Cyclic 3':5'-Nucleotide Phosphodiesterase

PURIFICATION, CHARACTERIZATION, AND ACTIVE FORM OF THE PROTEIN ACTIVATOR FROM BOVINE BRAIN*

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SUMMARY

A protein activator of cyclic 3':5'-nucleotide phosphodiesterase from bovine brain has been purified to homogeneity by the criteria of analytical polyacrylamide gel electrophoresis, polyacrylamide gel isoelectric focusing, Sephadex G-100 column chromatography, and analytical ultracentrifugation. The over-all purification was about 1700-fold with a yield of 7%.

The molecular weight of the activator is 15,000 as determined by several methods. Other physical properties are: sedimentation coefficient ($s_{20,w}$), 1.85 S; diffusion coefficient ($D_{20,w}$), 1.09 x 10^{-2} cm^2 per s; partial specific volume ($\bar{v}$), 0.72 cm^3 per g; frictional ratio ($f/f_0$), 1.20; and isoelectric point (pI), 4.3.

Amino acid analysis of the acid hydrolyzate of the activator showed high content of aspartic and glutamic acids compared to basic amino acids, in agreement with a low pI of 4.3. Sulfhydryl groups, cystine, and tryptophan were not detected. The NH$_2$-terminal residue was identified as valine. This, together with the fact that the molecular weight of 15,000 remained unchanged in 6 M guanidine hydrochloride, indicates that the activator is a single polypeptide.

The activator binds Ca$^{2+}$ specifically. A Scatchard plot of data obtained from equilibrium binding revealed four Ca$^{2+}$ binding sites per mole of activator; their dissociation constants ranged from 4 to 18 $\mu$M. Stimulation of phosphodiesterase by the activator required a low concentration of Ca$^{2+}$, and chelation of Ca$^{2+}$ by ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid rendered the activator inactive. The concentration of Ca$^{2+}$ needed to give half-maximum activation of phosphodiesterase was 3 $\mu$M. Ca$^{2+}$ alone did not activate phosphodiesterase. These results suggest that the active form of the activator is a Ca$^{2+}$-activator complex, in agreement with the findings of Teo and Wang (TEO, T. S., and WANG, J. H. (1973) J. Biol. Chem. 248, 5950).

In contrast to the heart activator (TEO, T. S., WANG, T. H., and WANG, J. H. (1973) J. Biol. Chem. 248, 588), the brain activator was stable in the presence of either 1 mM Mg$^{2+}$ or 1 mM EDTA. Staining of the activator with periodate-Schiff reagent or pyronine Y after electrophoresis on acrylamide gel was negative.

Bovine brain phosphodiesterase(s) hydrolyzes adenosine 3':5'-monophosphate (cyclic AMP) and guanosine 3':5'-monophosphate (cyclic GMP). At millimolar substrate concentration the rate of hydrolysis of cyclic AMP was greater than that of cyclic GMP, whereas at micromolar concentration, the reverse was found. Although the activator stimulated the hydrolysis of both nucleotides, it stimulated cyclic AMP hydrolysis more, regardless of the concentration used. The stimulation of the hydrolysis of both substrates was greater at micromolar concentrations than at millimolar.

Caffeine inhibited phosphodiesterase both in the presence and absence of the activator; this inhibition was not reversed by increased concentrations of the activator.

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\section*{Adenosine 3':5'-monophosphate has been shown to mediate the action of a wide variety of different hormones (1). Cyclic 3':5'-nucleotide phosphodiesterase(s) is the only enzyme (or enzyme system) that catalyzes the hydrolysis of cyclic AMP.¹ Thus, the enzyme plays an important role in determining the extent and duration of a hormone action.

Previous work from this laboratory showed that phosphodiesterase from bovine brain and other tissues lost activity upon purification (2-4). This loss resulted from the dissociation of an activator from the enzyme on a diethylaminoethylcellulose column (4, 5). Addition of an exogenous activator to the purified enzyme restored full enzymic activity. The activator increased $V_{max}$ and decreased $K_m$ of the purified enzyme for cyclic AMP. Activation of the enzyme by the activator followed a stoichiometric process (4). The activator is specific and lacks tissue or species specificity. It is present in excess over phosphodiesterase in all tissues examined, with the possible exception of human leukocytes (6).

One of the main interests in the protein activator has been its...
possible physiological role in regulating phosphodiesterase activity. We have undertaken two approaches. One was to correlate the distribution and the development of the activities of phosphodiesterase and its activator in different tissues. It was found that although their activities were not parallel in various tissues or during development of several tissues examined, they were parallel in subcellular fractions of isolated rat parenchymal cells and human blood platelets. Parallel activities of these two proteins in subcellular fractions may suggest physiological regulation of phosphodiesterase by the activator. But these studies are complicated by the presence of activator-dependent and activator-independent phosphodiesterase activities in some tissues, and therefore they did not allow definitive conclusions (6).

The other approach was to characterize the activator and to elucidate its mode of activation of phosphodiesterase. The existence of a protein activator for phosphodiesterase in mammalian tissues has now been reported from several laboratories (7-9). Kakiuchi and Yamazaki found that an activator from rat brain specifically activated the Ca\(^{2+}\) and Mg\(^{2+}\)-dependent phosphodiesterase (10). Teo et al. (11, 12) purified and characterized an activator from bovine heart.

This report describes a simple purification scheme that gives a homogeneous preparation of the activator from bovine brain. It summarizes some properties of the purified activator and presents evidence to indicate that the active form of the activator is a Ca\(^{2+}\)-activator complex. A preliminary account of this work has appeared (13).

**EXPERIMENTAL PROCEDURE**

Chemicals and Reagents—Cyclic AMP and cyclic GMP and their tritiated forms were obtained from Schwartz BioResearch. Lyophilized snake venom (Crotalus atrox), bovine serum albumin, sodium dodecyl sulfate, EGTA, horseradish peroxidase (type VII-L), cytochrome c (type VI), amino acids and their dansyl derivatives, and coarse DEAE-cellulose with a capacity of 0.9 meq per g were purchased from Sigma. Acrylamide, N,N'-methylenebisacrylamide, and N,N',N',N'-tetramethylenediamine were from Eastman Organic; acrylamide, sodium bisulfite, and RNAase from Worthington; ovalbumin and Coomassie brilliant blue from Mann; ammonium sulfate, magnesium sulfate, strontium chloride, magnesium metal, calcium carbonate, zinc oxide, sodium desoxycholate, sodium periodate, and Basic Fuchsin from Fisher; cal- cium chloride, cobalt chloride, manganous chloride, potassium meta-bisulfité, and zinc chloride from Baker; pyronine Y from Gurr, Ltd., London, England; and ampholine (pH 4 to 6) from LKB. Sephadex G-25 and G-100 were obtained from Pharmacia; myoglobin from Servac Laboratory, Ltd., Berkshire, England; double layer polyamide plates from Cheng Chin Trading Co., Ltd., Taipei, Taiwan; and ultrafiltration membrane, UF M, from Amicon. Calcium-45 chloride with a specific activity of 22 mCi per mg or 10.4 mCi per mg of calcium were products of Amersham and New England Nuclear, respectively. Manganese 54 chloride (100 mCi per mg of manganese) was from Amersham.

Preparation of Activator-deficient Phosphodiesterase—Phosphodiesterase was purified through the DEAE-cellulose column as described previously (3), and is referred to as the DEAE-enzyme. Briefly, fresh or frozen bovine cerebra were extracted with water, followed by pH fractionation, ammonium sulfate fractionation, calcium phosphate gel adsorption, and DEAE-cellulose column chromatography. The DEAE-enzyme was deficient in its activator, which had been dissociated from the enzyme on the cellulose column (3, 4). The specific activity of the enzyme, when fully activated by the activator, was about 0.5 pmoles of cyclic AMP hydrolyzed per mg of protein per min at 30° under standard assay conditions. Stimulation by the activator ranged from 2- to 6-fold.

Assay of Phosphodiesterase—Phosphodiesterase activity was determined by converting its reaction product, 5'-AMP, into adenosine and inorganic phosphate by snake venom 5'-nucleotidase and then measuring the inorganic phosphate released (14). The method employed a two-stage incubation (4) and was modified from the procedure of Butcher and Sutherland (15).

Briefly, the reaction mixture consisted of 40 mm Tris-chloride, pH 8.0, 0.1 mm MnCl\(_2\); 2 mm cyclic AMP; and an appropriate concentration of the enzyme in a volume of 0.5 ml. The first stage of incubation was started by adding cyclic AMP. After 10 min at 30°, the reaction was stopped by transferring the test tube to a boiling water bath and keeping it there for 2 min. After cooling the tube, 0.05 ml of snake venom (1 mg per ml) was added and the mixture was incubated for another 10 min. The second stage of incubation was terminated by adding 0.05 ml of 55% trichloroacetic acid, followed by 0.15 ml of ammonium molybdate in 5 N H\(_2\)SO\(_4\) and 0.7 ml of H\(_2\)O. The mixture was centrifuged and the clear supernantant was decanted into tubes containing 0.05 ml of a Fiske-Subbflow reagent (14). The blue color that developed after 10 min was measured spectrophotometrically at 660 nm.

**Fig. 1.** Dose-response curve of phosphodiesterase activation by the activator. The activator was assayed for its ability to stimulate the activity of the DEAE-enzyme from bovine brain. Unless stated otherwise, the reaction mixture of 0.5 ml contained 40 mm Tris-chloride, pH 8.0; 0.1 mm MnCl\(_2\); 60 μg of DEAE-enzyme; and an appropriate amount of activator. The reaction was started with cyclic AMP, and phosphodiesterase was determined by the two-stage incubation as described above. The activity over the basal activity of the DEAE-enzyme was defined as the stimulated activity. Fig. 1 shows the dose-response curve of phosphodiesterase activation by the activator. The amount of activator in routine assays was adjusted so that the stimulated activity was determined from the linear part of the curve, i.e., around the range of half-maximum stimulation. Although the dose-response curve of different DEAE-enzyme preparations might vary slightly, that of the same enzyme preparation was reproducible. For preparations containing phosphodiesterase, the inherent enzymic activity was measured separately, and the activity was properly corrected. One unit of the activator is defined as the amount of protein which stimulates the hydrolysis of 1 μmole of cyclic AMP per min above the basal level of the DEAE-enzyme. Specific activity is expressed as units per mg of protein.

**Protein Determination**—Unless otherwise stated, protein was determined with the biuret reagent containing sodium desoxycholate or as described by Lowry et al. (16). Bovine serum albumin served as a standard.

Analytical Acrylamide Gel Electrophoresis—Analytical disc electrophoresis without sodium dodecyl sulfate was run at room temperature. One hundred milliliters of 15% acrylamide gel solution contained 0.132 g of N,N'-methylenebisacrylamide, 0.616 g of Tris, 0.348 g of glycine, 0.0355 of N,N',N'-tetratemethyl-enediamine, and 0.0355 g of ammonium persulfate. The upper buffer contained Tris (3.16 g per liter) and glycine (3.48 g per liter), pH 8.0. The lower buffer contained Tris (14.6 g per liter) and 1 N HCl (60 ml per liter), pH 8.1. Protein was stained with

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*Note: The image contains a figure (Fig. 1) which is not described in the text. It is recommended to refer to the original source for a detailed analysis of the figure.*
Coomassie brilliant blue, glycoprotein with periodate-Schiff reagent (17), and ribonuclease A with pyronine Y (18).

Sodium dodecyl sulfate electrophoresis using 10% gel for estimation of minimum molecular weight was carried out at 22°C according to Weber and Osborn (19).

Isoelectric Focusing on Acrylamide Gel in 8 M Urea Isoelectric focusing modified from Catsimpoolas (20) was performed in an analytical disc electrophoresis apparatus at 22°C. The gel solution, which consisted of 5% acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.88% N,N',N'-tetramethylethylene diamine, 2% ampholines (pH 4 to 6), 0.001% riboflavine and 8 mM urea, was polymerized under a fluorescent lamp. The solution at the cathode (top) was 0.02 M NaOH and that at the anode (bottom) was 0.02 M HCl. A sample solution containing 20 μl of activator, 10 μl of 5% glycine, and 50 μl of 8 mM urea was layered on the top of the gel. Isoelectric focusing was performed at a constant voltage of 200 volts. The current dropped from the initial 2 mamps per tube to 0.3 mamp per tube at the end of 5 hours. One gel was stained for proteins with Coomassie brilliant blue. Another was sliced into 1-mm sections, and each section was extracted with 0.7 ml of water. The stained for proteins with Coomassie brilliant blue. Nevertheless, the Ca²⁺ in a reaction mixture reached about 2 μM, as determined by atomic absorption spectrophotometry.

A Beckman-Spinco analytical spectrophotometer, model 403, equipped with a graphite furnace, was used for the analysis of calcium, cobalt, magnesium, manganese, and zinc. The sensitivity of this instrument is about 0.01 ppm for these divalent cations.

Binding of Calcium-45 and Manganese-54 by Activator of Phosphodiesterase—The technique of equilibrium dialysis was used to determine the binding of calcium or manganese by the activator. One milliliter (320 μg) of activator was placed in cellulose dialysis tubing and dialyzed for 30 to 38 hours at 4°C against 100 ml of 25 mM Tris-HCl, pH 8.0, and various concentrations of divalent cations. At the end of dialysis, the radioactivity in 200 μl of the dialyzed sample and that in an equal volume of the buffer, were counted in duplicates. Bound Ca²⁺ or Mn²⁺ was determined by the difference of radioactivity between the dialyzed sample inside and the buffer solution outside the tubing.

RESULTS

Purification of Phosphodiesterase Activator

Fresh bovine brains were transported to the laboratory in packed ice, and brain stems and large blood clots were removed. The cleaned tissue was used immediately or stored at -20°C; no appreciable difference in activity was noted. When frozen brains were used, they were thawed at room temperature. All operations were carried out at 4°C unless stated otherwise.

Step 1: Homogenization—Six kilograms of cleaned brain were homogenized in 12 batches. Each batch of about 500 g of tissue was homogenized in 25 liters of 0.15 M NaCl. Four-milliliter fractions were collected at a flow rate of 24 ml per hour. The centrifugation was performed at 36,000 rpm at 4°C for 21 hours in an 8W rotor in a preparative ultracentrifuge (Spinco model L1). Fractions of 5 drops were collected manually. The molecular weight and the diffusion coefficient were estimated according to Sedlak and Lindsay (22).

Analytical Ultracentrifugation—The activator was dialyzed against 0.1 M NaCl solution and used at a protein concentration of 1 mg per ml. The dialyze was used as a reference solution. All experiments were performed at 20°C in a Beckman model E analytical ultracentrifuge with.an An-D rotor. For sedimentation velocity, the schlieren optics and a 12 mm double sector cell equipped with an aluminum-filled Epon centerpiece were used. The rotor speed was set at 56,100 rpm. For sedimentation equilibrium and synthetic boundary experiments, the interference optics and a cell with a 12-mm capillary synthetic boundary centerpiece were used. The rotor speed was set at 12,500 rpm.

Amino Acid Analysis and Identification of NH₂-Terminal Residue—The activator was hydrolyzed in 6 N HCl at 110°C in a sealed, evacuated tube for 24, 48, and 72 hours. Amino acid analysis was performed according to Moore and Stein (23) in a Beckman model 121 amino acid analyzer. Half-cystine was examined separately as cysteic acid following performic acid oxidation (24). Sulfhydryl groups were determined according to Sedlak and Lindsay (25). Tryptophan was estimated separately after alkaline hydrolysis (26).

The NH₂-terminal residue of the activator was identified by the method of d analyzation (27).

Removal of Ca²⁺ from Reagents—Chelex-100, a resin specific for sequestering divalent cations, was used to remove Ca²⁺ from all stock solutions. The resin was washed with 1 N HCl, 1 N NaOH, double glass-distilled water, and then packed into a plastic column (3 × 25 cm). The Chelex column reduced Ca²⁺ ions in the reagents to 0.02 ppm or 0.5 μM. Glass-distilled water contained less than 0.5 μM Ca²⁺; this level was not further reduced by passing through the Chelex column. A stock solution of imidazole contained barely detectable Ca²⁺ and was not passed through the column.

Calcium was removed from phosphodiesterase and its activator by incubating with 10 mM EGTA for 40 min at 4°C; the solution was desalted with a Sephadex G-25 (3 × 30 cm) column. Alternatively, EGTA-treated activator was extensively dialyzed against 20 mM Tris-chloride, pH 7.5. In experiments where the concentration of Ca²⁺ was critical, all glassware were used, and reagents were passed through a Chelex column to remove Ca²⁺.
FIG. 2. DEAE-cellulose column chromatography. About 14 liters of a boiled supernatant from 6 kg of bovine brain were applied to a DEAE-cellulose column (5 X 5.6 cm). Tubes 281 to 338 were pooled and concentrated by (NH₄)₂SO₄ precipitation. —, activity; — — —, protein; · · · · · · , gradient of (NH₄)₂SO₄. Other details are presented in the text.

The lower buffer and the elution buffer contained Tris (5.16 g per liter) and glycine (3.48 g per liter). The lower buffer and the elution buffer contained Tris (14.5 g per liter) and 1 N HCl (60 ml per liter). The concentration of the membrane holder buffer was 5 times that of the lower buffer. A preliminary electrophoresis at 25 ma was run for 10 hours to remove residual persulfate in the gel prior to loading the sample. The sample had been dialyzed against a so concentration of the upper buffer for 6 hours. The volume of the sample applied to the column was 8 ml containing about 12 to 15 mg of protein. Electrophoresis was initiated at a constant current of 25 ma. One hour later the current was increased to and maintained at 40 ma for about 15 hours. Fractions of 10 ml were collected at a rate of 1 ml per min. Fig. 3 shows the activity and protein profiles of the electrophoresis. Each fraction from the active peak was examined for purity by analytical polyacrylamide gel electrophoresis. Fractions with good purity were combined, concentrated through a UM-2 membrane, and dialyzed against Buffer A. The over-all purification of the activator from bovine brain was about 1700-fold with a yield of 7% (Table I). The electrophoretic protein patterns in various stages of purification are shown in Fig. 4.

**Homogeneity of Preparation**

The purified activator was homogeneous as determined by several criteria: (a) analytical polyacrylamide gel electrophoresis (see Fig. 4). A single protein band was also obtained in sodium dodecyl sulfate acrylamide gel electrophoresis (data not shown); (b) isoelectric focusing in 8 M urea (see Fig. 10); (c) sucrose gradient centrifugation (data not shown); (d) analytical ultracentrifugation (see Figs. 6 and 8); and (e) Sephadex G-100 chromatography (see Fig. 7A).

**Physical Properties**

**Molecular Weight**—Sodium dodecyl sulfate acrylamide gel electrophoresis (19) was used to estimate the minimum molecular weight of the activator. The plot of molecular weight versus the relative mobilities of the various proteins is shown in Fig. 5.

**Table I**

Purification of phosphodiesterase activator from bovine brain

The data presented in the table are based on 1 kg of brain tissue. The purification procedure is described in the text. Protein in the homogenate was determined by the biuret reagent and in subsequent steps by the spectrophotometric technique of Warburg and Christian (28).

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homogenate</td>
<td>102,000</td>
<td>22,300</td>
<td>0.22</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant</td>
<td>21,300</td>
<td>7,670</td>
<td>0.36</td>
<td>24.4</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>Boiled supernatant</td>
<td>1,100</td>
<td>4,090</td>
<td>3.67</td>
<td>18.1</td>
<td>16.7</td>
</tr>
<tr>
<td>4</td>
<td>DEAE-cellulose chromatography</td>
<td>11.5</td>
<td>3,500</td>
<td>300</td>
<td>15.7</td>
<td>909</td>
</tr>
<tr>
<td>5</td>
<td>Preparative electrophoresis</td>
<td>4.2</td>
<td>1,590</td>
<td>383</td>
<td>7.1</td>
<td>1,740</td>
</tr>
</tbody>
</table>

The relative mobility of the activator corresponded to a molecular weight of 15,000. The same value was obtained by sucrose gradient ultracentrifugation (22), with horseradish peroxidase as a marker (data not shown).

Analytical ultracentrifugation, a more accurate method of estimating molecular weight, was also used in these determinations. A value of 14,500 was calculated from sedimentation and diffusion coefficients, as compared to 15,000 from sedimentation equilibrium. These values agree well with those obtained from sodium dodecyl sulfate acrylamide gel electrophoresis and sucrose gradient centrifugation. Fig. 6 shows the sedimentation equilibrium analysis of the activator.

The amino acid composition of the activator has been determined (see Table IV). From the data presented in Table IV, the minimum molecular weight of the activator was calculated to be 18,920.

A molecular weight of 40,000 was obtained earlier (4) with a...
Fig. 4. Electrophoretic patterns on polyacrylamide gel of the proteins at various stages of purification. Protein was stained with Coomassie brilliant blue. The amount of protein in each gel, from left to right was: supernatant, 40 μg; boiled supernatant, 30 μg; DEAE-cellulose chromatography, 8 μg; and preparative electrophoresis, 5 μg.

 crude preparation of the activator using a Sephadex G-100 column. This figure is substantially greater than the values obtained with the purified activator in the present work. To eliminate the possibility that the higher molecular weight was caused by impurities in the crude preparation, we repeated the experiment on Sephadex G-100 using a highly purified activator. As shown in Fig. 7A, the activator appeared as a single symmetrical peak. In this experiment, the activator was eluted before trypsin inhibitor (mol wt 21,600) and after horseradish peroxidase (mol wt 40,000); the elution volume corresponded to a molecular weight of 18,920 from amino acid analysis. The frictional ratio suggests that the activator is a globular protein.

Partial Specific Volume and Frictional Ratio—The partial specific volume calculated from the data of amino acid analysis (31) was 0.72 cm³ per g. The frictional ratio was calculated to be 1.20 based on a molecular weight of 15,000 obtained by sedimentation equilibrium (32), and 1.11 based on a molecular weight of 18,920 from amino acid analysis. The frictional ratio suggests that the activator is a globular protein.

Isoelectric Point—The fact that the activator was eluted from a DEAE-cellulose column at about 0.25 M (NH₄)₂SO₄ suggests that the activator was acidic. Isoelectric focusing of the activator in analytical polyacrylamide gel containing 8 M urea (Fig. 10) gave a pI of 4.3.

Table III summarizes the physical parameters of the purified activator from bovine brain.

Chemical Properties

Nature of Activator—Susceptibility to trypsin but not to DNase or RNase suggested that the activator is a protein (4). As much as 870 μg of the purified activator in an analytical acrylamide gel after electrophoresis did not give a positive stain with the periodic acid–Schiff reagent (17), thus suggesting that the activator is probably not a glycoprotein. Staining of 1140 μg of the purified activator in an acrylamide gel with pyronine Y (18), a test for ribonucleic acid, was also negative. Glycoproteins from human erythrocyte membrane and ribonucleic acid from the yeast serving as controls were stained positively.

Amino Acid Composition and NH₄-terminal Group—The amino acid composition of the purified activator presented in Table IV is based on 1 mole of histidine. The data presented were averages of three samples after 24-hour hydrolysis. Values for threonine and serine were extrapolated to zero time based upon data obtained after 24, 48, and 72 hours of hydrolysis. One striking feature of the data is that one-third of the total amino acids is contributed from aspartic and glutamic acids following hydrolysis. Another feature is the absence of tryptophan and half-cystine. In a separate experiment, free sulphydryl groups were not detected by the method of Sedlack and Lindsay (25).

The NH₄-terminal residue was identified as valine by the method of dansylation (27). This, together with the data on molecular weight, suggests that the activator has one polypeptide chain.

Stability—The activator from bovine brain was stable. The
Fig. 5 (top left). Determination of molecular weight of phosphodiesterase activator by sodium dodecyl sulfate acrylamide gel electrophoresis. Electrophoresis was performed as described by Weber and Osborn (18). The markers are bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 45,000), pepsin (mol wt 35,000), soybean trypsin inhibitor (mol wt 21,500), myoglobin (mol wt 17,800), ribonuclease (mol wt 13,700) and cytochrome c (mol wt 11,700).

Fig. 6 (bottom left). Sedimentation equilibrium analysis of the activator. Purified activator (0.12 ml) in 0.1 M NaCl (1 mg per ml) and 0.16 ml of the reference solution were centrifuged for 24 hours as described in the text. The initial concentration in terms of fringe number was determined in a separate run using the synthetic boundary procedure. $J(z)$ is the fringe number equivalent to the concentration at the location whose magnified radial distance is $X$. The slope of the log $J(z)$ versus $X^2$ plot was used to calculate the molecular weight according to the equation:

$$M = 2.303 \cdot \frac{2RT}{(1 - \nu \omega^2)} \cdot \frac{m^2 d \log J(z)}{d(x^2)}$$

where $R$, $T$, $\omega$, $\nu$, $\rho$, and $m$ are, respectively, gas constant, absolute temperature, angular rotor speed, partial specific volume, density of the solvent, and the magnification factor of image on the picture with respect to the real object.

Fig. 7 (right). A, Sephadex G-100 column chromatography of the activator. Purified activator (2.1 mg) was chromatographed as described in the text. A fraction from each tube was assayed for activity. The basal activity was not corrected. B, Determination of molecular weight by Sephadex G-100 column chromatography using protein markers as described by Andrews (20). BSA, bovine serum albumin.
TABLE II

**Determination of molecular weight of phosphodiesterase activator from bovine brain**

Experimental details are described in the text.

<table>
<thead>
<tr>
<th>Method of determination</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation velocity and diffusion</td>
<td>14,500</td>
</tr>
<tr>
<td>Sedimentation equilibrium</td>
<td>15,000</td>
</tr>
<tr>
<td>Sucrose density gradient centrifugation</td>
<td>15,000</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
<td>15,000</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>18,920</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>31,000</td>
</tr>
</tbody>
</table>

Fig. 8. Sedimentation pattern of the activator. From left to right, the pictures were taken at 32 min, 96 min, and 144 min after the rotor had reached 56,000 rpm. A phase angle of 55° was used for the first two pictures and 45° for the last picture.

**Fig. 9.** Determination of the diffusion coefficient. Purified activator (0.12 ml) in 0.1 M NaCl (1 mg per ml) was used in the synthetic boundary experiment. Photographs were taken at 8-min intervals starting when the menisci in the two sectors were at the same level. The total fringe shift across the boundary, ΔJ, was determined for each picture. The difference between the magnified radial distances of the J/4th and the 3J/4th total fringe, ΔR, was measured. The slope of the plot (ΔR)² versus time was used to calculate the diffusion constant as described in the text.

Activity was essentially not affected when exposed to boiling for 4 min at pH 7 or 1.7, and to 8 M urea for 1 hour (4). The effect of Mg²⁺ on the stability of the activator was examined at 4°C. No loss of activity was observed at the end of 5 days, whether in the presence of Mg²⁺ or EDTA (Fig. 11). These results contrast sharply with those of Teo et al. (11), who found that an activator from bovine heart lost activity when stored at 4°C with or without Mg²⁺, and that the addition of 1 mM EDTA completely abolished the activity within 30 min. The cause for this discrepancy is not apparent.

Walsh et al. (33) described a heat-stable protein inhibitor of protein kinase with several properties similar to the activator of phosphodiesterase. Assays of exchanged purified samples did not show that the two entities substituted for each other.²

Metal Content of Activator—Mammalian phosphodiesterase(s) requires divalent cation to express full activity. Bovine brain

**Fig. 10.** Isoelectric focusing of phosphodiesterase activator on acrylamide gel containing 8 M urea. Thirty micrograms of the purified activator were subjected to isoelectric focusing as described in the text. At the end of focusing, the gel was sliced transversely into 1-mm slices. Each slice was extracted with 0.7 ml of H₂O in a small test tube, and the mixture was shaken for 1 hour in a metabolic shaker at 22°C. The pH was measured with a glass electrode. One-tenth milliliter of a 1 M Tris-Cl, pH 8.0, was then added to the mixture and extracted overnight. Fractions of 5 µl from each tube were assayed for activator activity. The inset shows the gel pattern of phosphodiesterase activator (5 µg) on acrylamide gel after isoelectric focusing. Protein was stained with Coomassie blue after extensive washing of the gel with 12% trichloroacetic acid.

**TABLE III**

**Physical properties of phosphodiesterase activator from bovine brain**

The frictional ratio was calculated based on a molecular weight of 15,000 from sedimentation equilibrium. Other experimental details are described in the text.

<table>
<thead>
<tr>
<th>Determination</th>
<th>Method used</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>s₂₀,₀, (sedimentation coefficient)</td>
<td>Sedimentation velocity</td>
<td>1.85 S</td>
</tr>
<tr>
<td>D₁₃,₀, (diffusion coefficient)</td>
<td>Analytical ultracentrifugation</td>
<td>1.09 x 10⁻⁴ cm²/s</td>
</tr>
<tr>
<td>佖 (partial specific volume)</td>
<td>Calculated from amino acid composition</td>
<td>0.72 cm²/g</td>
</tr>
<tr>
<td>f/f₀ (frictional ratio)</td>
<td>Calculated from D₁₃,₀, and molecular weight</td>
<td>1.20</td>
</tr>
<tr>
<td>pI (isoelectric point)</td>
<td>Isoelectric focusing</td>
<td>4.3</td>
</tr>
</tbody>
</table>

² D. A. Walsh and C. Ashby, personal communication.
TABLE IV
Amino acid composition of phosphodiesterase activator from bovine brain

The amino acid composition is based on 1 mole of histidine. The values for threonine and serine were extrapolated to zero time, based upon data obtained after 24, 48, and 72 hours of hydrolysis assuming first order kinetics. Half-cystine was determined as cysteic acid following performic acid oxidation, and tryptophan was determined after alkaline hydrolysis. For other details see the text.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>24</td>
</tr>
<tr>
<td>Threonine</td>
<td>14</td>
</tr>
<tr>
<td>Serine</td>
<td>5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>30</td>
</tr>
<tr>
<td>Proline</td>
<td>2</td>
</tr>
<tr>
<td>Glycine</td>
<td>13</td>
</tr>
<tr>
<td>Alanine</td>
<td>13</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>8</td>
</tr>
<tr>
<td>Methionine</td>
<td>11</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>9</td>
</tr>
<tr>
<td>Leucine</td>
<td>11</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>8</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 11 (left). Stability of the activator in the presence of 1 mM Mg2+ or 1 mM EDTA. The activator from Step 4 was dialed against 20 mM Tris-Cl, pH 7.5, to remove Mg2+ and was then stored in test tubes for various lengths of time at 4° at a protein concentration of 0.4 mg per ml. The buffer either contained 1 mM EDTA or 1 mM magnesium acetate without EDTA. At the time indicated, the solution was diluted 100 times. Fifty microliters of the diluted solution were assayed for the activator. The basal activity of the DEAE-enzyme was 0.321 A at 660 nm. Separate experiments showed that the trace amount of magnesium acetate or EDTA carried over from the stored solutions to the reaction mixture did not interfere with the assay.

Fig. 12 (center). Scatchard plot of Ca2+ binding by the protein activator of phosphodiesterase. \( \delta \), the moles of Ca2+ bound per mole of activator, was calculated from the difference in radioactivity in the protein solution inside the dialysis tubing and that in the buffer outside the tubing after equilibrium had been reached. \( A \) is the concentration of Ca2+ in the dialyzing buffer outside the dialysis tubing.

TABLE V
Divalent cations bound to activator of phosphodiesterase from bovine brain

Cations were measured using a Perkin-Elmer atomic absorption spectrophotometer as described under "Experimental Procedure." Preparation 4 was the same as preparation 3 except that it was treated with 10 mM EGTA and then dialyzed to remove divalent cations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Divalent cation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
<td>moles/mole activator</td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>0.02</td>
</tr>
</tbody>
</table>
amount of calcium bound to the activator might account for the calcium found in the partially purified enzyme.

To determine the maximum binding capacity of the activator for Ca\textsuperscript{2+}, we measured the amount of Ca\textsuperscript{2+} bound as a function of its concentration, using the technique of equilibrium dialysis. Fig. 12 depicts the Scatchard plot of the data from such an experiment. The curve was biphasic suggesting more than one set of binding sites with different affinities. The dissociation constants ranged from 4 to 18 \mu M. The intercept of the curve at the abscissa indicates a total of four binding sites per molecule of activator. Teo and Wang (12) reported that there were 3 to 4 binding sites on the heart activator.

**Effect of Divalent Cations on Binding of Ca\textsuperscript{2+} by Activators**—The fact that the activator contains only calcium suggests specificity of binding. Table VI shows the effect of other divalent cations to compete with Ca\textsuperscript{2+} for binding to the activator. Of the cations tested, Mn\textsuperscript{2+} was more effective, followed by Sr\textsuperscript{2+}. At 10 times the concentration of Ca\textsuperscript{2+}, Mn\textsuperscript{2+} decreased the binding of Ca\textsuperscript{2+} by about 40% at 100 times, all the cations examined were inhibitory.

Equilibrium dialysis showed that the activator also bound \textsuperscript{54}Mn\textsuperscript{2+} with a dissociation constant at least 10 times larger than that for Ca\textsuperscript{2+} (data not shown). One gram of brain tissue contains about 0.02 pmole of manganese and 2 pmoles of calcium (35). The relatively low tissue level of manganese and the lower affinity of the activator for Mn\textsuperscript{2+} would not favor its playing a physiological role in regulating phosphodiesterase activity.

The selective binding of Ca\textsuperscript{2+} is of particular interest in view of the earlier observation of Kakiuchi et al. (10), who found that a micromolar concentration of Ca\textsuperscript{2+} in the presence of millimolar Mg\textsuperscript{2+} renders rat brain phosphodiesterase more sensitive to the activator. They suggested the existence of a Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-dependent form of the enzyme.

**Effect of Ca\textsuperscript{2+} on Activity of Phosphodiesterase**—In our early study on the effect of divalent cations on phosphodiesterase activity, we found that EGTA partially inhibited phosphodiesterase activity (34). It was thought that inhibition by EGTA was due to chelation of the Ca\textsuperscript{2+} on the enzyme. The present finding that the activator binds Ca\textsuperscript{2+} specifically suggests that EGTA may chelate the Ca\textsuperscript{2+} on the activator. The effect of Ca\textsuperscript{2+} on the activation of phosphodiesterase by the activator was therefore examined. Fig. 13 shows that in the absence of exogenous Ca\textsuperscript{2+}, the activator increased the activity slightly. This stimulation was presumably due to a trace of Ca\textsuperscript{2+} contaminated in the reaction mixture (see below). With increasing Ca\textsuperscript{2+}, stimulation of phosphodiesterase increased, and at 100 \mu M Ca\textsuperscript{2+} the stimulation was about 5-fold. At optimal concentration of Ca\textsuperscript{2+}, 3 \mu g of activator were as effective as 40 \mu g. Thus, Ca\textsuperscript{2+} renders phosphodiesterase more responsive to the activator, in accord with the observation of Kakiuchi et al. (10). This point is better illustrated in Fig. 14, which shows the activity of phosphodiesterase as a function of Ca\textsuperscript{2+} concentration. In the absence of exogenous Ca\textsuperscript{2+}, 0.4 \mu g of activator caused a slight stimulation; the stimulation increased with increasing concentrations of Ca\textsuperscript{2+}, and at 100 \mu M, this amount of activator caused half-maximum stimulation. In the presence of higher activator concentration, for example, 40 \mu g, the concentration of exogenous Ca\textsuperscript{2+} to give half-maximum stimulation was about 1 \mu M. This experiment shows that the effectiveness of the activator is dependent on the concentration of Ca\textsuperscript{2+}, and vice versa.

The slight stimulation of phosphodiesterase by the activator in Figs. 13 and 14 in the absence of exogenous Ca\textsuperscript{2+} was attributed to the trace of Ca\textsuperscript{2+} present in the reaction mixture. EGTA, a metal chelator with preferential affinity for Ca\textsuperscript{2+} to Mg\textsuperscript{2+}, was used to establish this point. Fig. 15 shows the effect of EGTA on the stimulation of phosphodiesterase by an exogenous activator. With no EGTA, phosphodiesterase activity was considerably higher in the presence of activator than in its absence. Addition of 2 \mu M EGTA reduced the activity to the level of basal activity, which was not affected by EGTA. This concentration of EGTA was comparable to that of Ca\textsuperscript{2+} contaminated in the reaction mixture (see "Experimental Procedure"). This experiment showed that the activator was inactive unless Ca\textsuperscript{2+} was present. Since the activator binds Ca\textsuperscript{2+}, the true activator appears to be a Ca\textsuperscript{2+}-activator complex. A similar conclusion has been reached by Teo and Wang (12) on a bovine heart activator and by Kakiuchi et al. (36) on a rat brain activator.

Although the stimulation of phosphodiesterase by the activator was dependent on Ca\textsuperscript{2+} when Mg\textsuperscript{2+} was used in the reaction, the activator was inactive unless Ca\textsuperscript{2+} was present. Since the activator binds Ca\textsuperscript{2+}, the true activator appears to be a Ca\textsuperscript{2+}-activator complex. A similar conclusion has been reached by Teo and Wang (12) on a bovine heart activator and by Kakiuchi et al. (36) on a rat brain activator.

### Table VI

**Effect of divalent cations on binding of Ca\textsuperscript{2+} by activator of phosphodiesterase**

<table>
<thead>
<tr>
<th>Concentration of cation</th>
<th>Ca\textsuperscript{2+} bound in presence of other divalent cations</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(25 \mu M)</td>
<td>\begin{tabular}{l</td>
<td>l</td>
</tr>
<tr>
<td>(50 \mu M)</td>
<td>\begin{tabular}{l</td>
<td>l</td>
</tr>
<tr>
<td>(500 \mu M)</td>
<td>\begin{tabular}{l</td>
<td>l</td>
</tr>
</tbody>
</table>
were the same as in Fig. 13.

and that the stimulation was more pronounced at low substrate absence of an excess activator. Table VII shows that at millimolar range cyclic AMP is a better substrate than cyclic GMP, activator affects the relative rates of hydrolysis of the two nucleotides, we measured phosphodiesterase activity in the presence or absence of both Mg"+ and Ca"+ (data not shown).

The DEAE-enzyme from bovine brain catalyzes the hydrolysis of cyclic 3':5'-nucleotides with a purine but not a pyrimidine base (37). Cyclic GMP is the only other cyclic 3':5'-nucleotide found in tissues and physiological fluids. To examine whether the activator affects the relative rates of hydrolysis of the two nucleotides, we measured phosphodiesterase activity in the presence or absence of an excess activator. Table VII shows that at millimolar range cyclic AMP is a better substrate than cyclic GMP, whereas at micromolar concentrations, cyclic GMP is a better substrate. This was true whether the assay was performed in the presence or absence of the activator. Table VII also shows that the activator stimulated the hydrolysis of both nucleotides and that the stimulation was more pronounced at low substrate concentrations. Further, the stimulation was greater with cyclic AMP than with cyclic GMP, so that the activator changes the relative rate of hydrolysis of the two nucleotides in favor a cyclic AMP.

Effect of Caffeine on Activation of Phosphodiesterase by Activator—Methylxanthines inhibit phosphodiesterase and have been used extensively to potentiate the effects of hormones whose actions are thought to be mediated through the adenylate cyclase and cyclic AMP system. The question thus arises as to whether the activator protects the enzyme from inhibition by caffeine. Fig. 16 depicts an experiment in which phosphodiesterase was assayed in the presence of various concentrations of activator. The reaction mixture contained the usual components as described in the text and various concentrations of the activator (from Step 3 of the purification procedure) and caffeine as indicated.

![Fig. 15 (left). Effect of EGTA on activity of phosphodiesterase at different concentrations of protein activator. The procedures were the same as in Fig. 13.](http://www.jbc.org/)

![Fig. 16 (right). Inhibition of phosphodiesterase activity by caffeine in the presence of various concentrations of activator. The reaction mixture contained the usual components as described in the text and various concentrations of the activator (from Step 3 of the purification procedure) and caffeine as indicated.](http://www.jbc.org/)

**Table VII**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No activator</td>
<td>Plus activator</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM cyclic AMP</td>
<td>140</td>
<td>631</td>
</tr>
<tr>
<td>2 mM cyclic GMP</td>
<td>03</td>
<td>160</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM cyclic AMP</td>
<td>1.4</td>
<td>9.9</td>
</tr>
<tr>
<td>2 mM cyclic GMP</td>
<td>4.3</td>
<td>13.7</td>
</tr>
</tbody>
</table>

The data presented here established unequivocally that the activator is a protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, sucrose density gradient centrifugation, sedimentation equilibrium, and sedimentation velocity and diffusion all yielded a molecular weight of about 15,000 for the activator, a figure somewhat smaller than the 18,920 calculated from amino acid analysis. In contrast, the molecular weight estimated from Sephadex G-100 gel filtration was 31,000. That this figure indicates a dimeric form was considered unlikely because a comparable value was also obtained in a Sepharose 4B column equilibrated with 6 m guanidine hydrochloride. Other workers have noted that the technique of gel filtration gives

geneity. The procedure takes advantage of the fact that the activator is heat stable and acidic (4). The steps of heat treatment and DEAE-cellulose chromatography removed the bulk of contaminating proteins. The purification achieved at this stage is about 900-fold over the crude homogenate. Both steps allow the processing of kilogram quantities of tissue. In addition to being an effective purification step, the DEAE-cellulose column concentrates the activator from a large volume of dilute fluid. The bottleneck of the procedure is the last step, preparative electrophoresis, which has a capacity of about 15 mg of protein from Step 4. This amount of protein is equivalent to about 1 kg of bovine brain.

We have described a simple procedure for purifying the activator of phosphodiesterase from bovine brain to apparent homo-

**DISCUSSION**

The activator is heat stable and acidic (4). The steps of heat treatment and DEAE-cellulose chromatography removed the bulk of contaminating proteins. The purification achieved at this stage is about 900-fold over the crude homogenate. Both steps allow the processing of kilogram quantities of tissue. In addition to being an effective purification step, the DEAE-cellulose column concentrates the activator from a large volume of dilute fluid. The bottleneck of the procedure is the last step, preparative electrophoresis, which has a capacity of about 15 mg of protein from Step 4. This amount of protein is equivalent to about 1 kg of bovine brain.

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We have described a simple procedure for purifying the activator of phosphodiesterase from bovine brain to apparent homo-
apparently larger molecular weights (11). The reason for this discrepancy is not known.

The absence of half-cystine in amino acid analysis indicates that the activator does not possess a disulfide bridge. Because the molecular weight obtained from sodium dodecyl sulfate polyacrylamide gel electrophoresis is the same as that obtained from analytical ultracentrifugation and because valine is the only NH₂-terminal group, the activator probably possesses only one polypeptide. A fractional ratio of 1.1 to 1.2 indicates a globular protein. The prevalence of aspartic and glutamic acids accounts for the isoelectric point of 4.3.

There are several differences between the protein activator prepared from bovine brain and bovine heart (11). First, in amino acid composition, brain activator contains 1.2% proline, 0.6% histidine, and 6.5% methionine, whereas heart activator contains 2.4%, 0.86%, and 3.3% of these compounds, respectively. Second, although both preparations are heat stable, the heart activator is labile in the absence of Mg²⁺, whereas the brain activator is stable. Third, the heart preparation contains carboxylate, which was not detected in the brain preparation. In view of the fact that the activator lacks tissue and species specificity (4), these differences between activators from the brain and heart are surprising.

The procedure used to purify the activator from bovine heart (11) is slightly different from that described here. The last step of purification for the heart activator involved a Sephadex G-100 column. We have employed this technique as a last step in our earlier purification scheme and found it did not give a homogeneous preparation. Preparative acrylamide gel electrophoresis was therefore used, in spite of the fact that it had a smaller capacity and was more time consuming.

Early work with metal chelators has led to the suggestion that brain phosphodiesterase is a metalloenzyme (39). The present study reveals that the activator binds Ca²⁺ and suggests that the active form of the activator is a Ca²⁺-activator complex. Since tissue level of the activator is usually in excess of the enzyme (6), phosphodiesterase in vivo may be modulated by the Ca²⁺ flux as follows:

\[ \text{Ca}^{2+} + \text{Activator} \rightleftharpoons \text{Ca}^{2+}\text{-activator} \]

Phosphodiesterase in the brain is localized in the nerve endings (40). Increasing evidence indicates that cyclic AMP is closely involved with nervous activity (41). Addition of a depolarizing agent such as K+ to brain slices resulted in a 10-fold increase of tissue level of cyclic AMP (42). Depolarizing squid axons by increasing the external K⁺ concentration caused a 5-fold increase of Ca²⁺ influx (43). The increase of cyclic AMP may be temporally related to the increased Ca²⁺ influx when the nervous tissue is stimulated. The increase of Ca²⁺ may activate phosphodiesterase, which then restores the concentration of cyclic AMP to its prestimulated level.

Phosphodiesterase catalyzes the hydrolysis of cyclic AMP and cyclic GMP. The presence of one nucleotide interferes with the rate of hydrolysis of the other in a manner predictable from their kinetic constants. For example, the \( K_i \) of cyclic GMP as an inhibitor of cyclic AMP hydrolysis is comparable to its \( K_m \) as a substrate and vice versa (44). However, at micromolar substrate, the effect of cyclic GMP on the hydrolysis of cyclic AMP may be opposite to this. Beavo et al. (44) and Franks and Macmanus (45) showed that cyclic GMP increased the hydrolysis of cyclic AMP by phosphodiesterase from several tissues. The present work demonstrates that the activator increases the rate of hydrolysis of both cyclic AMP and cyclic GMP. The increase was more pronounced with cyclic AMP than with cyclic GMP, so that the activator changes the relative rate of hydrolysis in favor of cyclic AMP. Interpretation of these results is complicated by the fact that phosphodiesterase exists in multiple forms (46). Until the availability of a homogeneous preparation, studies on the regulation of phosphodiesterase activity should be interpreted with caution.

Acknowledgments—We are grateful to Dr. Andrew S. Kang for amino acid analysis, to Dr. William Groves for his help in our initial work with preparative electrophoresis, to Drs. Robert M. MacLeod and Shiv K. Soni for their assistance in performing ultracentrifugation, and to Dr. Jerry H. C. Wang for preprints of his work prior to publication. Mrs. Linda Smith provided us with excellent technical help and the Fineberg Packing Co., a generous supply of bovine brains.

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Cyclic 3' : 5'-Nucleotide Phosphodiesterase: PURIFICATION, CHARACTERIZATION, AND ACTIVE FORM OF THE PROTEIN ACTIVATOR FROM BOVINE BRAIN
Ying Ming Lin, Yung Pin Liu and Wai Yiu Cheung


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