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PARTIAL PURIFICATION AND PROPERTIES OF THE CYCLIC AMP AND THE CYCLIC GMP PHOSPHODIESTERASES OF BOVINE LIVER

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SUMMARY

The cyclic AMP phosphodiesterase of bovine liver was purified approximately 100-fold (assayed at a substrate concentration of 10^{-6} M cyclic AMP). The enzyme appeared to occur in two interconvertible forms of mol. wts of 120 000 and 240 000, respectively. They differed in their K_m values for cyclic AMP. The ionic strength of the buffer, the concentration of Mg^{2+}, and the presence of either cyclic AMP or cyclic GMP affected the predominance of one or the other form.

The cyclic GMP phosphodiesterase also had a mol. wt of approx. 240 000 but appeared to be distinct from the cyclic AMP phosphodiesterase.

A fraction, obtained after Sephadex G-200 filtration of crude extracts, of a mol. wt of approx. 360 000 showed both cyclic AMP and cyclic GMP phosphodiesterase activities when unphysiologically high concentrations of the two cyclic nucleotides were employed in the assay. The fact that high concentrations of cyclic GMP inhibited the activity of the cyclic AMP phosphodiesterase in this fraction suggested the existence of a molecular aggregate combining the activities of the two enzymes.

INTRODUCTION

The levels of adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) in any given tissue are controlled by the activities of the respective cyclases and phosphodiesterases. Adenyl cyclases, particularly with respect to their response to a variety of hormones, have been studied intensively. Relatively less is known about the cyclic nucleotide phosphodiesterases and their

Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; Buffer A, 0.01 M sodium phosphate (pH 7.5)–0.001 M $MgCl_2$–0.001 M β-mercaptoethanol; Buffer B, 0.01 M Tris–HCl (pH 7.8 at 4° C)–0.01 M β-mercaptoethanol–0.01 M $MgCl_2$–0.025 M NaCl; Buffer C, 0.01 M Tris–HCl (pH 7.8 at 4° C)–0.01 M β-mercaptoethanol–0.01 M $MgCl_2$–0.6 M NaCl.

role in regulation. The purification of cyclic AMP phosphodiesterase has proven difficult; this may be due in part to the fact that cyclic AMP phosphodiesterases, at least in certain tissues and organisms, require a proteinaceous activator[1-3]. Cyclic AMP phosphodiesterases of a number of tissues display two apparent K_m values for cyclic AMP[4-7] and upon gel electrophoresis of crude extracts, two or more bands showing cyclic AMP phosphodiesterase activity were found[8-10]. In rat brain[11] as well as in rat kidney and frog bladder[12] the two apparent K_m values seemed to be correlated with the activities of two proteins differing in size.

It has been reported[13-15,22] that partially purified cyclic AMP phosphodiesterase also hydrolyzed cyclic GMP and that cyclic GMP affected the rate of hydrolysis of cyclic AMP[13-17]. In certain cases cyclic AMP inhibited the hydrolysis of cyclic GMP and it was suggested that the two cyclic nucleotides were hydrolyzed by one enzyme that existed in several forms with different affinities for cyclic AMP and cyclic GMP[15,18].

We decided to purify the cyclic AMP phosphodiesterase from beef liver in view of the ready availability of this organ and the relative homogeneity of the material. We found that the cyclic AMP phosphodiesterase exists in at least two interconvertible forms which differ in their molecular weights and their K_m values for cyclic AMP. There was a distinct cyclic GMP phosphodiesterase and there also appeared to be a protein of a molecular weight of approx. 360 000 which hydrolyzed both cyclic AMP and cyclic GMP when these substrates were furnished at unphysiologically high concentrations.

METHODS

(1) Preparation of cyclic AMP and of cyclic GMP phosphodiesterases

(a) Crude extract and treatment with streptomycin sulfate. Fresh beef liver was obtained from a local slaughterhouse and kept on ice during transportation to the laboratory. All of the following steps were carried out at 4 °C. 250 g of the liver were cut into small pieces and added to 1000 ml of Buffer A; the material was homogenized for one minute in a Waring Blender, strained through a double layer of cheesecloth, and centrifuged for 30 min at 11 000 × *g*. A 10% solution of streptomycin sulfate (Sigma) was added dropwise, and with stirring, to the supernate to a final concentration of 2%; the pH was adjusted to 7.0. The extract was stirred gently for 60 min and centrifuged for 30 min at 35 000 × *g*. The supernate from this centrifugation was strained through glass wool so as to remove the fat. This preparation will be referred to as the crude extract. It was clear in appearance and contained 90–95% of the cyclic AMP phosphodiesterase activity found in the homogenate. The preparation was stored at −20 °C and lost approx. 30% of its activity in the first month of storage. Prior to use, it was dialyzed either against Buffer A or the buffer specified for individual experiments.

(b) Fractionation with acetone. Acetone, precooled to −20 °C, was added dropwise and with stirring to the undialyzed crude extract at pH 7 to a final concentration of 25%. Stirring was continued for 20 min and the extract centrifuged for 10 min at 35 000 × *g*. The pellet was resuspended to one-third of the original volume in Buffer A, dialyzed against the same buffer, and the precipitate removed by centrifugation. The cyclic AMP phosphodiesterase was purified 4–5-fold, compared with

its specific activity in the crude extract, by the precipitation with acetone. The enzyme, at this stage of purification, lost 80% of its activity when stored for one week at −20 °C, unless stabilized by a high concentration of either NaCl or $MgCl_2$.

(c) Ion exchange. A 3 cm × 50 cm column of DE-52 (Whatman Microgranular, preswollen) was equilibrated with Buffer A and loaded with the preparation obtained from the precipitation with acetone. Elution was stepwise with increasing concentrations of NaCl in Buffer A. The cyclic AMP phosphodiesterase was eluted at between 0.6 and 1.0 M NaCl. Fractions with high specific activity were pooled, concentrated to 10 mg of protein/ml by filtration through an Amicon UM-10 filter, and dialyzed against Buffer C. The spec. act. of this preparation indicated a 16–20-fold purification when compared with that of the crude extract. The enzyme retained 90% of its activity after storage at 4 °C in Buffer C for one month.

(d) Gel filtration. Gel filtration was carried out in 3 cm × 100 cm Sephadex G-200 (Pharmacia) columns; the flow rate was 24–25 ml/h and the $A_{280\ nm}$ was monitored with an Isco UV Analyzer (Model UA-2). The columns were calibrated with catalase (Boehringer, Mannheim), bovine serum albumin (Fraction V, Sigma), and RNAase (Bovine Pancrease, Type I-A, Sigma). Buffer C was employed in the filtration of the enzyme from the DE-52 column, whereas for the filtration of the crude extract and the acetone precipitate, Buffer B was used. Other modifications are cited in the context of the individual experiments. Enzyme preparations were dialyzed for 24 h against the buffer to be employed in a given experiment. Fractions were assayed routinely at two different substrate concentrations, 10^{-6} M and 10^{-3} M cyclic AMP, and at 10^{-7} M and 10^{-4} M cyclic GMP, respectively. Fractions showing high phosphodiesterase activity were pooled, concentrated to 5 mg of protein/ml, and stored at −20 °C.

(2) Assay of cyclic AMP and of cyclic GMP phosphodiesterase. The assay of the activities of both enzymes was based on a modification of the method of Brooker *et al.*[4]. The incubation mixture contained 0.05 M sodium phosphate, pH 7.5, 0.01 M β-mercaptoethanol, 0.01 M $MgCl_2$, 100 μg of bovine serum albumin (Fraction V, Sigma) and an appropriate concentration of the enzyme preparation. The enzyme was preincubated in this mixture for 15 min at 37 °C and the reaction started by the addition of either [^{3}H]cyclic AMP or [^{3}H]cyclic GMP. The specific radioactivity of the [^{3}H]cyclic AMP was 80 μCi/mmole when it was employed at a final concentration of 10^{-6} M and 0.08 μCi/mmole when employed at 10^{-3} M final concentration. In the case of [^{3}H]cyclic GMP the specific radioactivities were 800 μCi/mmole at 10^{-7} M and 0.8 μCi/mmole at 10^{-4} M, respectively. The final volume of the reaction mixture was 0.25 ml. The reaction was terminated by the immersion of the tubes in boiling water for 100 s. 100 μg of either snake venom (*Crotalus adamanteus*, Sigma) or of 5′-nucleotidase (purified from *C. adamanteus* venom, Grade II, Sigma) were then added in a volume of 0.05 ml and incubation continued for 30 min at 37 °C. At the end of this period 400 mg of washed Dowex 2-X8 (200–400 mesh, Cl^- form, Sigma) were added in 2.2 ml of water. The Dowex binds charged nucleotides; it does not bind adenosine or guanosine. After 30 min of contact with the Dowex, the incubation mixtures were centrifuged briefly and the radioactivity (corresponding to [^{3}H]adenosine or [^{3}H]guanosine, respectively) of 0.5 ml of the supernates determined by liquid scintillation counting. Under our conditions of assay rectilinearity with time prevailed until 40% of the cyclic AMP or 60% of the cyclic GMP were hydrolyzed.

The rates of the reactions were calculated from three time points representing duplicate determinations. Units of phosphodiesterase are presented as pmoles of cyclic AMP or cyclic GMP hydrolyzed per min at 37 °C. Specific activity is defined in terms of units/mg of protein. Protein was determined by the method of Lowry *et al.*[19] with bovine serum albumin (Fraction V, Sigma) serving as standard. [^{3}H]Cyclic AMP (14.3 Ci/mmole) and [^{3}H]cyclic GMP (0.63 Ci/mmole) were purchased from Schwarz–Mann (Orangeburg, N.Y.) and cyclic AMP and cyclic GMP from Sigma. [^{3}H]Cyclic GMP was employed without further purification, [^{3}H]cyclic AMP was purified by chromatography on Dowex (50W-X8, 200–400 mesh, H^+ form).

RESULTS

(1) Conditions for optimal activity

Both cyclic AMP and cyclic GMP phosphodiesterases require divalent cations for activity. The dependence of the two enzymes on Mg^{2+}, when sodium phosphate was employed as buffer, is shown in Fig. 1A. Optimal concentrations were 10^{-4} M for cyclic GMP phosphodiesterase and 10^{-2} M for cyclic AMP phosphodiesterase. Since cyclic GMP phosphodiesterase was not inhibited by the higher concentration, 10^{-2} M Mg^{2+} was employed routinely, Fig. 1B shows that the pH optimum of both activities was approximately pH 7.7, irrespective of whether sodium phosphate or Tris–HCl (not shown) buffer were employed. We found in preliminary experiments

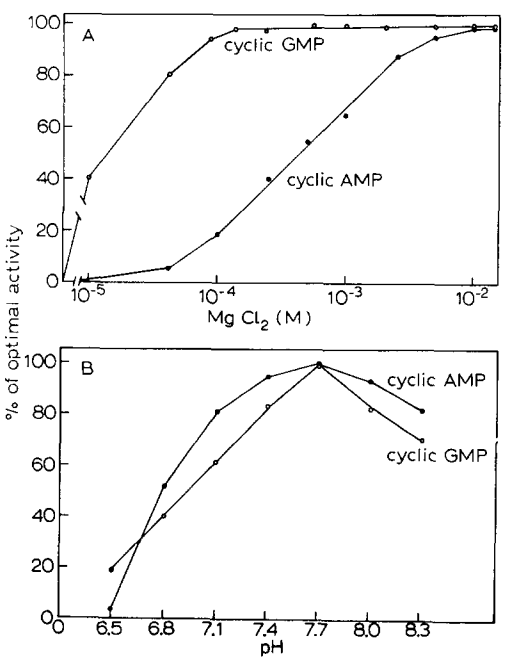


Fig. 1. A. Effect of concentration of Mg^{2+} on activity of cyclic AMP and cyclic GMP phosphodiesterases. $MgCl_2$ was added to a final concentration of 10^{-2} M for the nucleotidase reaction. B. Effect of pH on cyclic AMP and cyclic GMP phosphodiesterases. The pH was adjusted to 7.5 for the nucleotidase reaction. A crude extract was employed in the assays and the buffer was $5 \cdot 10^{-2}$ M sodium phosphate.

that reducing compounds stabilized enzymic activity. A variety of –SH compounds, including β-mercaptoethanol, dithiothreitol, GSH, and lipoic acid were effective, whereas NADH, NADPH, and ascorbic acid did not stabilize. Since –SH compounds protected against loss of activity when the enzymes were assayed at low concentrations of protein, β-mercaptoethanol as well as bovine serum albumin were included in assays routinely at concentrations of 0.01 M and 100 μg/0.25 ml, respectively.

(2) Michaelis constants

The cyclic AMP phosphodiesterases from most tissues examined show non-linear kinetics when a wide range of concentrations of cyclic AMP is tested. This finding has given rise to the concept of there being at least two cyclic AMP phosphodiesterases, so-called "low K_m" and "high K_m" enzymes. We therefore assayed the activity of cyclic AMP phosphodiesterase over a wide range of substrate concentrations. In the case of cyclic AMP phosphodiesterase two apparent K_m values were determined. When low concentrations of cyclic AMP were employed in the assay, a K_m of $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M was found (Fig. 2A); when higher concentrations of cyclic AMP were tested, we found a second K_m of $2 \cdot 10^{-4}$ M (Fig. 2B). Similarly, in the case of the cyclic GMP phosphodiesterase, two apparent K_m values were determined;

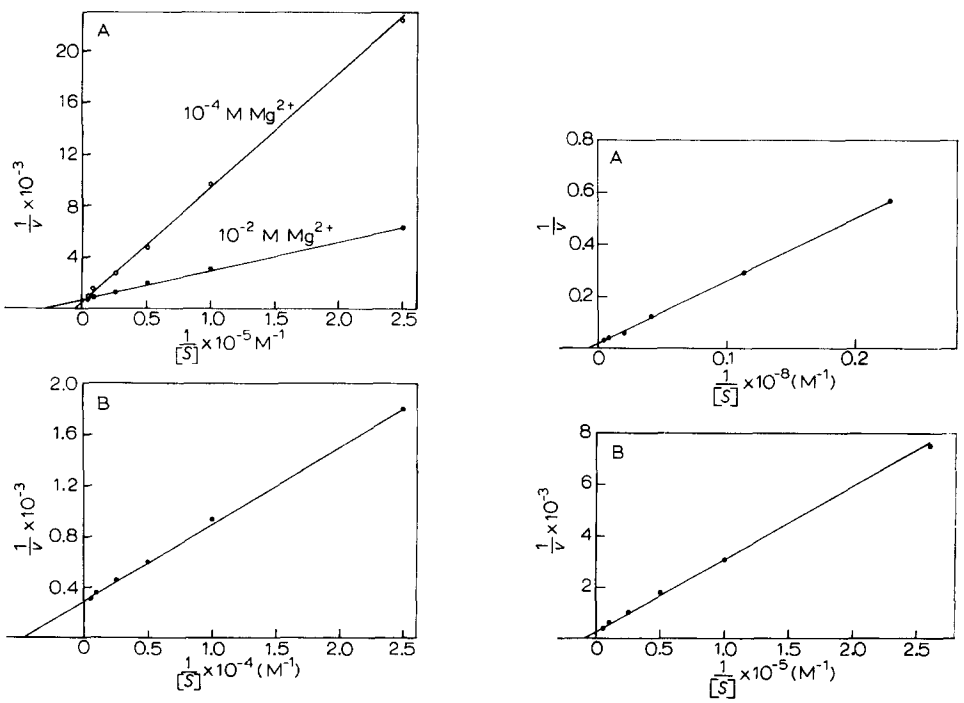


Fig. 2. The K_m values of cyclic AMP phosphodiesterase. A crude extract was employed in the assays. A. Concentrations of cyclic AMP, $4 \cdot 10^{-6}$ M to $2 \cdot 10^{-4}$ M; Mg^{2+}, 10^{-4} M and 10^{-2} M. B. Concentrations of cyclic AMP, $4 \cdot 10^{-5}$ M to $2 \cdot 10^{-3}$ M; Mg^{2+}, 10^{-2} M.

Fig. 3. The K_m values of cyclic GMP phosphodiesterase. A crude extract was employed in the assays. A. Concentrations of cyclic GMP, $4 \cdot 10^{-8}$ M to $2 \cdot 10^{-6}$ M. B. Concentrations of cyclic GMP, $4 \cdot 10^{-6}$ M to $2 \cdot 10^{-4}$ M.

one was at $2 \cdot 10^{-6}$–$3 \cdot 10^{-6}$ M cyclic GMP and the other at 10^{-4} M cyclic GMP (Figs. 3A, 3B). The experiments described in the figures were performed in sodium phosphate buffer; identical results were obtained when Tris–HCl was employed suggesting that the particular buffer used did not affect the apparent K_m values.

(3) Gel filtration of the crude extract on Sephadex G-200

Since the regulation of enzyme activity may be different at the low and high substrate concentrations, we assayed fractions at presumably physiological concentrations (cyclic AMP, 10^{-6} M; cyclic GMP, 10^{-7} M). Fig. 4A shows that when a crude extract was passed through Sephadex G-200 in Buffer B, cyclic AMP phosphodiesterase activity was found in a broad area corresponding to molecular weights ranging from approx. 115 000 to the exclusion volume (mol. wt > 400 000). A change in the buffer employed during gel filtration resulted in a different profile of cyclic AMP phosphodiesterase activity. Omission of β-mercaptoethanol (Fig. 4B) increased significantly the activity in the exclusion volume. Addition of 0.6 M NaCl (Fig. 4C) led to the appearance of a single peak of activity corresponding to a molecular weight of approx. 120 000. Reduction of the Mg^{2+} concentration from 10^{-2} M to 10^{-4} M

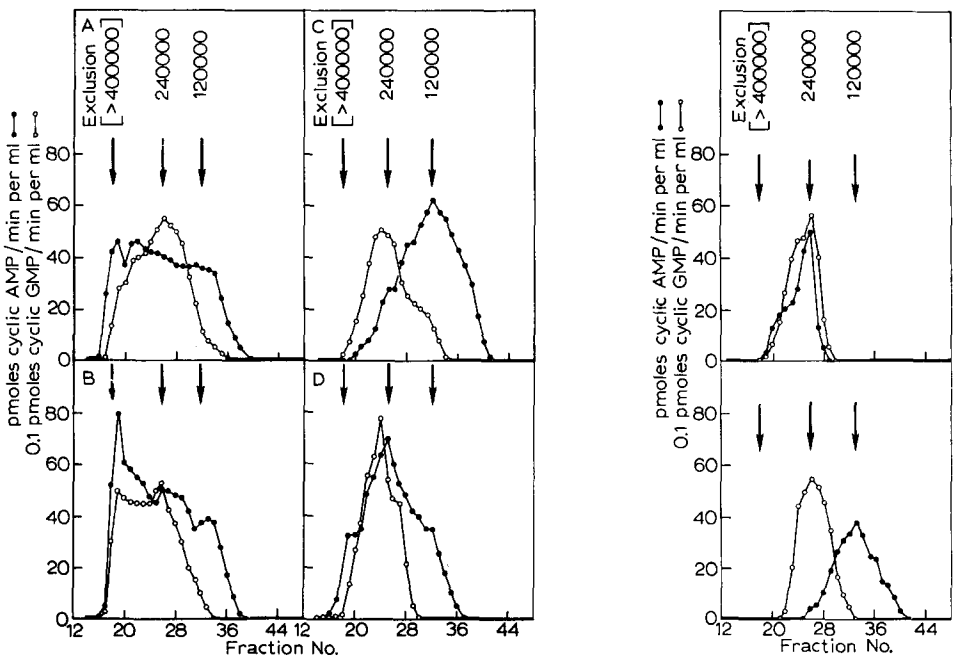


Fig. 4. Gel filtration of crude extract through Sephadex G-200 in Buffer B. A. Buffer B, unmodified. B. Buffer B, without β-mercaptoethanol. C. Buffer B, with added 0.6 M NaCl. D. Buffer B with low (10^{-4} M) concentration of Mg^{2+}. Cyclic AMP phosphodiesterase was assayed at 10^{-6} M cyclic AMP and cyclic GMP phosphodiesterase at 10^{-7} M cyclic GMP.

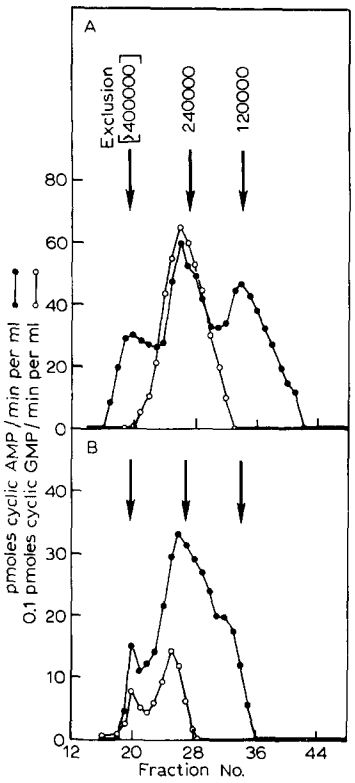
Fig. 5. Interconversion between two forms of cyclic AMP phosphodiesterase. Cyclic AMP phosphodiesterase was assayed at 10^{-6} M cyclic AMP and cyclic GMP phosphodiesterase at 10^{-7} M cyclic GMP. A. Gel filtration of crude extract through Sephadex G-200 in Buffer B containing $5 \cdot 10^{-4}$ M cyclic AMP. B. Fractions 20–27 (Fig. 5A) were pooled, concentrated, and reapplied to Sephadex G-200 in Buffer B containing 0.6 M NaCl.

resulted in a single peak of cyclic AMP phosphodiesterase activity with a molecular weight of approx. 240 000 (Fig. 4D). The lowering of the Mg^{2+} concentration also affected the "low K_m" of the enzyme: at a concentration of 10^{-4} M Mg^{2+} the K_m was $2 \cdot 10^{-4}$ M whereas it was $2 \cdot 10^{-5}$–$3 \cdot 10^{-5}$ M in the presence of 10^{-2} M Mg^{2+} (Fig. 2A).

These experiments show that cyclic AMP phosphodiesterase can occur in three forms: in the exclusion peak, *i.e.* molecular weight > 400 000, with a molecular weight of 240 000, and a molecular weight of 120 000. The fact that the use of a low concentration of Mg^{2+} in the assay gave rise to the high K_m cyclic AMP of $2 \cdot 10^{-4}$ M and that the use of the same low concentration of Mg^{2+} during gel filtration led to the preponderance of the 240 000 molecular weight form, suggested a correlation between the 240 000 molecular weight form and the high K_m. This possibility was explored further by gel filtration in presence of the substrate cyclic AMP. Fig. 5A shows the activity profile after chromatography in the presence of $5 \cdot 10^{-4}$ M cyclic AMP in the buffer. A single peak was found, corresponding to a molecular weight of 240 000. It was of interest to see whether this activity could be converted into forms with different molecular weights. It had been shown already that high concentrations of NaCl during gel filtration resulted in a single peak of activity with a molecular weight of 120 000 (Fig. 4C). Therefore the active fractions of the experiment in Fig. 5A were pooled, concentrated, and reapplied to the column with 0.6 M NaCl in the buffer. Fig. 5B demonstrates that the peak with the molecular weight of 240 000 was converted into the one with the molecular weight of 120 000. A series of experiments with the crude extract in the presence of decreasing concentrations of cyclic AMP in the buffer showed a gradual increase of activity at molecular weight 120 000 (not shown). When 10^{-7} M cyclic AMP was used during gel filtration, a distinct peak of activity at a molecular weight of 120 000 was found (Fig. 6A). In order to test the reversibility of this effect, the fractions with a molecular weight of approx. 120 000 were concentrated and reapplied to the column, this time in the presence of a high concentration of cyclic AMP ($5 \cdot 10^{-4}$ M). Fig. 6B shows that the activity now appeared in a peak corresponding to a molecular weight of 240 000. Cyclic GMP, if added to the buffer during gel filtration, had similar, though less pronounced effects (not shown).

We determined also the distribution of cyclic GMP phosphodiesterase activity. Cyclic GMP phosphodiesterase showed a single peak of activity corresponding to a molecular weight of 240 000; this was not affected by any of the modifications of the buffer used during gel filtration (Figs. 4–7).

In the experiments described up to this point, we used in the assay of cyclic AMP and cyclic GMP phosphodiesterases presumably physiological concentrations of the substrates. In the case of both enzymes, however, maximal specific activity was obtained with much higher substrate concentrations. Therefore we also determined the profile of activity after gel filtration, using cyclic AMP at 10^{-3} M and cyclic GMP at 10^{-4} M. Under these conditions of assay, both activities showed a single peak, coinciding at a molecular weight of approx. 360 000 (Fig. 7). The occurrence of this peak showing both activities was observed consistently and was not affected by changes in buffer.



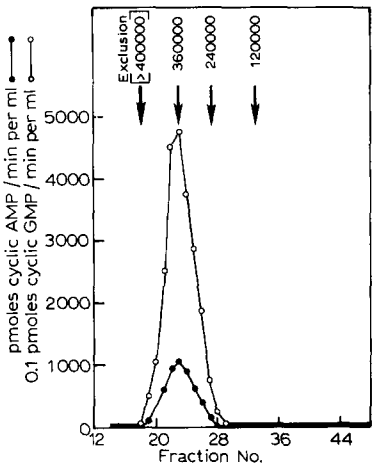


Fig. 6. Interconversion between two forms of cyclic AMP phosphodiesterase. Cyclic AMP phosphodiesterase was assayed at 10^{-6} M cyclic AMP and cyclic GMP phosphodiesterase at 10^{-7} M cyclic GMP. A. Gel filtration of crude extract through Sephadex G-200 in Buffer B containing 10^{-7} M cyclic AMP. B. Fractions 31–40 (Fig. 6A) were pooled, concentrated, and reapplied to Sephadex G-200 in Buffer B containing $5 \cdot 10^{-4}$ M cyclic AMP.

Fig. 7. Gel filtration of crude extract through Sephadex G-200 in Buffer B. Fractions assayed at high concentrations of substrate (10^{-3} M cyclic AMP; 10^{-4} M cyclic GMP).

(4) Purification of cyclic AMP phosphodiesterase

Acetone fractionation. Acetone fractionation led to a 4–5 fold purification of the cyclic AMP phosphodiesterase. This preparation also showed non-linear kinetics in the K_m determinations and the same values were found as in the case of the crude extract. After gel filtration through Sephadex G-200 in Buffer B (Fig. 8A) a single peak of molecular weight 240 000 was found, if fractions were assayed at 10^{-6} M cyclic AMP. The enzyme was unstable at this stage and lost 80% of its activity in one week during storage at −20 °C. NaCl (0.6 M) stabilized it and converted it into the form with a molecular weight of 120 000 (not shown). Fig. 8A shows that this preparation also contained cyclic GMP phosphodiesterase, though less than was present in the crude extract. It had the same apparent K_m as the cyclic GMP phosphodiesterase of the crude preparation.

Ion exchange fractionation on DE-52. The preparation from the preceding step was subjected to column chromatography on DE-52. Highest purification of the cyclic AMP phosphodiesterase was achieved with elution at between 0.6–1.0 M NaCl.

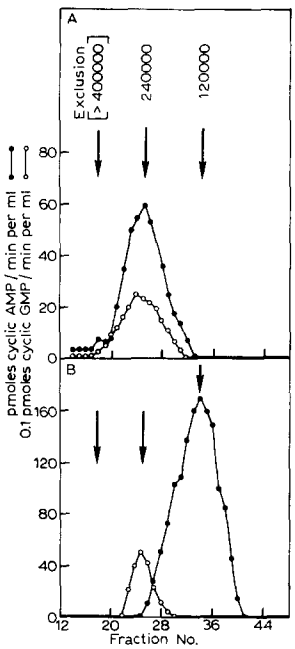


Fig. 8. A. Gel filtration through Sephadex G-200 of acetone-treated preparation. Buffer B was used and the fractions assayed with 10^{-6} M cyclic AMP and 10^{-7} M cyclic GMP, respectively. B. Gel filtration through Sephadex G-200 of the eluate from DE-52 column. Buffer C was employed and cyclic AMP phosphodiesterase assayed at 10^{-6} M cyclic AMP and cyclic GMP phosphodiesterase at 10^{-7} M cyclic GMP.

The preparation still contained a small amount of cyclic GMP phosphodiesterase. This is shown in the profile obtained after gel filtration of the eluate from DE-52 on Sephadex G-200 (Fig. 8B). There was still overlap in the peaks of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities, but it was possible by selective pooling and repetition of the gel filtration to separate cyclic AMP phosphodiesterase activity completely from that of the cyclic GMP phosphodiesterase. The 100-fold purified cyclic AMP phosphodiesterase still showed the two K_m values of $2 \cdot 10^{-5}$ M cyclic AMP and $2 \cdot 10^{-4}$ M cyclic AMP when assayed in the low and high ranges of cyclic AMP concentrations, respectively. Gel filtration in the presence of 1.5 M urea did not result in the formation of either cyclic AMP phosphodiesterase with a molecular weight of less than 120 000 or a cyclic GMP phosphodiesterase with a molecular weight of less than 240 000. The purification scheme is summarized in Table I.

Purified bovine brain cyclic AMP phosphodiesterase activator, kindly furnished by Dr W. Y. Cheung (St. Jude Children's Research Hospital, Memphis, Tenn.), had no effect on the activity of a 60-fold purified preparation of the cyclic AMP phosphodiesterase when tested under a variety of conditions.

(5) Effect of cyclic GMP on hydrolysis of cyclic AMP

We tested the effect of cyclic AMP on the hydrolysis of cyclic GMP using 10^{-7} M and 10^{-4} M cyclic GMP and a wide range of concentrations (10^{-8} M–$2 \cdot 10^{-4}$ M) of

TABLE I

PURIFICATION OF BEEF LIVER CYCLIC AMP PHOSPHODIESTERASE

Stage	*Total protein (mg)*	*Protein (mg/ml)*	*Total activity (units)*	*Spec. act. (units/mg protein)*	*Recovery (%)*	*Purification**
Homogenate	31 800	28.9	970 000	30.5	100	1
Streptomycin, supernate	26 800	22.3	874 000	32.6	90	1.1
Acetone precipitation	3 120	14.4	518 000	166	53	5.4
DE-52 eluate	780	10.0	390 000	500	40	16.4
First Sephadex G-200 eluate	167	5.0	311 000	1860	32	61
Second Sephadex G-200 eluate	59	4.5	185 000	3100	19	102

* Based on increase in specific activity when assayed with 10^{-6} M cyclic AMP.

cyclic AMP. Both crude extract and a partially purified preparation (Fig. 8B) of cyclic GMP phosphodiesterase were employed. We found no effect of cyclic AMP on the hydrolysis of cyclic GMP. Conversely cyclic GMP (10^{-8} to $2 \cdot 10^{-4}$ M) did not affect the hydrolysis of cyclic AMP when this was present at 10^{-6} or 10^{-4} M. The preparation of cyclic AMP phosphodiesterase used in this experiment was approximately 60-fold purified. Quite different results were obtained, however, when a crude extract was employed as source of cyclic AMP phosphodiesterase and when the concentration of cyclic AMP was high (10^{-4} M). Under these conditions, cyclic GMP at concentrations of above $5 \cdot 10^{-5}$ M inhibited (Fig. 9).

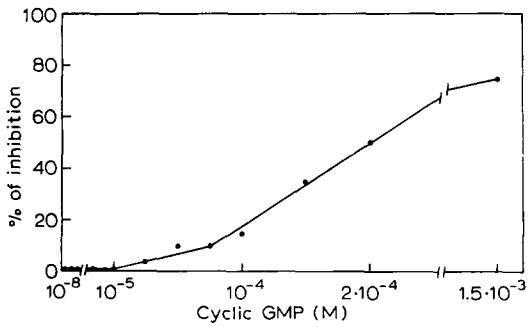


Fig. 9. Effect of cyclic GMP on the cyclic AMP phosphodiesterase of crude extract when assayed at high concentration (10^{-4} M) of cyclic AMP. The cyclic GMP was added together with the [^{3}H]cyclic AMP. Not more than 15% of the cyclic GMP was hydrolyzed in the course of the experiment at any of the concentrations of cyclic GMP employed.

DISCUSSION

Studies in other laboratories in which a variety of tissues were employed suggested that there were two cyclic AMP phosphodiesterase activities characterized by different K_m values[4-7]. We also observed two cyclic AMP phosphodiesterase activities with distinct K_m values in the crude extracts of beef liver (Figs. 2A, 2B). There are

at least two possible explanations for the anomalous kinetics. Either multiple binding sites on one enzyme with negative cooperativity (Levitzki and Koshland)[20] or the occurrence of two distinct forms of the enzyme with differing affinities for cyclic AMP could explain the findings.

The observations reported in this paper are in accord with the second hypothesis. We found two peaks showing cyclic AMP phosphodiesterase activity when a crude extract of liver was submitted to gel filtration and assayed at 10^{-6} M cyclic AMP, a presumably physiological concentration of the cyclic nucleotide. Molecular weights of approx. 120 000 and 240 000 could be assigned to the two fractions. If the cyclic AMP phosphodiesterase of crude extracts was assayed at the low concentration (10^{-4} M) of Mg^{2+}, a "high K_m" of approx. $2 \cdot 10^{-4}$ M cyclic AMP was obtained, even if the determination was carried out in the low range of cyclic AMP concentrations (Fig. 2A). Gel filtration in the presence of the low concentration of Mg^{2+} led to the appearance of only one peak showing cyclic AMP phosphodiesterase activity; its position corresponded to a molecular weight of approx. 240 000 (Fig. 4D). These findings suggest that the K_m of $2 \cdot 10^{-4}$ M cyclic AMP describes the activity of the 240 000 molecular weight form of the enzyme. By the same token, if gel filtration was carried out in the presence of a high concentration of cyclic AMP, only one peak of phosphodiesterase activity was obtained (Fig. 5A) also suggesting that high concentrations of cyclic AMP lead to the formation of the 240 000 molecular weight component.

If, however, gel filtration of crude extracts was carried out in either the presence of 10^{-2} M Mg^{2+} (Fig. 4A) or 0.6 M NaCl (Fig. 4C), another peak showing cyclic AMP phosphodiesterase activity appeared; its location corresponded to a molecular weight of 120 000. The presence during gel filtration of concentrations of cyclic AMP below 10^{-6} M did not lead to the formation of the 240 000 molecular weight form. This suggests that the K_m of $2 \cdot 10^{-5}$ M cyclic AMP, found when low concentrations of cyclic AMP were employed in the K_m determination, corresponds to the 120 000 molecular weight form. Clearly, the 120 000 and 240 000 molecular weight forms are interconvertible with the substrate cyclic AMP, itself, being one agent affecting the relative occurrence of the two forms (Figs 5 and 6). This presumably, explains the continued existence of two K_m values even when the isolated 120 000 and 240 000 molecular weight fractions were assayed. Thus fractions isolated after precipitation with acetone (p. 57) or after ion-exchange chromatography (p. 57) and having molecular weights of 240 000 and 120 000, respectively, still showed the two K_m values that were in crude extracts.

An analogous relationship was reported for the cyclic AMP phosphodiesterase of rat brain (Thompson and Appleman)[11] and for rat kidney and frog bladder (Jard and Bernard)[12].

Our findings indicate a different situation with respect to the cyclic GMP phosphodiesterase. If this enzyme was assayed at a concentration of 10^{-7} M cyclic GMP after the passage of a crude extract through Sephadex G-200, only one peak having activity was detected. It corresponded to a molecular weight of 240 000 (Fig. 4A) and, presumably, it was the activity in this peak that was characterized by the low K_m of $2 \cdot 10^{-6}$ M. When the fractions were assayed at the high substrate concentration of 10^{-4} M cyclic GMP, a new peak of activity with a molecular weight of 360 000 emerged (Fig. 7). We assume that this peak corresponds to the fraction with the high

K_m of 10^{-4} M cyclic GMP. None of the conditions which affected the occurrence of the two forms of the cyclic AMP phosphodiesterase had any effect on the cyclic GMP phosphodiesterases. It also appears that the cyclic GMP phosphodiesterase which is active at low substrate concentrations is distinct from the cyclic AMP phosphodiesterase despite the coincident molecular weights of 240 000. The evidence for this is based on the following: (a) the two enzymes can be separated from one another during purification (Fig. 8B); (b) conditions leading to the interconversion of the 120 000 and 240 000 molecular weight forms of cyclic AMP phosphodiesterase do not affect the molecular weight of the cyclic GMP phosphodiesterase; (c) at physiologically relevant concentrations of the substrates, cyclic GMP does not inhibit the hydrolysis of cyclic AMP and cyclic AMP does not inhibit the hydrolysis of cyclic GMP; (d) the concentrations of Mg^{2+} required for maximal rates of hydrolysis of cyclic GMP and cyclic AMP, respectively, differ by a factor of one hundred (Fig. 1A); (e) the two enzymes show differential sensitivity to several analogs of α-tocopherol (Schröder, J. and Rickenberg, H. V., unpublished). The last three points are not conclusive by themselves insofar as one could argue for the occurrence of one molecule with discrete binding sites for the two cyclic nucleotides.

The fact that cyclic GMP does inhibit cyclic AMP phosphodiesterase activity when high concentrations of both cyclic AMP and cyclic GMP are employed (Fig. 9) appears, at first sight, in conflict with the preceding statements regarding the discreteness of the two phosphodiesterases. However, as shown in Fig. 7, the fraction obtained after gel filtration of a crude extract which is maximally active when assayed at unphysiologically high concentrations of cyclic AMP (10^{-3} M) and cyclic GMP (10^{-4} M) elutes in an area corresponding to a molecular weight of approx. 360 000. This fraction shows both cyclic AMP and cyclic GMP phosphodiesterase activity and its behavior on the Sephadex column is unaffected by those changes in the concentration of ions or nucleotides which bring about the interconversions of the cyclic AMP phosphodiesterase fractions with molecular weights of 120 000 and 240 000. We speculate that the inhibition of the cyclic AMP phosphodiesterase by cyclic GMP which occurs when high concentrations of the two cyclic nucleotides are employed (Fig. 9) affects the activity of the 360 000 molecular weight species. It is conceivable that this fraction represents an aggregate of cyclic AMP phosphodiesterase (molecular weight 120 000) and of cyclic GMP phosphodiesterase (molecular weight 240 000) with low affinity for both of the cyclic nucleotides and hence detectable only, if the assay is carried out at high substrate concentrations. This peak of cyclic AMP and cyclic GMP phosphodiesterase activity was no longer detected after the crude extract had been treated with acetone. The postulated relationships between the different forms of the cyclic nucleotide phosphodiesterases are shown in Fig. 10.

Other investigators[2,8,13–15,21] found that even purified preparations hydrolyzed the two cyclic nucleotides. They employed 10^{-4} M or higher concentrations of cyclic AMP when monitoring for activity during purification and it appears possible that they selected for a species analogous to our postulated cyclic AMP–cyclic GMP phosphodiesterase aggregate.

In summary, our findings indicate the occurrence in beef liver of multiple forms of cyclic AMP phosphodiesterase characterized by differing molecular weights and differing K_m values for cyclic AMP. The 120 000 and 240 000 molecular weight forms of cyclic AMP phosphodiesterase are clearly interconvertible. Cyclic GMP phospho-

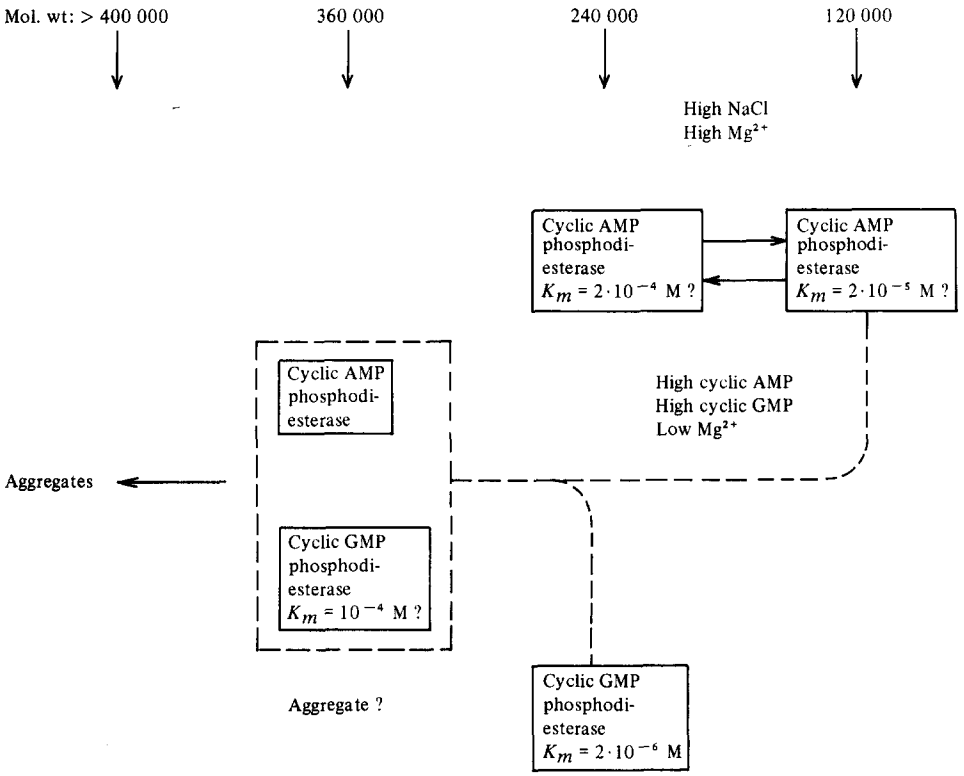


Fig. 10. Hypothetical relationships between the different forms of cyclic AMP and cyclic GMP phosphodiesterases.

diesterase appears to be distinct from the cyclic AMP phosphodiesterase, although in addition to the discrete phosphodiesterases there may also occur an aggregate with both cyclic AMP and cyclic GMP phosphodiesterase activities. These, however, seem to require unphysiologically high concentrations of substrate.

Whether the interconvertibility of the cyclic AMP phosphodiesterase between the high and the low molecular weight forms with high and low K_m values, respectively, plays any role in the regulation of the metabolism of cyclic AMP remains to be seen.

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