binding of either cyclic AMP or adenosine.

The binding protein had no cyclic AMP phosphodiesterase, adenosine deaminase, phosphofructokinase, or protein

kinase activities, nor does it inhibit the catalytic subunit of the cyclic AMP-dependent protein kinase.

Cyclic AMP probably exerts its effects by interacting with intracellular proteins. Much interest has been devoted to the binding of cyclic AMP to the regulatory subunit of cyclic AMP-dependent protein kinase (ATP: protein phosphotransferase EC 2.7.1.37) (1-4; for review, see ref. 5). Cyclic AMP receptors not related to protein kinase have recently been described in various eukaryotes (6-8).

We have reported the existence in mouse liver of a cyclic AMP binding protein not related to cyclic AMP-dependent protein kinase. The cyclic AMP binding sites were activated by treatment with  $Mg^{2+}$  ATP (9). We here describe the purification of this protein to apparent homogeneity and present some characteristics of the purified binding protein.

## MATERIALS AND METHODS

NMRI mice were purchased from Folkehelsen, Oslo. All nucleotides and nucleosides, bovine serum albumin, Hepes,<sup>1</sup> histone (Type II), and rabbit muscle phosphofructokinase were from Sigma Chemical Co. Human  $\gamma$ -globulin was from Kabi, Sweden. Casein (nach Hammerstein) was a product of Merck, Darmstadt. Cytochrome *c*, chymotrypsinogen, muscle aldolase, yeast alcohol dehydrogenase, aldolase, glycerolphosphate dehydrogenase, triosephosphate isomerase, and ferritin were obtained from Boehringer Mannheim Co. Sephadex G-150 (fine) and Sephadex G-200 (fine) were from Pharmacia, Sweden; DEAE-cellulose (DE 52) from Whatman Biochemicals Ltd. and Ampholine (pH 5-8) from LKB, Sweden. Polyethyleneimine-impregnated cellulose thin layer sheets (Polygram Cel 300 PEI, 0.1 mm) were purchased from Macherey Nagel & Co, Germany and polyethylene glycol 6000 from Fluka, Switzerland. Cyclic [ $^3H$ ]AMP (27 Ci/mmol) and [ $^2-^3H$ ]adenosine (21 Ci/mmol) were obtained from The Radiochemical Centre (Amersham), and the purity checked by thin-layer chromatography. [ $\gamma$ - $^{32}P$ ]ATP was prepared by a slight modification of the method of Glynn and Chappel (10). Other chemicals were of analytical grade.

**Thin-Layer Chromatography**—This was carried out at room temperature on polyethyleneimine-impregnated cellulose sheets. Three solvent systems were used: A, 0.5 M ammonium acetate:96% ethanol (5:2, v:v); B, 1.2 M LiCl; C, distilled water (cyclic AMP, AMP, and adenosine were separated in System A; ATP, ADP, AMP, and cyclic AMP in System B; AMP, adenosine, inosine, and adenine in Systems A and C).

**Measurement of Cyclic [ $^3H$ ]AMP and [ $^3H$ ]Adenosine Binding**—To

\* This work was supported by grants from the Norwegian Research Council for Science and the Humanities.

† Fellow of the Norwegian Research Council for Science and the Humanities.

<sup>1</sup> The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid. SDS, sodium dodecyl sulfate.

study the binding of ligands to the protein, the experimental conditions must be such that only minor changes in activation occur during the incubation. These demands were met by performing the incubations at 0° in the presence of 20% glycerol, conditions found to preserve the degree of activation. However, during the purification procedure, measurement of binding activity in separate fractions was performed at 30° for 1 h *i.e.* under nonequilibrium conditions. The measurement of cyclic AMP binding activity was conducted in the presence of Mg<sup>2+</sup> and KCl at 30°. In this way, cyclic AMP binding activity could be detected without prior treatment of the binding protein with Mg<sup>2+</sup> ATP. Therefore, two incubation conditions were used.

1. For studies of binding kinetics the samples and various concentrations of cyclic [<sup>3</sup>H]AMP (0.54 Ci/mmol) or [<sup>3</sup>H]adenosine (1.42 Ci/mmol) were incubated at 0° for 18 h in 15 mM Hepes buffer, pH 7.5, containing 20% glycerol and 10 mM 2-mercaptoethanol.

2. To measure the cyclic AMP or adenosine binding activity during the purification procedure, samples of 20 μl from the fractions were incubated in the presence of 10 μM cyclic [<sup>3</sup>H]AMP (0.54 Ci/mmol), 150 mM KCl, and 10 mM magnesium acetate in 15 mM Hepes buffer, pH 7.0 or 10 μM [<sup>3</sup>H]adenosine (1.42 Ci/mmol) in 15 mM Hepes buffer, pH 7.0 (Buffer A). The incubation proceeded for 60 min at 30°.

All incubations were performed in a volume of 120 μl and were stopped by pipetting an aliquot of 100 μl into 2 ml of ice-cold 80% saturated ammonium sulfate containing 100 μM unlabeled cyclic AMP or adenosine. After blending on a Vortex mixer, the protein was allowed to precipitate for 1 h at 0° and then was collected by suction through Millipore filters (HAWP, 0.45 μm). The filters were washed three times with 2 ml of 60% saturated ammonium sulfate and put in scintillation vials. The precipitate was dissolved in 1 ml 1% SDS and 7 ml of Diluene (Packard) added.

The background radioactivity was independent of the amount of protein in the sample. It was proportional to the amount of radioactivity passing through the filter; *i.e.* about 0.35% of the cyclic [<sup>3</sup>H]AMP or [<sup>3</sup>H]adenosine applied was retained on the filters in the absence of binding protein.

To exclude the possibility that radioactive ligand was bound or trapped during or after precipitation, 50 μl of 20 μM <sup>3</sup>H-labeled ligand was mixed with the ammonium sulfate (2 ml), and then 50 μl of receptor protein (1 mg/ml) was added. The retention of radioactivity by the Millipore filters was not above background.

The following experiments were conducted to reassure that ligand (cyclic AMP or adenosine) was not released from the binding protein during or after precipitation. The binding protein (0.8 mg/ml) was incubated for 1 h at 30° in the presence of 10 μM cyclic [<sup>3</sup>H]AMP, 150 mM KCl and 10 mM Mg<sup>2+</sup>-acetate in Buffer A or 10 μM [<sup>3</sup>H]adenosine in Buffer A. An aliquot of 400 μl was applied to a Sephadex G-25 column (0.9 × 15 cm) equilibrated with 15 mM Hepes buffer, pH 7.5, and eluted with the same buffer. Fractions of 0.3 ml were collected and 20-μl aliquots counted (Fig. 1, *inset*). After the column had been calibrated, new incubations of binding protein with cyclic [<sup>3</sup>H]AMP or [<sup>3</sup>H]adenosine were performed. The fractions, eluted in a position corresponding to the material excluded from the column (Fractions 15 to 18, Fig. 1), were pooled (1.2 ml). Samples of 100 μl were counted directly or added immediately to ice-cold 80% ammonium sulfate as described. Precipitation was allowed to proceed for the time indicated on the abscissa of Fig. 1, the precipitate collected on Millipore filters and the radioactivity measured. The experimental results (for both cyclic [<sup>3</sup>H]AMP and [<sup>3</sup>H]adenosine) presented in Fig. 1, show that almost total recovery of the radioactivity excluded by Sephadex G-25 was obtained with the ammonium sulfate precipitation Millipore filtration technique. No carrier protein was necessary to give quantitative recovery of protein-bound radioactive ligand, even with dilute solutions.

The radioactivity bound to the protein, excluded from the Sephadex G-25 column (see above), was identified as cyclic [<sup>3</sup>H]AMP or [<sup>3</sup>H]adenosine by thin layer chromatography when the receptor pro-

tein was incubated with cyclic [<sup>3</sup>H]AMP or [<sup>3</sup>H]adenosine, respectively.

Using the ammonium sulfate precipitation Millipore filtration technique as reference, 5 to 25% of cyclic [<sup>3</sup>H]AMP and about 75% of [<sup>3</sup>H]adenosine bound (to both activated and unactivated receptor protein) was recovered by the following method. Aliquots (100 μl) from the incubation mixture were added to and mixed with 3 ml of ice-cold 10 mM sodium phosphate buffer, pH = 6.0, and after 1 min, filtered through Millipore filters and washed three times with 3 ml of the same buffer. When 15 mM Hepes buffer, pH = 7.5, was used, low recovery (2 to 10%) was obtained for both cyclic AMP and adenosine.

The buffer passing through the filters was collected and immediately mixed with 3 times its volume of 100% saturated ice-cold ammonium sulfate (containing unlabeled ligand). After 1 h at 0°, the mixture was filtered and the radioactivity measured as described. About 20% of the [<sup>3</sup>H]adenosine-receptor complex was recovered when the reaction mixture was added to and filters washed in 10 mM sodium phosphate, pH = 6.0. (Low and variable recovery (2 to 20%) of cyclic [<sup>3</sup>H]AMP was obtained under these conditions.) In this way, 90 to 95% of both [<sup>3</sup>H]adenosine and cyclic [<sup>3</sup>H]AMP bound could be detected when the buffer was 15 mM Hepes, pH = 7.5.

These experiments suggest that at pH = 6.0 about 75% of the binding protein was retained on the Millipore filter. Adenosine was bound to the protein whereas cyclic AMP was rapidly released. At pH = 7.5, both adenosine and cyclic AMP were bound to the receptor protein, but the receptor protein passed through the filter.

**Assay for Enzyme Activities** — Protein kinase activity was assayed essentially as described (11) by determining the amount of phosphate incorporated into histone (0.67 mg/ml) or casein (5 mg/ml) from [<sup>γ</sup>-<sup>32</sup>P]ATP (30 μM).

Phosphodiesterase activity was measured by determining the hydrolysis of 10 μM cyclic [<sup>3</sup>H]AMP in the presence of 10 mM magnesium acetate, 150 mM KCl in Buffer A. The reaction was run for 1 h at 30° and was stopped by heating. Cyclic [<sup>3</sup>H]AMP was separated from its reaction products by thin layer chromatography (System A). The cyclic AMP, AMP, and adenosine zones were cut out and counted by liquid scintillation.

Adenosine deaminase activity was measured by following the change of absorbance at 265 nm resulting from the conversion of adenosine to inosine (12) or by determination of the conversion of [<sup>3</sup>H]adenosine to [<sup>3</sup>H]inosine by thin layer chromatography (System C).

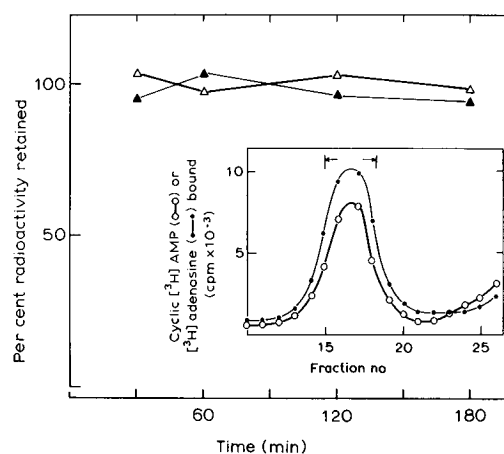


Fig. 1. Separation of the free and protein-bound radioactive ligand by Sephadex G-25 filtration and recovery of the excluded radioactivity after ammonium sulfate precipitation and collection of the protein on Millipore filters. Experimental details are given in the text. *Inset* shows binding of [<sup>3</sup>H]adenosine (●—●) or cyclic [<sup>3</sup>H]AMP (○—○) to the receptor protein as determined by Sephadex G-25 chromatography. In separate experiments the effluent corresponding to Fractions 15 to 18 were pooled and equal portions (100 μl) were counted directly or allowed to precipitate in 80% ammonium sulfate for the time indicated on the *abscissa*. [<sup>3</sup>H]Adenosine (▲—▲) or cyclic [<sup>3</sup>H]AMP (△—△) recovered after ammonium sulfate precipitation and filtration through Millipore filters as per cent of counts per min counted directly, is plotted against time of precipitation.

<sup>2</sup>The cyclic AMP-adenosine binding protein existed in its inactive form when not exposed to Mg<sup>2+</sup>ATP. In the presence of KCl and Mg<sup>2+</sup>, and high concentration of (radioactive) cyclic AMP, the binding of the nucleotide to the unactivated binding protein gradually increased with time when the incubation was performed at high temperature (30°). This phenomenon, currently under investigation, is interpreted as an activation of the protein by cyclic AMP under the conditions used. The amount of cyclic AMP bound was linear with the concentration of protein.

Phosphofructokinase activity was determined as described by Massey and Deal (13). The reaction was started by adding sample to be tested (5 to 40  $\mu$ l) or 20  $\mu$ l of fructose 6-phosphate (0.1 M) to 500  $\mu$ l of 50 mM Tris/HCl, pH 8.0, containing 5 mM  $MgCl_2$ , 50 mM KCl, 50 mM 2-mercaptoethanol, 1 mg of aldolase, 0.5 mg of glycerol phosphate dehydrogenase:triosephosphate isomerase mixture (10:1), 20 mM NADH, 2 mM ATP, and if the reaction was initiated by addition of enzyme, 4 mM fructose 6-phosphate.

**Preparation of Free Catalytic and Regulatory Subunit of Cyclic AMP-dependent Protein Kinase**—Protein kinase (isoenzyme PK I) from mouse liver was prepared through DEAE-cellulose chromatography as described (11). The holoenzyme was incubated for 2 h at 0° in the presence of 800 mM KCl, 0.1% bovine serum albumin in Buffer A, and 500  $\mu$ l of the incubate was applied to a Sephadex G-150 column (0.9  $\times$  40 cm) equilibrated with the same buffer. Fractions of 0.3 ml were collected and assayed for cyclic AMP binding activity and protein kinase activity as described in legend to Fig. 4. The protein kinase activity, independent of cyclic AMP, eluted well behind the cyclic AMP binding activity, indicating dissociation of the holoenzyme into the free catalytic and the regulatory subunits.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis of the native binding protein was performed at different pH and gel concentrations (5%, 7.5%, and 9%) and 3% cross-linking in tubes (75  $\times$  5 mm). The protein (5 to 80  $\mu$ g) was applied to the gel in 20% sucrose. The electrophoresis proceeded at 4° for 1 to 4 h (depending on the gel concentration) at  $\frac{1}{2}$  to 1 mA per gel. The following buffer systems were used: A, 20 mM phosphate buffer, pH 7.0; B, 15 mM Tris/phosphate, pH 7.8; C, 15 mM Tris/glycine, pH 8.9. The gels were stained in Coomassie blue and destained in 7% acetic acid.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber *et al.* (14) using 10  $\mu$ g of protein per tube. The electrophoresis was run in tubes (75  $\times$  5 mm) for 4 h using 6 mA per gel. Gel concentration was 7.5%. The electrophoretic mobility of the denatured receptor was compared to that of the following standard proteins: Bovine serum albumin ( $M_n = 68,000$ ), catalase ( $M_n = 58,000$ ), human  $\gamma$ -globulin heavy chain ( $M_n = 50,000$ ), ovalbumin ( $M_n = 43,000$ ), glyceraldehyde-3-phosphate dehydrogenase ( $M_n = 36,000$ ), chymotrypsinogen ( $M_n = 25,700$ ), human  $\gamma$ -globulin light chain ( $M_n = 23,500$ ).

**Isoelectric Focusing**—This was carried out by using a 110-ml column (LKB Instrument). The amount of carrier ampholyte used, with a pH range of 5 to 8 was 3% (w/v) in a 0 to 48% (w/v) sucrose gradient. The electrofocusing was initiated at 200 V; as the current decreased, the voltage was increased and maintained at 700 V. The duration of electrofocusing was 48 h.

**Sucrose Gradient Centrifugation**—Samples (200  $\mu$ l) were loaded on the top of a 10-ml linear (10 to 25% w/v) sucrose gradient. Human hemoglobin (4.5 S) was used as an internal marker, and human  $\gamma$ -globulin (7 S) and  $\gamma$ -globulin dimer (11 S) as external markers. Centrifugation was performed at 40,000 rpm for 24 h at 4° in an International B-60 ultracentrifuge. Fractions of 0.5 ml were collected and assayed for adenosine and cyclic AMP binding activity.

**Analytical Gel Chromatography**—A sample of 1.5 ml was applied to a Sephadex G-200 column (1.6  $\times$  67 cm) equilibrated with Buffer A which in some experiments contained 150 mM KCl or 20% glycerol or both. Elution was carried out with the same buffer at reverse flow (4.5 ml/h). Blue dextran was used to determine the void volume. The column was calibrated with marker proteins of known molecular weight.

**Analytical Ultracentrifugation**—Sedimentation velocity experiments were performed at 20° in a MSE Centriscan 75 ultracentrifuge. The sedimentation coefficient,  $s_{20,w}$ , was determined by centrifugation of a homogeneous solution of protein (0.5 mg/ml) in 15 mM Hepes, pH 7.0, at 60,000 rpm. The absorbance at 280 nm in the cell was measured during centrifugation using a split-beam photoelectric scanner (15).

**Determination of Protein**—Protein was estimated by the method of Klungsøyr (16) using bovine serum albumin as standard.

## RESULTS

### Purification

All steps were carried out at 0–4°.

**Step I: Preparation of Tissue Extract**—Mice of the NMRI strain were killed by cervical dislocation and their livers were rapidly removed and put into liquid  $N_2$ , where they were kept

until use. Frozen livers (400 g) were thawed, finely minced with scissors, and homogenized (1/4 w/v) with three strokes at 465 rpm in a Teflon glass homogenizer in 15 mM Tris/HCl buffer, pH 7.6, containing 10 mM EDTA, 5 mM 2-mercaptoethanol, and 0.25 M sucrose (Buffer B). The homogenate was centrifuged for 40 min at  $20,000 \times g_{av}$ .

**Step II: Polyethylene Glycol Fractionation**—Polyethylene glycol 6000 (130 g) was slowly added to 1080 ml of supernatant solution from Step I and dissolved under constant stirring for 1 h. The solution was then centrifuged for 40 min at  $20,000 \times g_{av}$ . The pellet, containing no adenosine binding activity, was discarded, and 194 g of polyethylene glycol was added to the supernatant. After centrifugation ( $20,000 \times g_{av}$  for 40 min), the pellet so obtained was dissolved in 400 ml of 10 mM Tris/HCl, pH 7.5, 4 mM EDTA, and 5 mM 2-mercaptoethanol (Buffer C).

**Step III: DEAE-cellulose (DE 52) Chromatography**—The solution obtained from Step II was applied to a DEAE-cellulose (DE 52) column (2.4  $\times$  31 cm) equilibrated with Buffer C, and the column was washed with the same buffer. Fractions of 20 ml were collected. The cyclic AMP-adenosine binding activity eluted as a sharp peak after washing with about 500 ml of buffer (Fig. 2).

**Step IV: Ammonium Sulfate Fractionation**—Fractions 32 to 40 (Fig. 2) were pooled (160 ml). Ammonium sulfate fractionation (33 to 60%) was performed by slowly adding solid ammonium sulfate with stirring to the solution. The precipitates were collected by centrifugation for 40 min at  $20,000 \times g_{av}$ .

**Step V: Sephadex G-150 Chromatography**—The ammonium sulfate precipitate from Step IV was dissolved in 7 ml of Buffer A and applied to a Sephadex G-150 column (2.6  $\times$  90 cm) equilibrated with the same buffer. Fractions of 7 ml were collected. The cyclic AMP-adenosine binding activity eluted as a sharp symmetrical peak and the fractions containing the main activity (70%) were pooled (35 ml).

**Step VI: Isoelectric Focusing**—This was performed as described under "Materials and Methods." The solution from Step V (35 ml) was diluted in the middle three-fifths of the sucrose gradient. After completion of the focusing, 2-ml fractions were collected and assayed for adenosine and cyclic AMP binding activity (Fig. 3). Fractions 17 to 23 were pooled (12 ml) and concentrated by ammonium sulfate precipitation. The precipitate was dissolved in 2 ml of Buffer A and desalted by passage through a Sephadex G-25 column (0.9  $\times$  9 cm) equi-

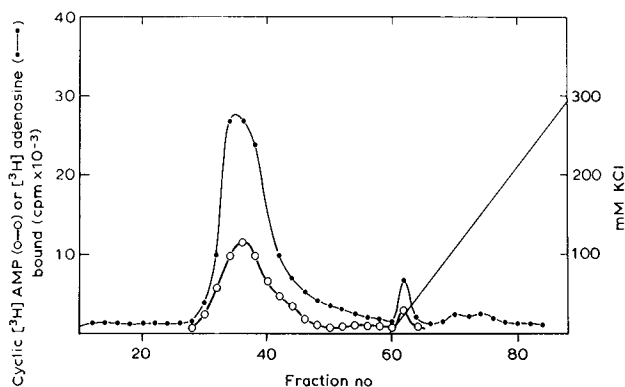


Fig. 2. DEAE-cellulose chromatogram of cyclic AMP-adenosine binding protein. Step II preparation of binding protein was chromatographed on a DEAE-cellulose column (2.4  $\times$  31 cm) as described in the text, and the fractions (20  $\mu$ l) were assayed for adenosine binding activity (●—●) and cyclic AMP binding activity (○—○).

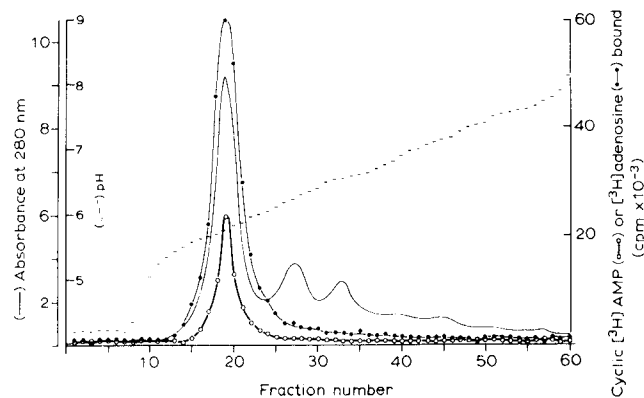


FIG. 3. Isoelectric focusing of cyclic AMP-adenosine binding protein. Step V preparation was subjected to isoelectric focusing in a 110-ml LKB column as described in the text. Isoelectric pH was 5.7 as determined in four separate experiments (range: pH = 5.68 to pH = 5.72). [ $^3\text{H}$ ]Adenosine bound, (●—●); cyclic [ $^3\text{H}$ ]AMP bound, (○—○).

brated with the same buffer.

**Step VII: Sucrose Gradient Centrifugation**—This was performed as described under "Materials and Methods." Samples of 20  $\mu\text{l}$  from each fraction were subjected to polyacrylamide gel electrophoresis in 9% gel at pH 8.9. Fractions 5 to 8 (Fig. 4A) giving one single band, were pooled and concentrated by ammonium sulfate precipitation and desalted by passage through a Sephadex G-25 column (0.9  $\times$  9 cm) equilibrated with Buffer A. The preparation was stored in liquid  $\text{N}_2$  until use.

#### Notes on Recovery and Purification

Data on the recovery of the binding protein through the various steps were impossible to obtain because crude preparations contained appreciable amounts of adenosine deaminase and cyclic AMP binding protein associated with protein kinase. The fact that the binding properties may change widely depending on the pretreatment of the binding protein (see below) made it difficult to make comparable quantization of the binding protein during different steps in the purification procedure.

About 80% of the cyclic AMP-dependent protein kinase was recovered in the pellet obtained between 0 and 11 g of polyethylene glycol/100 ml. The fraction (11 to 24 g of polyethylene glycol/100 ml) containing the main adenosine binding activity was applied to the DEAE-cellulose column from which it was eluted with start buffer, (Fig. 2) whereas the small amount of protein kinase left in the preparation after Step II was eluted with a linear gradient from 0 to 300 mM KCl (data not shown).

The amount of adenosine deaminase detected in the preparation after Step IV eluted as a peak well behind the cyclic AMP-adenosine binding activity on the Sephadex G-150 column (Step V). The ultraviolet absorbing contaminants after Step V focused essentially as two main peaks between pH 6 and 8 (Fig. 3). The cyclic AMP-adenosine binding protein was 90% pure after Step VI as judged by analytical ultracentrifugation, and the contaminants (three to four bands on polyacrylamide gel electrophoresis) sedimented in the 4 to 7.5 S region of the sucrose gradient (Step VII).

From 400 g of liver about 30 mg of homogeneous binding protein was obtained.

#### Homogeneity

Purity was checked by polyacrylamide gel electrophoresis of the native binding protein at various gel concentrations and in

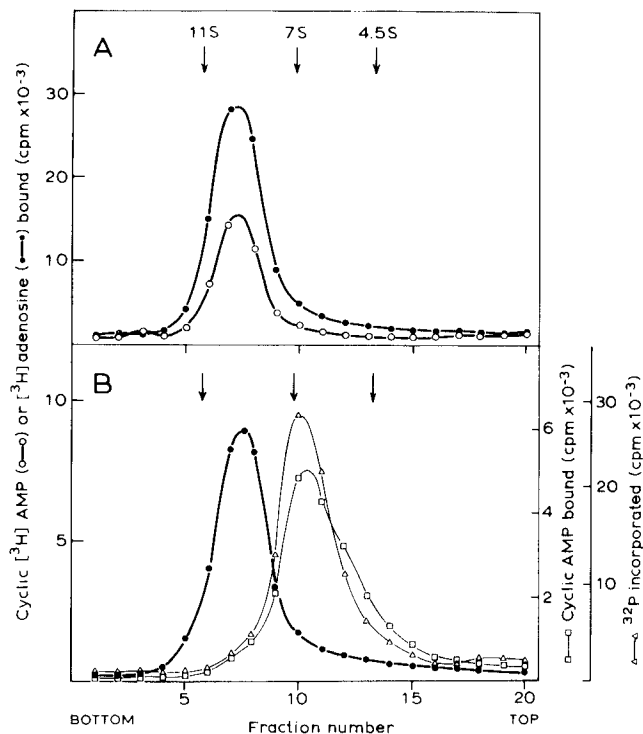


FIG. 4. A, sedimentation of highly purified binding protein (from Step VI) in a sucrose gradient. The sucrose gradient centrifugation and the determination of adenosine (●—●) and cyclic AMP (○—○) binding activity were performed as described under "Materials and Methods." B, sucrose gradient centrifugation of crude cytosol prepared immediately after animal sacrifice. Livers were homogenized in Buffer B (1/5, w/v) and the homogenate centrifuged at 100,000  $\times g$  av for 1 h. A sample (2 ml) of the supernatant was passed through a Sephadex G-25 (medium) column (0.9  $\times$  9.0 cm) equilibrated with Buffer A and 200  $\mu\text{l}$  of the effluent was subjected to sucrose gradient centrifugation as described under "Materials and Methods." Fractions of 500  $\mu\text{l}$  were collected and 20- $\mu\text{l}$  aliquots were taken for determination of protein kinase activity ( $\Delta$ — $\Delta$ ). The reaction was run for 10 min in a final volume of 120  $\mu\text{l}$ . Determination of the high affinity cyclic AMP binding activity associated with protein kinase ( $\square$ — $\square$ ) was performed by incubating 20  $\mu\text{l}$  aliquots in the presence of  $10^{-9}$  M cyclic [ $^3\text{H}$ ]AMP (27 Ci/mmol) in Buffer A for 6 h at 4° in a final volume of 120  $\mu\text{l}$ . The reaction was stopped by pipetting 100  $\mu\text{l}$  of the incubate into 2 ml ice-cold 80% saturated ammonium sulfate, and the cyclic AMP bound determined by collecting the precipitate on Millipore filters as described. Determination of adenosine binding activity (●—●) associated with the cyclic AMP-adenosine binding protein was performed as described under "Materials and Methods." Vertical arrows indicate the position of human hemoglobin (4.5 S), human  $\gamma$ -globulin (7.0 S), and human  $\gamma$ -globulin dimer (11 S).

different buffer systems as described under "Materials and Methods." A sharp single band was observed. Fig. 5, A and B show the gel electrophoresis at alkaline pH. A single band was observed on SDS-polyacrylamide gel electrophoresis (Fig. 5C). The purified protein sedimented as a single boundary on analytical ultracentrifugation, indicating a high degree of purity (data not shown).

#### Stability

The receptor protein was stable for months when stored in liquid  $\text{N}_2$ . Repeated freezing and thawing did not affect the binding characteristics. The protein was stable for at least 3 weeks at 4° in 15 mM Hepes buffer, pH 7.0. The binding capacity for adenosine and cyclic AMP decreased after incubating the receptor in the presence of pronase, indicating its protein nature.

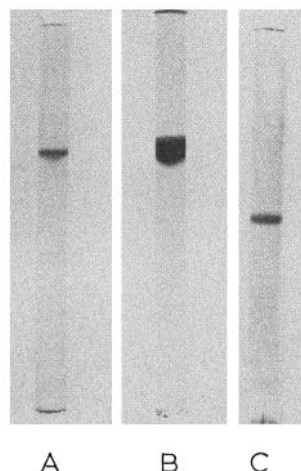


FIG. 5. Polyacrylamide gel electrophoresis. Native binding protein (Step VII) was subjected to electrophoresis in polyacrylamide gel (9%) using alkaline buffer (pH = 8.9). A, 5  $\mu$ g of protein; B, 50  $\mu$ g of protein; C, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cyclic AMP-adenosine binding protein performed as described under "Materials and Methods" using 10  $\mu$ g of protein.

#### Physical Properties

The isoelectric point was at pI = 5.7 as determined in four separate experiments (Fig. 3).

The binding protein eluted from a Sephadex G-200 column corresponding to a Stokes radius of about 48 Å (Fig. 6) and sedimented as 9 S on sucrose gradient centrifugation (Fig. 4A).

The molecular size as determined by either of these methods was independent of the ionic strength (150 or 600 mM KCl or NaCl was included in the buffer during the experiments) and protein concentration (varied in the range 0.1 to 11 mg/ml). The presence of 10% glycerol did not affect the elution or sedimentation behavior (relative to marker proteins).

Untreated binding protein and binding protein activated by treatment with  $Mg^{2+}$ ATP (as described in legend to Fig. 7) were subjected to sucrose gradient centrifugation. The degree of activation of the binding protein was checked in these experiments after completion of the centrifugation to exclude the possibility of inactivation. No difference in the sedimentation behavior was detected when the unactivated and the activated binding protein were compared.

Fig. 4B shows the sedimentation of the binding protein in freshly prepared cytosol, indicating that the 9 S form is not a result of proteolysis during purification. (For comparison, the cyclic AMP binding activity associated with protein kinase activity is also shown in the figure).

The pure binding protein was subjected to analytical ultracentrifugation as described under "Materials and Methods." The data from the sedimentation velocity experiments were plotted as time versus logarithm of distance from center of rotation. The linear plot obtained was consistent with a sedimentation coefficient ( $s_{20,w}$ ) of  $8.8 \cdot 10^{-13}$  s.

From the equation  $M_n = 6 \pi \eta N S a / (1 - \nu \rho)$  where  $\eta$  = viscosity of medium,  $\rho$  = density of medium, and  $N$  = Avogadro's number, the molecular weight ( $M_n$ ) could be calculated by use of the experimentally determined Stokes radius ( $a$ ) and  $s_{20,w}$ . Assuming a partial specific volume ( $\nu$ ) of 0.73 cm<sup>3</sup>/g, the molecular weight was calculated to be 180,000.

The mobility of the protein compared to that of standards on

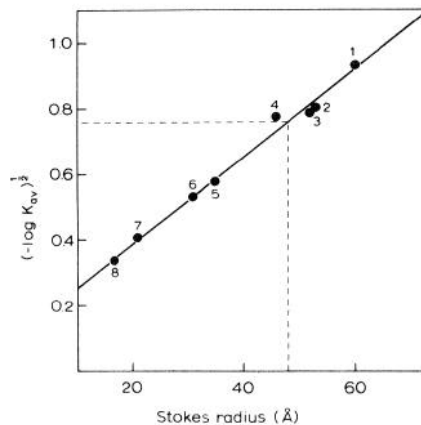


FIG. 6. Estimation of Stokes radius of cyclic AMP-adenosine binding protein by Sephadex G-200 chromatography. Samples (1.5 ml) were chromatographed as described under "Materials and Methods." The elution position of standard molecular weight markers was determined by absorbance while the elution of the receptor protein was determined by assay of cyclic AMP and adenosine binding activity. Treatment of the data as described by Laurent and Killander (17) indicate the Stokes radius of the cyclic AMP-adenosine receptor was 48 Å. The standard protein markers used were: 1), ferritin (60 Å); 2), human IgG (53 Å); 3), catalase (52 Å); 4), aldolase (46 Å); 5), bovine serum albumin (35 Å); 6) human hemoglobin (31 Å); 7), chymotrypsinogen (21 Å); 8), cytochrome c (17 Å).

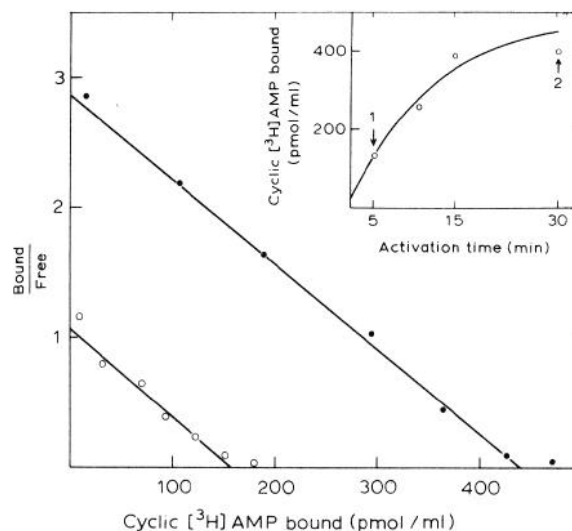


FIG. 7. Scatchard plot (18) for the binding of cyclic  $[^3H]$ AMP to the protein activated to different degrees. Cyclic AMP-adenosine binding protein (2 mg/ml) was incubated at 30° for 30 min in the presence of 9 mM ATP in 15 mM HEPES pH = 7.0 containing 150 mM KCl and 10 mM  $Mg^{2+}$ -acetate. Aliquots of 30  $\mu$ l were taken at 5, 10, 15, and 30 min (inset), cooled, and applied to Sephadex G-25 columns (0.45  $\times$  6 cm) equilibrated with 15 mM HEPES pH = 7.5 containing 20% glycerol and 10 mM 2-mercaptoethanol. Temperature was kept at 4°. Protein was collected quantitatively in 200  $\mu$ l of effluent and diluted, and the binding of cyclic  $[^3H]$ AMP (0.54 Ci/mmol) was measured at saturating concentration (10  $\mu$ M) of cyclic  $[^3H]$ AMP. Inset shows the binding capacity for cyclic AMP as a function of time of activation. After 5 min ( $\circ$ ) and 30 min ( $\bullet$ ) of activation samples were taken for binding studies. The results are presented as Scatchard plots.

SDS-polyacrylamide gel electrophoresis as described under "Materials and Methods" indicated that the binding protein is composed of polypeptides with the same molecular weight of 45,000.

Preliminary Studies on the Activation of the Binding Protein by  $Mg^{2+}ATP$

The untreated purified protein bound adenosine with apparently low affinity and high capacity (*dashed line*, Fig. 8). The amount of cyclic AMP bound, with an apparent dissociation constant of  $1 \times 10^{-7}$  to  $3 \times 10^{-7}$  M, was 2 to 10% of the adenosine binding capacity (data not shown).

An increase in binding capacity for cyclic AMP after preincubation in the presence of  $Mg^{2+}ATP$  and a monovalent cation ( $K^+$ ) (9) was confirmed on the purified binding protein. As most of the binding kinetic data presented in this work are based on experiments performed on binding protein treated with  $Mg^{2+}ATP$ , this process, tentatively termed activation, was studied in some detail.

The binding protein was preincubated in the presence of  $Mg^{2+}ATP$  and KCl for increasing periods of time (Fig. 7). ATP was separated from the binding protein by Sephadex G-25 filtration. (High concentrations of ATP interfered with the binding studies which might be expected as ATP competes with cyclic AMP for the site specific for this nucleotide, as shown in the following paragraph.) After Sephadex G-25 filtration, the binding protein was appropriately diluted and incubated in the presence of radioactive ligand. The incubation was performed at  $0^\circ$  in 15 mM Hepes buffer, pH 7.5 containing 20% glycerol, conditions found to preserve the degree of activation as judged by stability of the binding properties with respect to time. Equilibrium of the binding reaction was reached within 12 h of incubation. The amount of cyclic AMP and adenosine bound was linear with respect to concentration of protein (under conditions where only a small fraction of the total radioactive ligand was bound).

The binding capacity for cyclic AMP increased in a time-dependent manner upon activation (Fig. 7, *inset*). The affinity for cyclic AMP (apparent  $K_d = 1.5 \times 10^{-7}$  M) was independent of the degree of activation (Fig. 7). These results indicate that activation made more sites available for cyclic AMP. Activa-

tion did not increase the total binding capacity for adenosine, but high affinity sites for adenosine appeared (Fig. 8).

The possibility existed that ATP was decomposed during the preincubation and that activation was effected by the decomposition products. Binding protein was preincubated in the presence of  $[^3H]ATP$  (13 mCi/mmol) under the conditions described in the legend to Fig. 7 and samples from the preincubate were subjected to thin layer chromatography (System B). Less than 3% of the total ATP was converted to ADP and nondetectable amounts to AMP and adenosine. The binding protein was not activated when preincubated in the presence of phosphate or pyrophosphate (1, 10, and 150 mM of the sodium salts) instead of ATP. ADP and AMP were apparently poor activators (9).

To check if the activation process was accompanied by a phosphorylation of the binding protein, the latter (3 mg/ml) was incubated in the presence of 6 mM  $[\gamma\text{-}^{32}P]ATP$  (2.5 mCi/mmol), 150 mM KCl, and 10 mM magnesium acetate for 30 min at  $30^\circ$ . Samples of 30  $\mu$ l were applied to a Sephadex G-25 column (0.45  $\times$  6 cm) equilibrated with 15 mM Hepes buffer, pH 7.5, containing 20% glycerol. Radioactivity was excluded from the gel clearly separated from free ATP, indicating association of radioactive  $^{32}P$  with the protein. The excluded radioactivity was pooled (150  $\mu$ l) and 25  $\mu$ l (about 8000 cpm) subjected to SDS-polyacrylamide gel electrophoresis as described under "Materials and Methods." The gel was sliced and counted. No radioactivity could be detected corresponding to the protein band. Furthermore, when 100  $\mu$ l of the excluded radioactivity was spotted on Whatman filters and washed in 5% ice-cold trichloroacetic acid as described for assay of protein kinase activity (11), no incorporation of  $^{32}P$  could be detected. These results indicate that activation was not accompanied by phosphorylation, but that ATP bound to the receptor protein. Furthermore, about 50% of the  $[\gamma\text{-}^{32}P]ATP$  bound was removed by filtration through a column containing charcoal, which supports the suggestion that  $^{32}P$  was not covalently bound.

There was a correlation between the degree of activation of cyclic AMP binding sites and the concentration of ATP present during the activation. The results obtained with the partly purified binding protein (9) were confirmed using the homogeneous receptor. The binding capacity for cyclic AMP increased between  $5 \times 10^{-4}$  M and  $10^{-2}$  M ATP. Studies on the activation by adenine derivatives other than ATP met with the problem that these derivatives, being potent inhibitors to cyclic AMP binding, cause serious interference with the subsequent measurement of cyclic AMP bound to the receptor.

We have tried to correlate the degree of activation with the amount of ATP bound to the receptor protein. When ATP was partly removed (50%) by treatment with charcoal prior to incubation in the presence of cyclic  $[^3H]AMP$ , the binding capacity for cyclic AMP did not decrease. Thus, there was no relation between ATP binding and the degree of activation as judged by cyclic AMP binding. The extension of these studies were hampered by a low apparent affinity for ATP under the conditions of activation. Furthermore by preincubating in the presence of  $[\gamma\text{-}^{32}P]ATP$ , it was possible to demonstrate that more than 95% of the ATP bound was dissociated from the receptor protein during incubation (18 h at  $0^\circ$ ) in the presence of  $^3H$ -labeled ligand.

#### Specificity of Cyclic AMP Binding Site

The double reciprocal plot of the data presented in Fig. 9, was consistent with adenosine, AMP, ADP, and ATP competi-

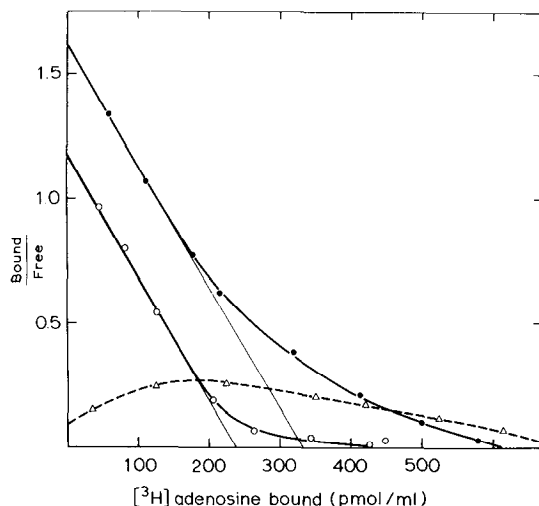


FIG. 8. Scatchard plot for the binding of  $[^3H]$ adenosine (1.42 Ci/mmol) to nonactivated binding protein and to the activated protein, in the absence and presence of cyclic AMP. Cyclic AMP-adenosine binding protein was activated for 30 min and desalted on a Sephadex G-25 column as described in legend to Fig. 7. The binding of  $[^3H]$ adenosine was measured at 40  $\mu$ g of protein/ml incubate. Data on  $[^3H]$ adenosine binding to the nonactivated receptor ( $\Delta$ — $\Delta$ ), activated receptor in the absence ( $\bullet$ — $\bullet$ ) or presence ( $\circ$ — $\circ$ ) of 100  $\mu$ M cyclic AMP are shown.

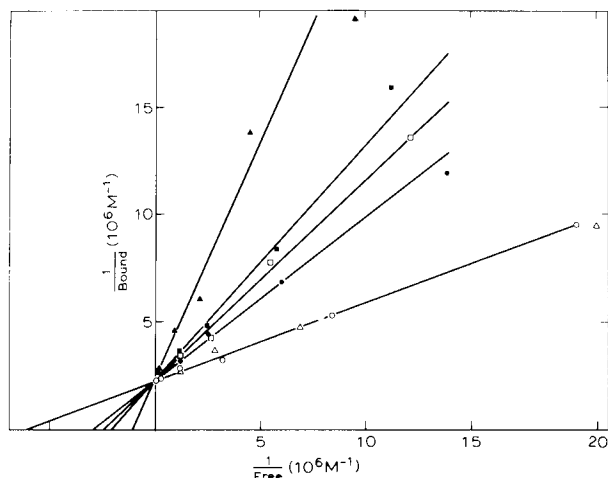


FIG. 9. Effect of inhibitors on the binding of cyclic [ $^3\text{H}$ ]AMP to the cyclic AMP-adenosine binding protein. Double reciprocal plot for the binding of cyclic [ $^3\text{H}$ ]AMP to the activated (as described in legend to Fig. 7) receptor protein (40  $\mu\text{g}/\text{ml}$ ) in the presence of 4  $\mu\text{M}$  adenosine ( $\blacktriangle$ ), 4  $\mu\text{M}$  AMP ( $\square$ ), 4  $\mu\text{M}$  ADP ( $\bullet$ ), 20  $\mu\text{M}$  ATP ( $\blacksquare$ ), 4  $\mu\text{M}$  adenine ( $\triangle$ ), or no inhibitor ( $\circ$ ).

tively inhibited the binding of cyclic [ $^3\text{H}$ ]AMP to the activated receptor protein indicating that these adenine derivatives were bound to the same site as cyclic AMP. Adenine did not compete for this site. The inhibition constant ( $K_i$ ) calculated from the equation (19):

$$K_i = \frac{K_d \text{ cyclic AMP} (I)v}{v_{\max}(\text{cyclic AMP}) - [( \text{cyclic AMP} ) + K_d \text{ cyclic AMP}]v}$$

where  $v$  = amount of cyclic AMP bound and  $(I)$  = concentration of competing molecule, was about  $8 \times 10^{-7}$  M for adenosine,  $1.2 \times 10^{-6}$  M for AMP,  $1.5 \times 10^{-6}$  M for ADP, and higher than  $5 \times 10^{-6}$  M for ATP. (The inhibition constant for unlabeled cyclic AMP was  $1.5 \times 10^{-7}$  M.) Cyclic GMP, GMP, guanosine, cyclic IMP, IMP, and inosine did not inhibit the binding of cyclic [ $^3\text{H}$ ]AMP.

#### Binding of Adenosine to Activated Receptor Protein

A Scatchard plot for the binding of [ $^3\text{H}$ ]adenosine to the binding protein activated as described in the legend to Fig. 7, was hyperbolic (Fig. 8), indicating the existence of more than one site binding adenosine. The part of the curve with the steepest slope corresponded to a dissociation constant of about  $2 \times 10^{-7}$  M. To test the specificity of the high affinity site, the binding at 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]adenosine was measured in the presence of increasing concentrations of inhibitors (Table I). Under the experimental conditions described, only about 50% of the binding could be suppressed by cyclic AMP which was the most effective inhibitor. This could be interpreted as competition between cyclic AMP and adenosine for only a part of the adenosine binding sites. This interpretation was supported by studies on binding of adenosine in the presence of a concentration of cyclic AMP (100  $\mu\text{M}$ ) high enough to saturate the cyclic AMP site. Under these conditions the steepest part of the Scatchard plot for adenosine binding showed the same slope as in the absence of cyclic AMP, whereas the part corresponding to low affinity was suppressed by cyclic AMP (Fig. 8, filled symbols). This might represent inhibition of the binding of adenosine to the site shown preferentially to bind cyclic AMP. To test the specificity of the high affinity binding for adenosine not suppressed by cyclic AMP, the binding of [ $^3\text{H}$ ]adenosine at

TABLE I

#### Effect of purine derivatives on the binding of [ $^3\text{H}$ ]adenosine

The binding of [ $^3\text{H}$ ]adenosine to the activated receptor protein (activated as described in legend to Fig. 7) was measured in the presence of various purine derivatives at concentrations indicated in the table. The concentrations were 0.1  $\mu\text{M}$  and 40  $\mu\text{g}/\text{ml}$  for [ $^3\text{H}$ ]adenosine and receptor protein respectively.

Addition	$\mu\text{M}$	Pmol [ $^3\text{H}$ ]adenosine bound per ml of incubate
Control		22.3
Adenosine	10	2.2
	50	1.4
Cyclic AMP	4	12.2
	10	10.7
	50	10.6
Adenine	4	19.0
	10	18.5
	50	12.5
AMP	4	14.0
	10	10.6
	50	9.2
ADP	4	17.0
	10	12.8
	50	9.5
ATP	4	24.0
	10	23.0
	50	20.0
Guanosine	50	23.0
GMP	50	22.0
Cyclic GMP	50	21.2
Inosine	50	22.3
IMP	50	20.0
Cyclic IMP	50	24.2

TABLE II

#### Specificity of the high affinity adenosine binding not suppressed by cyclic AMP

Receptor protein was activated as described in the legend to Fig. 7 except that the concentration of binding protein was 4 mg/ml and the preincubation was allowed to proceed for 60 min at 30°. Measurement of [ $^3\text{H}$ ]adenosine binding was performed at 80  $\mu\text{g}$  binding protein per ml and 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]adenosine. In addition to 100  $\mu\text{M}$  cyclic AMP present in all incubations, various adenine derivatives (at concentrations indicated in the table) were added. The binding of [ $^3\text{H}$ ]adenosine with only 100  $\mu\text{M}$  cyclic AMP and buffer (control) was taken as 100%.

Addition	$\mu\text{M}$	[ $^3\text{H}$ ]Adenosine bound % buffer control
Control		100%
Adenosine	5	15
	50	4.1
Adenine	5	70
	50	15
AMP	5	86
	50	74
ADP	5	95
	50	107
ATP	5	102
	50	103

a concentration of 0.1  $\mu\text{M}$  was measured in the presence of 100  $\mu\text{M}$  unlabeled cyclic AMP and possible (as judged from the results presented in Table I) inhibitors of adenosine binding (adenine, AMP, ADP, and ATP). The binding of adenosine to the high affinity site was inhibited by adenine and to a slight degree by AMP, whereas ADP and ATP were ineffective (Table II).

TABLE III

Effect of free regulatory subunit or cyclic AMP-adenosine binding protein on the phosphotransferase activity of free-catalytic subunit of cyclic AMP-dependent protein kinase

Free-catalytic and regulatory subunits from cyclic AMP-dependent protein kinase isoenzyme I, were prepared as described under "Materials and Methods." The preparation of regulatory subunit was desalted by passing through Sephadex G-25 column (0.9 × 6 cm) equilibrated with Buffer A containing 0.1% bovine serum albumin. To 25- $\mu$ l samples of catalytic subunit 25  $\mu$ l of regulatory subunit (able to bind 6 pmol of cyclic AMP/ml) or cyclic AMP-adenosine binding protein (concentration indicated in the table) or Buffer A (containing 0.1% bovine serum albumin) was added. The mixture was made 10  $\mu$ M in ATP and 5 mM in Mg<sup>2+</sup> by adding 10  $\mu$ l and preincubating for 40 min at room temperature. Samples of 25  $\mu$ l were taken for measurement of protein kinase activity in the absence and presence of 2  $\mu$ M cyclic AMP in a total volume of 125  $\mu$ l. The reaction was run for 10 min.

Catalytic subunit preincubated in the presence of	pmol of phosphate incorporated/ml		Ratio -/+
	-Cyclic AMP	+Cyclic AMP	
Buffer control	430	370	1.16
Regulatory subunit	172	453	0.38
Cyclic AMP-adenosine binding protein			
0.6 mg/ml	380	369	1.03
1.2 mg/ml	365	420	0.87
2.3 mg/ml	470	461	1.02

#### Test for Enzymic Properties

The pure preparation of binding protein had no adenosine deaminase or cyclic AMP phosphodiesterase activity. Under conditions optimal for phosphotransferase activity catalyzed by cyclic AMP-dependent protein kinase or casein kinase, neither the unactivated nor the activated binding protein phosphorylated casein (5 mg/ml) or histone (0.7 mg/ml) to any significant extent.

The phosphotransferase activity of the free catalytic subunit of cyclic AMP-dependent protein kinase has been shown to be inhibited by the addition of the regulatory subunit from the same enzyme ((20) and Table III). The cyclic AMP-adenosine binding protein did not inhibit the protein kinase activity of the free catalytic subunit of cyclic AMP-dependent protein kinase (PK I) under conditions (in the presence of Mg<sup>2+</sup>ATP) where recombination with the free regulatory subunit occurred (Table III). These results serve to differentiate the cyclic AMP-adenosine binding protein from the regulatory subunit of cyclic AMP-dependent protein kinase.

As phosphofructokinase has been shown to bind cyclic AMP with about the same affinity for this nucleotide as the binding protein described by us (19, 21), it should be noted that neither the unactivated nor activated form of the binding protein had any phosphofructokinase activity, and neither form inhibited the phosphofructokinase from rabbit skeletal muscle added to the assay mixture.

#### DISCUSSION

This paper reports the existence of a cyclic AMP-adenosine binding protein in mouse liver. The binding protein was purified to apparent homogeneity as judged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate and by analytical ultracentrifugation. The binding protein appears to be tetrameric with a molecular weight of 180,000. The molecular size seems to be independent of ionic

strength and concentration of protein. The same molecular form was detected in freshly prepared, crude liver extract. This indicates structural stability of the cyclic AMP-adenosine binding protein.

Measurement of the binding activity involved ammonium sulfate precipitation of the binding protein and collection of the precipitate on Millipore filters. Experiments presented indicate that this method gives a true estimate of the amount of protein-bound ligand. The method can be performed under conditions preserving the degree of activation and is less laborious than the gel filtration technique (22) and equilibrium dialysis.

The binding of cyclic AMP to the regulatory subunit of protein kinase can be measured by the Millipore filtration technique described by Gilman (23). It was of interest to investigate whether the binding of cyclic AMP and adenosine to the cyclic AMP-adenosine binding protein could be detected by a similar method not involving ammonium sulfate precipitation. When the assay of Gilman was adopted, 75% of the adenosine binding activity was detected. Cyclic AMP seemed to be rapidly liberated from the binding protein under these conditions (sodium phosphate buffer, pH 6.0). When 15 mM Hepes, pH = 7.5, was used instead of the phosphate buffer, low recovery was obtained for both cyclic [<sup>3</sup>H]AMP and [<sup>3</sup>H]adenosine bound to the receptor protein. The reason seemed to be poor retention of the binding protein on the Millipore filter. The following observations support this explanation. The ligand-receptor complex was recovered in the buffer passing through the filter. Both cyclic AMP and adenosine bind to the receptor molecule under similar conditions as judged by the gel filtration technique (Fig. 1).

An increased capacity for cyclic AMP binding after preincubating a crude preparation of the binding protein in the presence of Mg<sup>2+</sup>ATP has been presented in a recent communication (9). In the present work it was demonstrated that treatment with Mg<sup>2+</sup>ATP activated a high affinity site for adenosine, and an increased capacity for cyclic AMP binding after such treatment was confirmed on the pure binding protein. Thus, activation is not effected by a contaminating factor. Recently, autophosphorylation of cyclic AMP-dependent protein kinase with subsequent changes in binding properties of the regulatory subunit has been reported (24-26). Our results indicate that autophosphorylation is not the mechanism of activation of the cyclic AMP-adenosine binding protein from mouse liver. Furthermore, sucrose gradient centrifugation did not reveal changes in sedimentation behavior upon activation. Thus, dissociation or aggregation does not accompany activation of the binding protein.

As radioactive ATP was excluded from Sephadex G-25 columns after preincubation with the binding protein, ATP probably binds to the protein under conditions of activation. Experiments presented suggest that no direct relation exists between binding of ATP and activation, as partial removal of ATP did not decrease the binding capacity for cyclic AMP. ATP seems to competitively inhibit the binding of cyclic [<sup>3</sup>H]AMP to the binding protein (Fig. 9). This may indicate binding of ATP to the cyclic AMP binding site. Whether ATP interaction with this or another site is a prerequisite to activation remains to be established.

Studies on the activation of the cyclic AMP-adenosine binding protein showed that its binding properties can change widely. If the ratio of cyclic AMP to adenosine binding capacity is taken as a measurement of activation, this varies between 0.05 and about 1. Assuming that the binding protein is



subjected to similar changes in binding properties in the cell, one may speculate whether this protein modulates the effects of cyclic AMP. Studies on the mutual interactions of binding sites under conditions approaching those believed to exist in the cell, may give some insight into the functional role of activation.

Binding studies on the activated receptor protein indicate that cyclic AMP binds (with an apparent dissociation constant of  $1.5 \times 10^{-7}$  M)<sup>3</sup> to one main site (Figs. 7 and 9). Assuming that the inhibition constant ( $K_i$ ) is the same as the dissociation constant ( $K_d$ ) for one particular inhibitor of cyclic AMP binding, one might expect that adenosine binds to the cyclic AMP binding site with a  $K_i$  4 to 5 times higher than that for cyclic AMP. Competition studies (Table I) suggest heterogeneity of adenosine binding sites, only part of the adenosine binding was inhibited by cyclic AMP. A Scatchard plot for adenosine binding in the absence and presence of 100  $\mu$ M cyclic AMP (Fig. 8) showed suppression of the part of the curve corresponding to low affinity ( $K_i$  about  $10^{-6}$  M) binding of adenosine. These data were interpreted as adenosine binding to at least both the cyclic AMP site and to a site with high affinity for adenosine ( $K_i = 2 \times 10^{-7}$  M). Competition studies in the presence of 100  $\mu$ M cyclic AMP (Table II) showed that the latter site binds adenosine with high specificity for this adenine derivative. The high specificity of the adenosine binding site points to the possibility of adenosine as a modulator of intracellular effects of cyclic AMP. The binding kinetic studies presented here do not exclude the possibility that part of the inhibition of cyclic AMP binding effected by adenosine may be explained by altered affinity for cyclic AMP when the adenosine binding site is occupied.

Recently, the existence of two types of cyclic AMP binding sites, one of which had a  $K_d$  of  $1.6 \times 10^{-7}$  M, has been reported in cells of *Dictyostelium discoideum* (27). These findings, together with a report on ATP activation of cyclic AMP receptors in the same species (28), should be taken into account when the possibility of activation of cyclic AMP receptors in intact mammalian cells is considered.

Several workers have reported on the existence in eukaryotic cells of cyclic AMP binding factors not related to protein kinase (6-8). These factors have one or all of the following characteristics. The sedimentation coefficient is about 9 S (6-8); the dissociation constant for cyclic AMP is about  $10^{-7}$  M (7, 8); adenine derivatives compete for the cyclic AMP site (7, 8). Specific adenosine binding activity has been described in rat liver (29). These reports, together with the observation that cyclic AMP-adenosine binding proteins are present in bovine adrenal cortex, human renal cortex, and human renal cell carcinoma,<sup>4</sup> indicate that the cyclic AMP-adenosine binding proteins are widely distributed.

The adenosine-cyclic AMP binding factors purified to homogeneity from rabbit erythrocytes by Yuh and Tao (7) have similar binding properties to the binding protein described by us. The binding factors from rabbit erythrocytes were suggested to be pentamers and of higher molecular weight

<sup>3</sup> We have earlier reported (9) that half-maximal saturation was obtained at 2  $\mu$ M cyclic AMP, and the cyclic AMP binding was not inhibited by addition of adenosine. Experiments presented here demonstrate that adenosine is a powerful inhibitor of cyclic AMP binding. The reason for this discrepancy may be that the incubations were performed under conditions (30°) not preserving the degree of activation, and the crude receptor-preparation used contained adenosine deaminase.

<sup>4</sup> S. O. Døskeland, T. M. Fosberg, and P. M. Ueland, unpublished observations.

(240,000) than the cyclic AMP-adenosine binding protein from mouse liver. The factors from rabbit erythrocytes bound more adenosine than cyclic AMP with a ratio of 3 to 8:1 (7, 30). The authors commented upon the low binding capacity for cyclic AMP (about 0.44 molecule of cyclic AMP was bound to each molecule of receptor). The possibility that a large population of the proteins might be in an inactive form and the possible requirement for cofactors for optimal binding were in fact discussed. For comparison, it could be mentioned that about 2 molecules of cyclic AMP were bound to 1 molecule of binding protein from mouse liver when the receptor was treated with  $Mg^{2+}$ -ATP as described in legend to Fig. 7.

The functional role of the cyclic AMP-adenosine binding protein is unknown. Increasing evidence is accumulating suggesting that cyclic AMP and adenosine metabolism in mammalian cells is interrelated (31-36). Adenosine has been proposed as a metabolic and physiological regulator (34, 37-39). The interaction of adenosine with its intracellular receptor sites may mediate some of its metabolic effects.

*Acknowledgments*—We wish to thank Dr. E. Slinde and Professor T. Flatmark for the use of their electrofocusing equipment and Dr. J. Berg and Professor K. Kleppe for their help in the sedimentation velocity experiments. We are grateful to Professor K. Kleppe for a critical reading of the manuscript and to Dr. I. F. Pryme for correcting its English content. The technical assistance of G. Kvalheim is highly appreciated.

#### REFERENCES

- Gill, G. N., and Garren, L. D. (1970) *Biochem. Biophys. Res. Commun.* 39, 335-343
- Kumon, A., Yamamura, H., and Nishizuka, Y. (1970) *Biochem. Biophys. Res. Commun.* 41, 1290-1297
- Reimann, E. M., Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1971) *Biochem. Biophys. Res. Commun.* 42, 187-194
- Erlichman, J., Hirsch, A. H., and Rosen, O. M. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 731-735
- Krebs, E. G. (1972) *Curr. Top. Cell. Regul.* 5, 99-133
- Chambaut, A. M., Leray, F., and Hanoune, J. (1971) *FEBS Lett.* 15, 328-334
- Yuh, K.-C. M., and Tao, M. (1974) *Biochemistry* 13, 5520-5526
- Tsuzuki, J., and Kiger, J. A., Jr. (1975) *Biochim. Biophys. Acta* 393, 225-235
- Døskeland, S. O., and Ueland, P. M. (1975) *Biochem. Biophys. Res. Commun.* 66, 606-613
- Glynn, I. M., and Chappell, J. B. (1964) *Biochem. J.* 90, 147-149
- Ueland, P. M., and Døskeland, S. O. (1976) *Biochem. J.* 157, 117-126
- Kalckar, H. M. (1947) *J. Biol. Chem.* 167, 461-475
- Massey, T. H., and Deal, W. C. (1975) *Methods Enzymol.* 43C, 99-110
- Weber, K., Pringle, J. R., and Osborn, M. (1972) *Methods Enzymol.* 26C, 3-28
- Schachman, H. K., and Edelman, S. L. (1966) *Biochemistry* 5, 2681-2705
- Klungsoyr, L. (1969) *Anal. Biochem.* 27, 91-98
- Laurent, T. C., and Killander, J. (1964) *J. Chromatog.* 14, 317-330
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* 51, 660-672
- Kemp, R. G., and Krebs, E. G. (1967) *Biochemistry* 6, 423-434
- Kumon, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y. (1972) *J. Biol. Chem.* 247, 3726-3735
- Setlow, B., and Mansour, T. E. (1972) *Biochemistry* 11, 1478-1486
- Hummel, J. P., and Dreyer, W. J. (1962) *Biochim. Biophys. Acta* 63, 530-532
- Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U. S. A.* 67, 305-312
- Erlichman, J., Rosenfeld, R., and Rosen, O. M. (1974) *J. Biol. Chem.* 249, 5000-5003
- Rosen, O. M., and Erlichman, J. (1975) *J. Biol. Chem.* 250, 7788-7794
- Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G.

- (1975) *J. Biol. Chem.* **250**, 7795-7801
27. Green, A. A., and Newell, P. C. (1975) *Cell* **6**, 129-136
28. Mato, J. M., and Konijn, T. M. (1975) *Dev. Biol.* **47**, 233-235
29. Hsu, H. H. T. (1975) *Proc. Soc. Exp. Biol. Med.* **149**, 698-701
30. Tao, M., Yuh, K.-C., and Hosey, M. M. (1974) in *Lipmann Symposium on Energy, Regulation and Biosynthesis in Molecular Biology* (Richter, D., ed) pp. 636-646, Walter de Gruyter, New York
31. Fain, J. N., Pointer, R. H., and Ward, W. F. (1972) *J. Biol. Chem.* **247**, 6866-6872
32. Schwabe, U., Ebert, R., and Erbler, H. C. (1973) *Naunyn-Schmiedebergs Arch. Pharmacol.* **276**, 133-148
33. Huang, M., and Daly, J. W. (1974) *Life Sci.* **14**, 489-503
34. Schwabe, Y., Schönhöfer, P. S., and Ebert, R. (1974) *Eur. J. Biochem.* **46**, 537-545
35. Haslam, R. J., and Rosson, G. M. (1975) *Mol. Pharmacol.* **11**, 528-544
36. Hjemdahl, P., and Fredholm, B. B. (1976) *Acta Physiol. Scand.* **96**, 170-179
37. Berne, R. M., Rubio, R., Dobson, J. G., and Curnish, R. R. (1971) *Circ. Res.* **28**, I-115-I-119
38. Osswald, H. (1975) *Naunyn-Schmiedebergs Arch. Pharmacol.* **288**, 79-86
39. Fredholm, B. B. (1976) *Acta Physiol. Scand.* **96**, 422-430