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# CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE

## EFFECT OF DIVALENT CATIONS

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SUMMARY

1. A partially purified preparation of bovine brain phosphodiesterase contained approx. 1 mole each of $Ca^{2+}$ and $Zn^{2+}$ per 250 000 g of protein. Extensive dialysis of the enzyme preparation against an EDTA solution reduced the metals to about half their contents before dialysis.

2. The brain enzyme depended on divalent cations for activity. $Mg^{2+}$ was slightly stimulatory and $Cu^{2+}$ was inhibitory. $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$ were stimulatory at low ($\leqslant$ 0.1 mM) but inhibitory at high concentrations ($\geqslant$ 1 mM). Therefore, the terms stimulatory and inhibitory are to be used arbitrarily since a cation may be either stimulatory or inhibitory depending on its concentration. In this study $Mn^{2+}$ (0.1 mM) and $Ca^{2+}$ (1 mM) will be used as stimulatory and inhibitory cations, respectively.

3. Kinetic analysis indicated that a stimulatory cation (0.1 mM $Mn^{2+}$) increased the $K_{mapp}$ and $v_{max}$ of phosphodiesterase, whereas an inhibitory cation (1 mM $Ca^{2+}$) decreased them. The simplest mechanism appears to be that the metal ions principally affect the rate of dissociation of the enzyme-substrate complex to its product and free enzyme.

4. The optimal pH of phosphodiesterase varied with an exogenous metal. It was observed at pH 8.3 in the absence of added cation, at pH 8 in the presence of 0.1 mM $Mn^{2+}$, and at pH 7.6 in 1 mM $Ca^{2+}$. The effectiveness of a divalent cation varied with pH. Whereas the stimulation by 0.1 mM $Mn^{2+}$ was pronounced at a neutral pH, the inhibition by 1 mM $Ca^{2+}$ was severe at an alkaline pH.

5. The effect of a mixture of paired divalent cations on phosphodiesterase was studied. The stimulation afforded by a pair of stimulatory cations was invariably less than the sum of that by the individual cations. On the other hand, the effect of a mixture of a stimulatory cation and an inhibitory cation was comparable to that given by the stimulatory cation alone.

6. EDTA and ATP were potent inhibitors of phosphodiesterase, with 50%

Abbreviation: EGTA, ethyleneglycol-bis($\beta$-aminoethyl ether)-*N*,*N*'-tetraacetate.

inhibition at 7 $\mu$M and 30 $\mu$M, respectively. Inhibition of phosphodiesterase by EDTA was completely reversed by $Mn^{2+}$, but that by ATP was only partially reversed.

7. Imidazole stimulated phosphodiesterase activity, and the imidazole stimulated activity was greater in the presence than in the absence of $Mn^{2+}$. This was true over a wide range of imidazole concentrations.

8. Phosphodiesterase was fully active in the crude stage, and was relatively inactive when purified, unless supplemented with a specific protein activator which was removed from the enzyme during the course of purification. $Mn^{2+}$ increased the activity of the non-stimulated enzyme as well as the enzyme after full stimulation by the activator.

9. The brain enzyme also hydrolyzed the other naturally-occurring cyclic GMP. The effectiveness of divalent cations on the hydrolysis of cyclic AMP and cyclic GMP appeared to vary with the substrate. $Mn^{2+}$ was more effective for the hydrolysis of cyclic AMP whereas $Mg^{2+}$ was more effective for that of cyclic GMP.

## INTRODUCTION

The synthesis of cyclic AMP from ATP is catalyzed by adenyl cyclase, and its hydrolysis to 5'-AMP by cyclic 3',5'-nucleotide phosphodiesterase[1]. In the brain, the relative activity of adenyl cyclase to phosphodiesterase is about 1 to 100 or less[2]. In spite of such a disparity of the two enzymic activities, cyclic AMP levels in the rabbit brain changed markedly within seconds after decapitation[3], indicating a rapid turnover of the nucleotide. Since the conversion of cyclic AMP to 5'-AMP by phosphodiesterase is the only physiological mechanism known to terminate the action of the nucleotide, an understanding of the means regulating phosphodiesterase activity assumes significance.

Brain phosphodiesterase is partly soluble and partly particulate. A considerable portion of the soluble enzyme may have derived from the synaptoplasm. The majority of the particulate activity is associated with the microsomes, and is revealed only in the presence of a detergent[4]. The enzyme is fully active in the crude stage, and becomes relatively inactive when purified, unless supplemented with an exogenous protein activator which has been removed from the enzyme during the course of purification[5].

Phosphodiesterase requires a divalent cation for maximal activity, and is inhibited by EDTA, nucleoside triphosphate, citrate, and other metal chelators[6], suggesting that the active forms of phosphodiesterase is a metal enzyme complex. The present communication described further the effect of divalent cations on the activity of bovine brain phosphodiesterase.

## MATERIALS AND METHODS

### *Chemicals*

$(NH_4)_2SO_4$ and Tris were ultra pure grades from Mann BioResearch. Tris was recrystallized 3 times before use. Snake venom *(Crotalus atrox)* was a lyophilized preparation from Sigma. All other reagents were of analytical grade. Divalent cations were either salts of sulfate or of chloride. Double glass-distilled water was used throughout.

*Preparation and assay of phosphodiesterase*

Phosphodiesterase was prepared from bovine brain cerebra as described previously[7]. The purification procedure consisted of extraction of the brain tissue with water, followed by pH precipitation, $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption, and DEAE-cellulose chromatography. Previous work from this laboratory showed that phosphodiesterase lost activity upon purification, due to the gradual removal of a protein activator from the enzyme. Most of the work described in this study was performed with a phosphodiesterase purified through the step of calcium phosphate gel. At this stage of purification, the enzyme retained sufficient activator, and the activity was essentially independent of an exogenous activator. When purification was carried one step further through a DEAE-cellulose column, more activator was removed, and the purified enzyme was relatively inactive unless supplemented with an exogenous activator[8]. All preparations were usually dialyzed against 20 mM Tris–HCl (pH 7.5) with several changes over a 24-h period. This buffer was used routinely unless otherwise stated. Occasionally, the enzyme was dialyzed first against Tris–HCl containing 4 mM EDTA, and then dialyzed against the usual Tris–HCl buffer to remove the EDTA from the previous dialysis. Both dialysis procedures gave essentially similar results in the assay of phosphodiesterase activities.

The enzyme was assayed according to a 2-stage procedure, using 5′-nucleotidase of snake venom *(Crotalus atrox)* to convert the product of the reaction, 5′-AMP into adenosine and inorganic phosphate, which was determined colorimetrically[7].

Alternatively, the activity was followed titrimetrically. The phosphate moiety of cyclic AMP possesses one ionizable species while that of 5′-AMP possesses two. As cyclic AMP is hydrolyzed by phosphodiesterase to 5′-AMP, a stoichiometric amount of proton is generated. The amount of an alkali added to maintain the pH of an unbuffered reaction mixture gives a continuous measure of the rate of hydrolysis[9].

*Protein determination*

Proteins were measured either with the Biuret reagent, using bovine serum albumin as a standard, or according to the spectrophotometric technique of WARBURG AND CHRISTIAN[10].

*Analysis of metals*

Metals were determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 403. Enzyme samples were adjusted 8–13 mg protein/ml of the Tris–HCl buffer, and were analyzed for metals directly in this solution.

## RESULTS

*Metal content of phosphodiesterase partially purified from bovine brain*

Most of the work described in this paper used an enzyme preparation purified through the step of calcium phosphate gel[7]. The purified enzyme was heterogeneous in acrylamide gel electrophoresis, and attempts to achieve further purification have not been successful. Gel filtration chromatography indicated that the brain enzyme had a molecular weight of 200 000 or greater. The metal contents of six of such preparations are presented in Table I. Three of these samples (Samples 4–6) have been dialyzed extensively against a Tris–HCl buffer containing 4 mM EDTA before

TABLE I

METAL CONTENTS OF A PARTIALLY PURIFIED PHOSPHODIESTERASE FROM BOVINE BRAIN

Phosphodiesterase was purified through the calcium phosphate gel according to a procedure described previously[7]. Samples 1–3 were dialyzed as usual against a 20 mM Tris–HCl (pH 7.5). Samples 4–6 were first dialyzed for 50 h against Tris–HCl buffer containing 4 mM EDTA and then against a Tris–HCl buffer containing no EDTA. The protein concentrations of the samples were: (1) 8.6 mg/ml, (2) 10.3 mg/ml, (3) 13.8 mg/ml, (4) 12.0 mg/ml, (5) 10.5 mg/ml, and (6) 13.5 mg/ml. The Tris–HCl buffer contained a trace amount of $Ca^{2+}$ (0.7 $\mu$g/ml or 1.7 $\mu$M) and no detectable amounts of $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$. The values of $Ca^{2+}$ for the protein samples had been corrected for the amount of $Ca^{2+}$ present in the Tris–HCl buffer.

| *Sample* | *Metal content (moles/250 000 g protein)* | | | | |
|---|---|---|---|---|---|
| | $Mg^{2+}$ | $Ca^{2+}$ | $Mn^{2+}$ | $Co^{2+}$ | $Zn^{2+}$ |
| 1 | 0.10 | 0.99 | 0.001 | 0.03 | 0.77 |
| 2 | 0.06 | 0.90 | 0.003 | 0.01 | 0.91 |
| 3 | 0.07 | 1.02 | 0.002 | — | 0.86 |
| 4 | 0.04 | 0.29 | 0.004 | 0.03 | 0.21 |
| 5 | 0.06 | 0.41 | 0.003 | 0.01 | 0.39 |
| 6 | 0.07 | 0.48 | 0.003 | | 0.34 |

its final dialysis against a Tris–HCl buffer to remove the EDTA from the previous dialysis. Of the metals analyzed only $Ca^{2+}$ and $Zn^{2+}$ were found in appreciable amounts. Note that the concentration of $Ca^{2+}$ and $Zn^{2+}$ was approx. 1 mole per 250 000 g protein and decreased to less than 0.5 mole after extensive dialysis against the Tris–EDTA buffer. This suggested that the metals were bound to the protein with different affinities. The amount of $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ was negligible in these preparations.

In the assay of phosphodiesterase the amount of protein present in the incubation mixture was usually 100 $\mu$g/ml. It can be estimated that the contribution of each of these metals to the reaction system from this amount of protein would be less than 1 $\mu$M.

*Effect of divalent cations*

Phosphodiesterase of different tissues requires divalent cations for optimal activity. Fig. 1 shows the effect of various divalent cations on the activity of bovine brain phosphodiesterase. $Mg^{2+}$ was stimulatory; $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$ were stimulatory at 0.1 mM or lower and were inhibitory at 1 mM or above, while $Cu^{2+}$ inhibited phosphodiesterase activity. The basal activity in the absence of an exogenous cation in this experiment was 40% of the activity in 0.1 mM $Mn^{2+}$. It should be noted that the basal activity varied from preparation to preparation and could not be suppressed by extensive dialysis against a Tris–EDTA buffer.

This experiment shows that a metal divalent cation can be either stimulatory or inhibitory, depending on its concentration. Therefore, the terms stimulatory and inhibitory are to be used arbitrarily in the present work.

According to the data of HANIG AND APRISON[12], the tissue level of metals in the cerebral cortex of rabbit brain may be estimated to be: $Mg^{2+}$, 7.9 mM; $Ca^{2+}$, 1.6 mM; $Mn^{2+}$, 0.03 mM; and $Zn^{2+}$, 0.3 mM*. Since the intracellular concentration of

* These values were calculated by this author from the original data of HANIG AND APRISON[12].

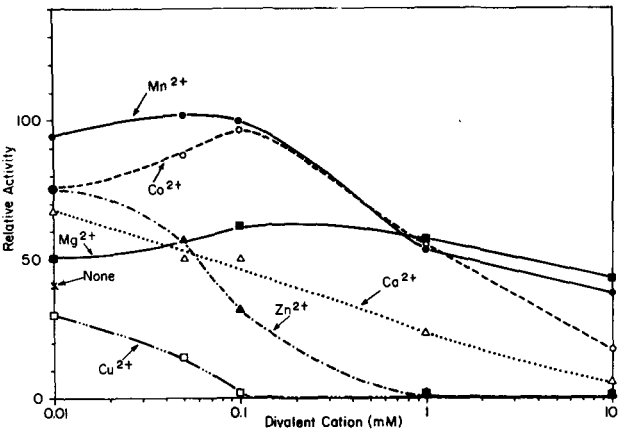


Fig. 1. The effect of various divalent cations on the activity of phosphodiesterase as a function of cation concentration. The reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 2 mM cyclic AMP, 75 μg enzyme, and divalent cations as indicated. At the end of 10 min, the reaction was terminated in a boiling water bath. After thermal equilibration, 0.1 mg snake venom *(Crotalus atrox)* and various amounts of EDTA were added for another 10-min incubation. The amount of EDTA added varied with the concentration of the cations and final concentrations of cations and EDTA were comparable. EDTA was added to protect the venom's 5′-nucleotidase activity from inhibition by heavy metal ions. EDTA itself has no appreciable effect on 5′-nucleotidase activity. The reaction of the second incubation was arrested by the addition of 0.1 ml of 55% trichloroacetic acid. The inorganic phosphate released was determined colorimetrically[7].

$Ca^{2+}$ in the various parts of the mammalian brain is in the mM range[12], $Ca^{2+}$ at 1 mM will be treated subsequently as an inhibitory divalent cation.

*Effect of a mixture of paired divalent cations*

The combined effect of a mixture of a pair of stimulatory cations on phosphodiesterase activity was studied. Table II shows the effect of paired combinations of

TABLE II

INTERACTION BETWEEN A PAIR OF STIMULATORY DIVALENT CATIONS ON PHOSPHODIESTERASE ACTIVITY

The reaction mixture of 1.0 ml contained 40 mM Tris–HCl (pH 8.0), 118 μg enzyme, 2 mM cyclic AMP, and divalent cations as indicated. Phosphodiesterase was assayed as described in the legend to Fig. 1.

| *Addition (mM)* | *Activity (nmoles/ 10 min)* |
|---|---|
| 1. None | 69 |
| 2. $Mn^{2+}$ (0.1) | 101 |
| 3. $Co^{2+}$ (0.1) | 124 |
| 4. $Zn^{2+}$ (0.01) | 104 |
| 5. $Mg^{2+}$ (1) | 90 |
| 6. $Mn^{2+}$ (0.1) + $Co^{2+}$ (0.1) | 99 |
| 7. $Mn^{2+}$ (0.1) + $Zn^{2+}$ (0.01) | 85 |
| 8. $Mn^{2+}$ (0.1) + $Mg^{2+}$ (1) | 86 |
| 9. $Co^{2+}$ (0.1) + $Zn^{2+}$ (0.01) | 99 |
| 10. $Co^{2+}$ (0.1) + $Mg^{2+}$ (1) | 110 |
| 11. $Zn^{2+}$ (0.01) + $Mg^{2+}$ (1) | 87 |

TABLE III

INTERACTION BETWEEN $Mg^{2+}$ AND $Ca^{2+}$ ON PHOSPHODIESTERASE ACTIVITY

The reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 75 $\mu$g enzyme, 2 mM cyclic AMP, and divalent cations as indicated. The first stage of incubation was 10 min and the second stage 30 min. A longer second incubation was adopted in these experiments to substitute for the use of EDTA as described in the legend to Fig. 1.

| *Addition (mM)* | *Activity (nmoles/mg per min)* |
|---|---|
| 1. None | 258 |
| 2. $Mg^{2+}$ (1) | 260 |
| 3. $Mg^{2+}$ (10) | 234 |
| 4. $Ca^{2+}$ (1) | 46 |
| 5. $Ca^{2+}$ (10) | 18 |
| 6. $Mg^{2+}$ (1) + $Ca^{2+}$ (1) | 268 |
| 7. $Mg^{2+}$ (1) + $Ca^{2+}$ (10) | 171 |
| 8. $Mg^{2+}$ (10) + $Ca^{2+}$ (1) | 256 |
| 9. $Mg^{2+}$ (10) + $Ca^{2+}$ (10) | 248 |

$Mn^{2+}$, $Co^{2+}$, $Zn^{2+}$ and $Mg^{2+}$ on phosphodiesterase activity at their optimal concentrations. The stimulation by the paired combinations was invariably less than that by the more effective individual cation or even by any one of the pair. However, the effect of a mixture of stimulatory cations and an inhibitory cation was governed by that of the stimulatory cation. As shown in Table III, $Ca^{2+}$ strongly inhibited the activity of phosphodiesterase, and the inhibition was completely reversed by an equimolar concentration of $Mg^{2+}$. Even when the concentration of $Ca^{2+}$ was 10 times that of $Mg^{2+}$ the stimulatory effect of $Mg^{2+}$ predominated.

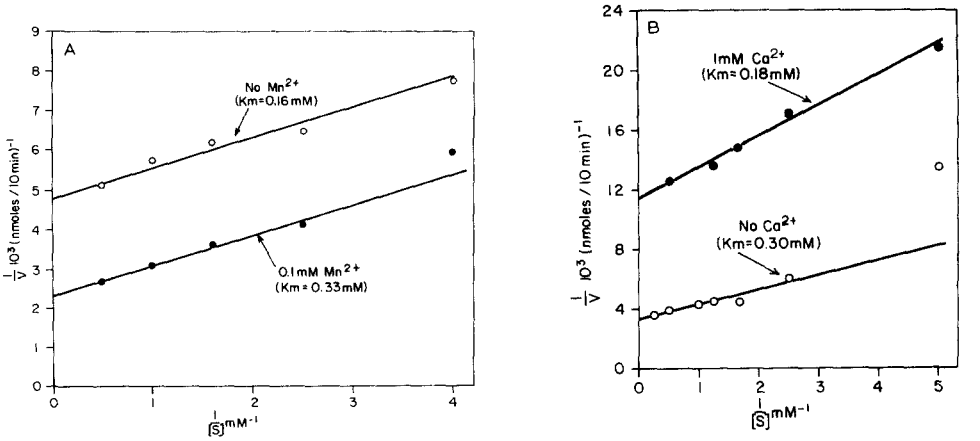


Fig. 2. A. The nature of stimulation of phosphodiesterase activity by $Mn^{2+}$. Phosphodiesterase activity was assayed with and without $Mn^{2+}$ as in Fig. 1. The data are expressed as double reciprocal plots, and the lines drawn by visual approximation to best fit. B. The nature of inhibition of phosphodiesterase activity by $Ca^{2+}$. Phosphodiesterase was assayed as in Fig. 1.

*Mechanism of stimulation or inhibition by divalent cations*

The effect of a stimulatory divalent cation on the activity of phosphodiesterase was examined by the Lineweaver–Burk plot. Fig. 2A shows that in the absence of any added divalent cations, the apparent Michaelis constant is 0.16 mM, and the $v_{max}$ is 21 nmoles/min; in the presence of $Mn^{2+}$, the $K_{mapp}$ is increased to 0.33 mM and the $v_{max}$ to 46 nmoles/min. The effect of $Mg^{2+}$, $Zn^{2+}$ and $Co^{2+}$ on the $K_{mapp}$ and $v_{max}$ of phosphodiesterase (not shown here) was qualitatively similar to that of $Mg^{2+}$. All $K_{mapp}$ and $v_{max}$ were derived graphically from the kinetic data.

The effect of an inhibitory cation such as $Ca^{2+}$ on phosphodiesterase activity is shown in Fig. 2B. $Ca^{2+}$ decreased the $K_{mapp}$ from 0.3 to 0.18 mM, and the $v_{max}$ from 30 to 9 nmoles/min.

The $K_{mapp}$ of phosphodiesterase ranged between 0.15–0.30 mM. In spite of the range, the qualitative effect of the divalent cations was reproducible. A stimulatory divalent cation increased the $K_{mapp}$ and $v_{max}$ of phosphodiesterase whereas an inhibitory cation decreased them.

*Effect of metal chelators*

EDTA inhibits phosphodiesterase of rabbit brain[13], rat brain[6], bovine brain[11], and fish brain[14]. Other compounds that also inhibit the enzyme include the nucleoside triphosphates and diphosphates, inorganic pyrophosphate and polyphosphates, citrate and other dicarboxylic acids[6]. These compounds chelate divalent cations to various extents. It has been postulated that phosphodiesterase is a metalloenzyme, and that the inhibition by these compounds is exerted through chelation of the metal on the enzyme molecule[6].

Table IV shows the inhibition of phosphodiesterase by the usual metal chelators. EDTA was most potent, followed by EGTA, *O*-phenanthroline and quinoline. 100 $\mu$M EDTA suppressed phosphodiesterase activity completely while 100 $\mu$M EGTA exerted about 50% inhibition. At the same concentration, the inhibition caused by *O*-phenanthroline or quinoline was 25% or less.

The residual phosphodiesterase activity in the presence of EGTA is of interest. It is known that EGTA exhibits a selective affinity for $Ca^{2+}$. The data in Table I

TABLE IV

INHIBITION OF PHOSPHODIESTERASE BY METAL CHELATORS

The assay was performed as described in the legend to Table III. Phosphodiesterase used was 450 $\mu$g.

| *Additions ($\mu$M)* | *Inhibition (%)* |
|---|---|
| 1. None | 0 |
| 2. EDTA (15) | 49 |
| 3. EDTA (100) | 100 |
| 4. EGTA (15) | 37 |
| 5. EGTA (100) | 46 |
| 6. *O*-Phenanthroline (15) | 9 |
| 7. *O*-Phenanthroline (100) | 25 |
| 8. Quinoline (15) | 1 |
| 9. Quinoline (100) | 17 |

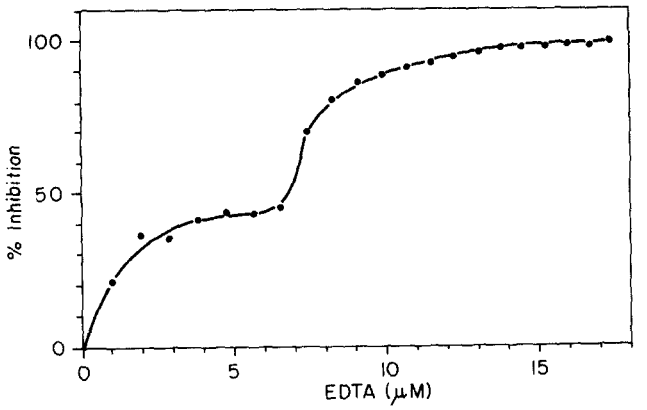


Fig. 3. Inhibition of phosphodiesterase activity as a function of EDTA concentration. Phosphodiesterase activity was followed titrimetrically in a micro version of the Metrohm Combi-Titrator 3D. Assay was carried out in a final volume of 1.5 ml containing 40 mM NaCl, 240 mg protein, 1 mM cyclic AMP and other additions as indicated. The pH of the reaction mixture was maintained at pH 8.0 by the addition of 2 mM NaOH. The enzyme preparation was dialyzed as usual in the Tris–HCl buffer.

showing the decrease of $Ca^{2+}$ and $Zn^{2+}$ in the enzyme preparation after extensive dialysis suggests that there are at least two binding sites for the metals with different affinities. The inhibition observed in the presence of EGTA might be due to chelation of the less firmly bound $Ca^{2+}$, and the activity resistant to EGTA to the more firmly bound $Ca^{2+}$. Alternatively, the residual activity might be attributable to the bound $Zn^{2+}$ that was not chelated by EGTA, the assumption being that both the bound $Ca^{2+}$ and $Zn^{2+}$ were necessary for phosphodiesterase activity.

Fig. 3 depicts the inhibition of phosphodiesterase as a function of EDTA concentration. Half maximal inhibition was obtained at 7 $\mu$M EDTA. The biphasic shape of the inhibition curve was routinely reproducible, and indicated differential interaction between the EDTA and the metals that are probably bound to the enzyme with different affinities. This is in line with the data in Table I, which shows that

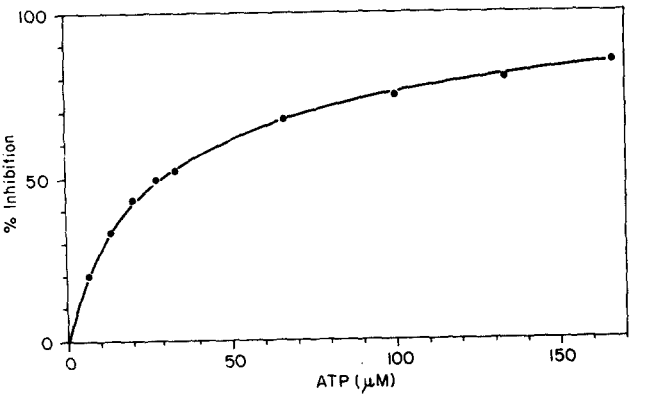


Fig. 4. Inhibition of phosphodiesterase activity as a function of ATP concentration. Phosphodiesterase was assayed titrimetrically as described in Fig. 3, using 350 mg protein. The enzyme preparation was dialyzed as usual in the Tris–HCl buffer.

TABLE V

REVERSAL OF EDTA OR ATP INHIBITION BY $Mn^{2+}$

Phosphodiesterase was measured titrimetrically as described in the legend to Fig. 4, using 240 $\mu$g protein.

| *Expt. No.* | *Addition (mM)* | *Activity (nmoles/min)* |
|---|---|---|
| 1 | None | 12.1 |
| | EDTA (1) | 0 |
| | EDTA (1), $Mn^{2+}$ (2) | 12.7 |
| 2 | None | 11.0 |
| | ATP (0.9) | 0 |
| | ATP (0.9), $Mn^{2+}$ (2) | 4.7 |

extensive dialysis of the enzyme preparation against EDTA lower its content of $Ca^{2+}$ and $Zn^{2+}$ both of which stimulate the activity of phosphodiesterase at low concentrations.

As mentioned earlier, ATP and other nucleoside triphosphates were potent inhibitors of phosphodiesterase. 1 mM ATP exerted 50% inhibition in the presence of 1.8 mM $Mg^{2+}$ (ref. 15). In the absence of any added divalent cation, phosphodiesterase was more sensitive to ATP inhibition. Fig. 4 shows the inhibition of phosphodiesterase as a function of ATP concentration. Unlike the biphasic inhibition curve of EDTA, the ATP curve was hyperbolic. Half maximal inhibition was reached at 30 $\mu$M ATP. The potency of other nucleoside triphosphates under similar conditions were comparable to that of ATP (not shown).

*Reversal of EDTA or ATP inhibition by $Mn^{2+}$*

Table V shows that the inhibition of phosphodiesterase by EDTA is completely reversible by $Mn^{2+}$. Presumably, the inhibition was released following the chelation of EDTA by the exogenous $Mn^{2+}$.

Although the inhibition by EDTA was fully relieved by $Mn^{2+}$, that by ATP was only partially reversible. Even when the concentration of $Mn^{2+}$ was in excess over that of ATP, the activity was about 40% that of the uninhibited enzyme. This experiment suggested that the inhibition by ATP might be more complicated than simple metal chelation.

*Effect of $Mn^{2+}$ or $Ca^{2+}$ on phosphodiesterase activity as a function of pH*

In the presence of $Mn^{2+}$, phosphodiesterase of bovine brain exhibits maximal activity at pH 8, Fig. 5A. When $Mn^{2+}$ was omitted from the reaction mixture, the activity was lower throughout the pH range, and the optimal pH was shifted from 8 to 8.3. At pH 7 the basal activity was negligible, but the stimulatory effect by $Mn^{2+}$ was most pronounced. As the pH was increased, the stimulation decreased; and at pH 10 it became negligible.

The effect of $Ca^{2+}$ on phosphodiesterase activity as a function of pH is shown in Fig. 5B. $Ca^{2+}$ depressed the activity of phosphodiesterase at all pH and the optimal pH was lowered to 7.6. Unlike the stimulation by $Mn^{2+}$, which diminished with increasing pH, the inhibition by $Ca^{2+}$ increased with increasing pH.

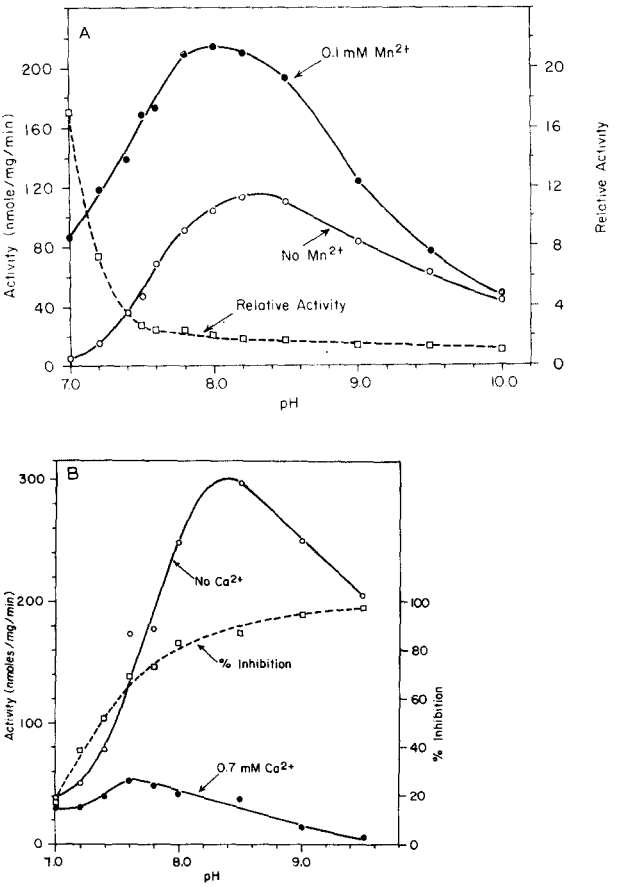


Fig. 5. A. Stimulation of phosphodiesterase by $Mn^{2+}$ as a function of pH. Phosphodiesterase was assayed by the two stage procedure[7]. The reaction mixture of 0.5 ml contained 40 mM Tris–HCl, 75 $\mu$g protein, 2 mM cyclic AMP and where indicated, 0.1 mM $Mn^{2+}$. B. Inhibition of phosphodiesterase by $Ca^{2+}$ as a function of pH. Phosphodiesterase was assayed as in A.

*Effect of $Mn^{2+}$ on the stimulation of phosphodiesterase by imidazole*

Stimulation of phosphodiesterase by imidazole was first noted by BUTCHER AND SUTHERLAND[16] on a beef heart enzyme. Subsequently, stimulation was also observed on the enzyme from dog heart[17], rat brain[6], and bovine brain[11]. Fig. 6 shows the effect of different concentrations of imidazole on the enzymic activity in the presence and absence of $Mn^{2+}$. In its absence 10 mM imidazole caused maximal stimulation, and higher concentrations were less effective or even inhibitory. In fact, 200 mM imidazole suppressed all activity. When 0.1 mM $Mn^{2+}$ was present, the activity of phosphodiesterase was higher at all imidazole concentrations, and the maximal activity was observed at 20 mM. Higher concentrations were inhibitory, but the inhibitory effect was partly overcome by $Mn^{2+}$. At 200 mM phosphodiesterase activity was still comparable to the control that contained no imidazole and $Mn^{2+}$. The ratio of the activities with and without $Mn^{2+}$ increased with increasing concentrations of imidazole.

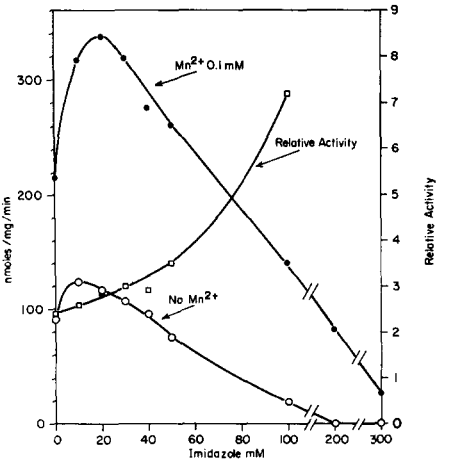


Fig. 6. The effect of different concentrations of imidazole on phosphodiesterase activity in the presence or absence of $Mn^{2+}$. Phosphodiesterase was assayed as described in Fig. 5A.

$NH_4^+$ also stimulated phosphodiesterase[17], and the stimulatory effect was similar to imidazole.

*Effect of $Mn^{2+}$ on the activity of purified phosphodiesterase*

The experiments described so far were performed with enzyme preparations that contained sufficient activator, which were independent of exogenous activator for activity. The question arises whether a purified enzyme that is dependent on exogenous activator for activity requires $Mn^{2+}$; also, whether the effects of $Mn^{2+}$ and the activator are interrelated. Table VI shows that $Mn^{2+}$ enhances the activity of the non-stimulated as well as the stimulated enzyme; and it also shows that the effects of $Mn^{2+}$ and the activator are synergistic.

The purified enzyme could be stimulated by snake venom[7] and proteolytic enzymes[13] and $Mn^{2+}$ enhanced the activity of the enzyme after maximal stimulation by these agents. Note that the process of the stimulation of phosphodiesterase by proteolytic enzymes is time dependent, whereas that by the activator is concen-

TABLE VI

EFFECT OF $Mn^{2+}$ ON THE ACTIVITY OF PURIFIED PHOSPHODIESTERASE

The reaction mixture of 0.5 ml contained 40 mM Tris–HCl (pH 8.0), 105 μg of the purified enzyme, 2 mM cyclic AMP and other additions as indicated. The phosphodiesterase protein activator was purified about 1000-fold from bovine brain cerebra (Y. M. LIN AND W. Y. CHEUNG, unpublished experiment). Phosphodiesterase was assayed as described previously[7].

| *Additions* | *Activity (nmoles/tube per 10 min)* |
|---|---|
| 1. None | 38 |
| 2. $Mn^{2+}$ (0.1 mM) | 66 |
| 3. Activator (1 μg) | 247 |
| 4. $Mn^{2+}$ (0.1 mM), activator (1 μg) | 340 |

TABLE VII

SUBSTRATE SPECIFICITY OF PHOSPHODIESTERASE FROM BOVINE BRAIN

All substrates were 2 mM and phosphodiesterase was assayed as described previously[7].

| *Substrate* | *Activity (nmoles/mg per min)* | *Relative activity* |
|---|---|---|
| *Purine base* | | |
| Cyclic AMP | 288.8 | 100 |
| $N^6$,$O^{2'}$-Dibutyryl cyclic AMP | 0 | 0 |
| Cyclic IMP | 86.3 | 30 |
| Cyclic GMP | 56.5 | 20 |
| *Pyrimidine base* | | |
| Cyclic CMP | 0 | 0 |
| Cyclic UMP | 4.7 | 2 |
| Cyclic TMP | 0 | 0 |

tration dependent. In spite of the apparently different mode of stimulation, both processes lead to a decreased $K_{mapp}$ of the enzyme for cyclic AMP (ref. 11).

These experiments show that both the activated and the non-activated enzymes exhibit a similar dependency on $Mn^{2+}$ for activity. They also indicate that the metal requirement of the activated enzyme is independent of the process by which the enzyme is stimulated.

*Effect of divalent cations on the hydrolysis of cyclic GMP*

Phosphodiesterase of bovine brain hydrolyzed cyclic purine nucleotides with the following relative rates: cyclic AMP (100), cyclic IMP (30), cyclic GMP (20), and $N^6$,$O^{2'}$-dibutyryl cyclic AMP (0). Cyclic nucleotides with a pyrimidine base were virtually not hydrolyzed, Table VII. Of these compounds cyclic GMP is the only compound other than cyclic AMP that has been found in biological systems. Table VIII shows that the effect of divalent cations on the hydrolysis of cyclic GMP differs

TABLE VIII

EFFECT OF DIVALENT CATIONS ON THE HYDROLYSIS OF CYCLIC GMP

Phosphodiesterase was assayed as described previously[7].

| *Expt. No.* | *Additions (mM)* | *Activity (nmoles/tube per 10 min)* |
|---|---|---|
| 1 | *Substrate:* cyclic AMP | |
| | None | 160 |
| | $Mn^{2+}$ (1) | 197 |
| | $Mg^{2+}$ (1) | 169 |
| | $Ca^{2+}$ (1) | 61 |
| 2 | *Substrate:* cyclic GMP | |
| | None | 43 |
| | $Mn^{2+}$ (1) | 35 |
| | $Mg^{2+}$ (1) | 69 |
| | $Ca^{2+}$ (1) | 11 |

from that of cyclic AMP. $Mn^{2+}$ decreased the hydrolysis of cyclic GMP while it increased the hydrolysis of cyclic AMP. On the other hand, $Mg^{2+}$ was a much more effective cation for the hydrolysis of cyclic GMP than for that of cyclic AMP. $Ca^{2+}$ inhibited the hydrolysis of both nucleotides. It thus appears that the effect of divalent cations may vary with the substrates.

## DISCUSSION

The presence of metals in the enzyme preparation even after prolonged dialysis against EDTA and the inhibition by metal chelators on the activity of phosphodiesterase activity lend credence to our earlier postulate that the active form of the enzyme is a metal enzyme complex[6]. An enrichment of the bound metal(s) parallel to the enzyme purification would strengthen this thesis. Unfortunately, the failure to achieve substantial purification beyond the stage of present preparations makes a close correlation difficult.

Phosphodiesterase of different tissues requires a divalent cation for maximal activity. It appears that this requirement is a property common to phosphodiesterase from all sources[11]. Not all divalent cations are effective; some are stimulatory, others are inhibitory. A divalent cation may be stimulatory or inhibitory depending on its concentration, examples are $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$ and $Zn^{2+}$. Further, the effectiveness of a stimulatory or inhibitory cation varies with the pH. The stimulation by $Mn^{2+}$ is marked at a neutral pH whereas the inhibition by $Ca^{2+}$ is severe at an alkaline pH. In view of the relatively low tissue level of $Mn^{2+}$ and the relative high level of $Ca^{2+}$ in the brain, $Mn^{2+}$ (0.1 mM) and $Ca^{2+}$ (1 mM) have been used arbitrarily as stimulatory and inhibitory cations, respectively.

Kinetic experiments show that a stimulatory cation such as $Mn^{2+}$ increases the $K_{m\text{app}}$ and the $v_{\max}$ of phosphodiesterase and an inhibitory cation such as $Ca^{2+}$ decreases them. According to equations depicting classical kinetics, an enzyme (E) interacts with its substrate (S) to form an unstable intermediate (ES) which dissociates to give the free enzyme and its product (P) in the following manner:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P \tag{1}$$

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \tag{2}$$

$$v_{\max} = k_{+2} \cdot [E] \tag{3}$$

where $k_{+1}$, $k_{-1}$, and $k_{+2}$ are rate constants as designated in Eqn. 1, and $[E]$ in Eqn.3 is the concentration of the enzyme. Since a cation affects the $K_{m\text{app}}$ and $v_{\max}$ in the same direction, (see Eqns. 2 and 3), its major effect appears to be on $k_{+2}$, and not $k_{+1}$, or $k_{-1}$. An increase of $k_{+2}$ would facilitate the breakdown of the $ES$ complex to give the product of the reaction. Conversely, a decrease of $k_{+2}$ would diminish the breakdown of this complex. The present investigation does not exclude the possibility that $Mn^{2+}$ or $Ca^{2+}$ may also effect $k_{+1}$ or $k_{-1}$. For our discussion, however, we have assumed that the effects on them are negligible.

It should be noted that the above kinetic equations are simplified versions of

what may be more realistic representations of the sequence of reaction mechanisms. Water is a second substrate of the reaction catalyzed by phosphodiesterase (Eqn. 4),

$$\text{Cyclic AMP}^{-} + H_2O \xrightarrow{\text{Phosphodiesterase}} 5'\text{-AMP}^{2-} + H^{+} \quad (4)$$

and has been ignored in the kinetic discussion. The interaction between the substrates and the enzyme may proceed by a simultaneous or a sequential addition of the two substrates. According to theoretical considerations of CLELAND[18] these kinetic data would be consistent with an ordered sequential mechanism with cyclic AMP as the probable first substrate to interact with the enzyme. For a theoretical treatment of the kinetics of the enzyme-catalyzed reactions involving more than one substrate or product, the reader is referred to the original work of CLELAND[18].

The decreased effectiveness of a mixture of a pair of stimulatory divalent cations suggests competition of the two for some common sites, resulting in a diminished $k_{+2}$. Similarly, protection of a stimulatory cation for an inhibitory cation may be regarded as simple equilibrium effects with the two cations competing for some common sites. A greater affinity of the stimulatory cation over the inhibitory cation will give an apparent protective effect. Thus, the activity of phosphodiesterase is not only dependent on a specific divalent cation, but also on the simultaneous presence of other divalent cations.

The effect of metal chelators such as EDTA and ATP is thought to sequester the metal on the enzyme. The relief of EDTA inhibition by exogenous $Mn^{2+}$ is presumably due to chelation of the EDTA, which would otherwise act on the metal of the enzyme. Although ATP is a metal chelator, its inhibition on phosphodiesterase was not completely reversed by exogenous $Mn^{2+}$. Since the logarithms of the stability constant of $Mn^{2+}$-EDTA and $Mn^{2+}$-ATP are high, in the order of 12 and 5, respectively[19], one would expect that in the presence of an excess $Mn^{2+}$ there would not be any significant quantities of unsequestered EDTA nor ATP available to chelate the metal ion of the enzyme. This explains the effective reversal of the inhibition by EDTA. The fact that the ATP inhibition was only partially relieved by excess $Mn^{2+}$ suggested that the effect of ATP was probably more complicated than a simple chelation of the metal ion on the enzyme. This conclusion is supported by our earlier kinetic work, which showed that the ATP inhibition was of a mixed type[15].

Although phosphodiesterase depended on $Mn^{2+}$ for optimal activity, the dependency was not absolute. An enzyme preparation which had been dialyzed against EDTA still retained considerable basal activity, suggesting that at least some of the metal on the enzyme molecule was bound firmly. This is in agreement with the finding in Table I which shows that about half of the metals associated with the enzyme preparation is removed after extensive dialysis against EDTA.

KAKIUCHI AND YAMAZAKI[20] recently noted the stimulatory effect of micromolar concentrations of $Ca^{2+}$ on the activity of a rat brain phosphodiesterase. They also observed that excess EGTA did not inhibit completely the enzymic activities, and postulated the occurrence of two separate phosphodiesterase activity, the $Ca^{2+}$ dependent and the $Ca^{2+}$ independent. The present study concerns the effect of the inhibition of $Ca^{2+}$ at a mM concentration whereas the Japanese workers emphasize that at a $\mu$M range. Because of the apparently opposite effects depending on the

concentration of $Ca^{2+}$ the results from the two laboratories complement rather than contradict each other.

The different effectiveness of $Mn^{2+}$ and $Mg^{2+}$ on the hydrolysis of cyclic AMP and cyclic GMP may mean that the degradation of these nucleotides is regulated independently of one another. Indeed, recent studies from other laboratories show that the tissue levels of cyclic AMP and cyclic GMP change independently, indicating that their metabolism is under different control mechanisms[21,22].

There is increasing evidence that phosphodiesterase is critical in the regulation of tissue levels of cyclic AMP. The present work on the effect of divalent cations on the activity of phosphodiesterase adds to the increasing awareness that the enzyme is subject to intricate control mechanisms at the cellular level[11,15,24–28].

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