Cyclic 3',5'-Nucleotide Phosphodiesterase

EVIDENCE FOR AND PROPERTIES OF A PROTEIN ACTIVATOR

WAI YIU CHEUNG

From the Laboratory of Biochemistry, St. Jude Children's Research Hospital and the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38101

SUMMARY

Purification of cyclic 3',5'-nucleotide phosphodiesterase of bovine brain cerebrum resulted in partial loss of activity, due to dissociation of an activator (or cofactor) from the enzyme. A systematic study showed that as purification proceeded, the activator was removed from phosphodiesterase in a stepwise fashion. Fractions representing phosphodiesterase at different stages of purification contained different concentrations of the activator in relation to phosphodiesterase. The fractions obtained in the initial stages contained excess or sufficient activator relative to the enzyme, and they required no exogenous cofactor for optimal activity. The fraction obtained in the last stage of purification was deficient in the co-factor, and phosphodiesterase was relatively inactive unless supplemented with an exogenous activator.

The activator was sensitive to proteolytic enzymes, but not to RNase or DNase, indicating that it was a protein. The protein was resistant to 8 M urea, and to boiling at pH 1.7. Boiling at pH 12.3 obliterated all activity. Gel filtration indicated that the protein had a molecular weight of 40,000.

Kinetic analysis showed that stimulation was independent of the time of preliminary incubation with the activator, but dependent on its concentration in the reaction mixture. In contrast, stimulation of the enzyme by trypsin was a catalytic process. Although mechanistically different, both agents increased the $V_{max}$ of the enzyme and decreased its $K_m$ for adenosine 3',5'-monophosphate.

An activator and a relatively inactive phosphodiesterase were also isolated from human brain, porcine brain, and rat brain as well as from bovine heart. An activator from one tissue cross-activated effectively a purified phosphodiesterase from another tissue, indicating a lack of tissue specificity. However, the effect of the activator was specific; none of a variety of proteins mimicked its action.

A heated purified preparation lost its enzymic activity, but was capable of stimulating another preparation which had not been heated. It was concluded that the residual activity associated with the purified enzyme was due to an incomplete removal of the activator from the enzyme.

Adenosine 3',5'-monophosphate mediates the action of a variety of different hormones. Its tissue level is critically related to the intensity and duration of hormonal action (1). Since cyclic 3',5'-nucleotide phosphodiesterase catalyzes its hydrolysis to 5'-AMP, thereby terminating its action, a study of the factors affecting phosphodiesterase activity assumes significance.

Previous work from this laboratory showed that phosphodiesterase was partly soluble and partly particulate. The particulate activity was associated mostly with a microsomal fraction. The majority of the microsomal activity appeared latent, and was not accessible to cyclic AMP unless previously exposed to a detergent, Triton X-100 (2). The enzyme was strongly inhibited by nucleoside triphosphates, citrate ions, ethylenediaminetetraacetate (3, 4), and p-hydroxymercurial benzoate (5). Guanosine 3',5'-monophosphate and inosine 3',5'-monophosphate were also effective inhibitors (6).

The activity of phosphodiesterase can be regulated in another manner. The enzyme, as extracted from the bovine brain cerebrum, was fully active, but became relatively inactive upon purification. The purified enzyme was reactivated by prior incubation with snake venom or proteolytic enzymes (7, 8). The crude enzyme, however, was not affected by these agents. Combination experiments showed that the activity of a mixture of the crude and the purified enzyme was greater than the summed activities of the individual preparations. An activator of phosphodiesterase was isolated from the crude homogenate, and it effectively reconstituted the activity of the purified enzyme. The loss of phosphodiesterase activity in the purified fraction was due to removal of the activator from the enzyme (9).

This communication describes experiments indicating the presence of the activator in brain extracts and its subsequent removal from the enzyme during the process of purification. Some properties of the activator and the mechanism of activation of the purified enzyme are given. A large scale preparation of the activator from bovine brain cerebra is also presented. Preliminary accounts have appeared elsewhere (5, 10, 11).

EXPERIMENTAL PROCEDURE

Chemicals and Reagents—Cyclic AMP was obtained from Schwarz BioResearch. Lyophilized snake venom (Crotalus

1 The abbreviation used is: adenosine 3',5'-monophosphate, cyclic AMP.
The method is essentially that of Butcher and Sutherland (12), employing snake venom as a source of 5′-nucleotidase to convert the product of phosphodiesterase, viz., 5′-AMP to adenosine and inorganic phosphate. The inorganic phosphate released was determined according to Fiske and SubbaRow (13). The present procedure, using a two-stage incubation, incorporated slight modifications of the one described previously (8). The separation of the incubation into two stages was necessary, because snake venom stimulated the purified phosphodiesterase. In the first stage of the incubation, the reaction mixture of 0.5 ml contained 40 mM Tris-chloride, pH 8.0, 0.1 mM MnCl₂, 2 mM cyclic AMP, and an appropriate concentration of enzyme. The reaction was initiated by the addition of cyclic AMP. At the end of 10 min at 30° the tubes containing the reaction mixture were transferred to a boiling water bath for 2 min to terminate the reaction. After thermal equilibration to 30°, 0.05 ml of snake venom (1 mg per ml) was added for a second 10-min incubation. The reaction was stopped by the addition of 0.05 ml of 55% trichloroacetic acid, which was followed by 0.75 ml of water and 0.15 ml of 2.5% ammonium molybdate in 5 N H₂SO₄. Denatured proteins were removed by centrifugation. The clear supernatant fluid was decanted into clean tubes containing 0.05 ml of the reducing Fiske-SubbaRow reagent. The blue color developed was measured at 690 mμ. When snake venom was used to stimulate the purified enzyme (as in Fig. 1), 50 μg of the venom was present in the reaction mixture, which was previously incubated for 10 min before the first stage incubation was initiated with cyclic AMP. Previous experiments established that the purified enzyme was fully activated under this condition (7). Subsequent steps were similar to those described above. A word of technical interest should be added. The protein sediment removed by the centrifugation was yellowish, and the subsequent loss of color was proportional to the amount of proteins removed in the sediment. In experiments involving addition of more proteins to some tubes, and less to the others, an equivalent amount of sample protein was added to those tubes after the reaction of the second stage incubation had been terminated. This treatment provided a basis for compensating the loss of color due to unequal amounts of protein in the reaction mixture. All assays were performed in duplicates, under conditions that the rate was linear with the time of incubation and the concentration of protein. All data were corrected for a control that contained all components except phosphodiesterase.

Alternatively, enzymic activity was followed by a continuous titrimetric technique which takes advantage of the fact that the phosphate moiety of cyclic AMP possesses one dissociable species at a neutral pH range while that of 5′-AMP has two. As cyclic AMP is hydrolyzed by phosphodiesterase to 5′-AMP, a stoichiometric quantity of proton is generated. The rate of an alkali added to the unbuffered reaction mixture to maintain a constant pH provides a direct and continuous measure of the hydrolysis of cyclic AMP (14).

Preparation of Brain Activator—In the initial phase of this work, the activator was prepared from a DEAE-cellulose column (see Fig. 1). The tubes after those containing phosphodiesterase were pooled, the combined eluates were lyophilized, and the lyophilized sample was dialyzed extensively against 20 mM Tris-chloride, pH 7.5. Dialysis resulted in some denatured proteins, which were removed by centrifugation. The amount of activator obtained in this way was small.

Subsequent studies showed that the effectiveness of the activator was virtually unchanged after brief exposure to a boiling water bath. The thermal stability offered a convenient means to prepare a much larger quantity of the activator.

Fresh bovine brain, obtained from a local slaughterhouse, was cleaned in water and the cerebrum was isolated at room temperature. This tissue was used fresh or stored at −20°. No appreciable change of activity was noted in using either the fresh or frozen tissue. Cerebra (500 g, fresh weight) were homogenized in 1500 ml of glass-distilled water chilled to 0°. The homogenate was adjusted to pH 5.9, and spun at 13,000 x g for 30 min. The sediment was discarded. The supernatant was heated in 500-ml portions in a 2-liter stainless steel beaker immersed in a large boiling water bath. The solution was stirred and the temperature was rapidly brought to 95 to 99°. The elevated temperature was maintained for 5 min, and the beaker was transferred to an ice slurry for quick cooling. Denatured proteins were removed by centrifugation. This process was repeated and the boiled supernatant fluid was pooled. The pooled solution was concentrated by ultrafiltration through a UM-10 membrane at 4° under a nitrogen atmosphere at 50 p.s.i. The filtrate was discarded and the concentrated solution was dialyzed extensively against 20 mM Tris-chloride, pH 7.5, at 4°. Denatured proteins in the dialyzed solution were removed by centrifugation and the supernatant fluid was adjusted to 10 mg of protein per ml with the same buffer. A 50 to 60% (NH₄)₂SO₄ fraction was prepared, using solid (NH₄)₂SO₄. This represents about 100-fold purification from the crude homogenate, with a yield of 10%. An attempt to achieve further purification is in progress.

Preparations from both procedures effectively stimulated the activity of the purified enzyme. Present evidence does not suggest differences between the two preparations. Most of the work described here was done using a boiled pH 5.9 supernatant.
The activity over and above that of the purified phosphodiesterase is the stimulated activity attributable to the action of the cofactor. A preliminary experiment determined the amount of purified phosphodiesterase used in each assay so that the enzyme was in excess of the stimulatory capacity of the activator (see Fig. 3).

Estimation of Molecular Weight—A Sephadex G-100 column (2.5 x 64.5 cm) was calibrated according to the method of Andrews (15), using cytochrome c, soybean trypsin inhibitor, ovalbumin, and bovine serum albumin as markers. The column was equilibrated with 20 mM Tris-chloride, pH 7.6, containing 100 mM NaCl. Filtration experiments were run at 22°C.

Protein Determination—In the initial stages of purification, proteins were measured with the biuret reagent containing sodium deoxycholate. Bovine serum albumin was used as a standard. Proteins at later stages of purification were estimated spectrophotometrically according to the method of Warburg and Christian (16).

RESULTS

Partial Loss of Phosphodiesterase Activity during Process of Purification—Earlier attempts to purify phosphodiesterase from rat and bovine brain often encountered unexplained loss of enzymic activity. Drummond and Ferrott-Yee (17) experienced similar difficulties in their work with rabbit brain. Subsequently, we found that the loss of activity was manifested only at a later stage of the purification procedure. Table I shows a typical pattern of the specific activity of bovine brain phosphodiesterase at the different stages of purification. The specific activity was increased from 50 in Fraction a to 340 in Fraction d. Further purification resulted in a precipitous loss of activity (Fraction e).

A recent report from this laboratory described the preparation of a partially inactive phosphodiesterase from bovine brain cerebrum and its subsequent activation by snake venom (8). It was shown that the enzyme lost activity upon purification and that exposure of the purified enzyme to snake venom prior to assay markedly enhanced phosphodiesterase activity. Pronounced stimulation was observed only with the fraction eluted from the DEAE-cellulose column, and not with fractions obtained before this purification step. It was concluded that an activator of phosphodiesterase was removed from the enzyme during the course of purification, and that the removal of the activator caused the purified enzyme to be dependent on the venom for activity.

Resolution of Phosphodiesterase into Activator and Partially Inactive Enzyme—Fig. 1 depicts the elution pattern of the enzyme from a DEAE-cellulose column and shows phosphodiesterase activity in its stimulated and nonstimulated state. The activity in the peak tubes was very much dependent on venom but that in the tubes trailing the peak was not. In fact, the activity of these tubes (e.g. tubes 69 to 73) was essentially the same whether or not venom was present during the incubation prior to assay. This suggested that the enzyme in these tubes retained sufficient activator and that the latter was eluted after the enzyme. Tubes were pooled as indicated in Fig. 1 to test this possibility. Fraction I was mostly inactive, and Fraction II exhibited no phosphodiesterase activity (Table II). However, Fraction II augmented the activity of Fraction I 6-fold. Note that the purified enzyme in the peak tubes of Fig. 1 was stimulated by the venom to a comparable extent.

<table>
<thead>
<tr>
<th>Fraction (tubes a through e)</th>
<th>Activity (mmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Homogenate</td>
<td>54.1</td>
</tr>
<tr>
<td>(b) pH 5.9 Supernatant</td>
<td>91.3</td>
</tr>
<tr>
<td>(c) 30 to 60% (NH₄)₂SO₄</td>
<td>181.5</td>
</tr>
<tr>
<td>(d) Calcium phosphate gel eluate</td>
<td>342.0</td>
</tr>
<tr>
<td>(e) DEAE-cellulose eluate</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Table I

Phosphodiesterase was purified according to a procedure described previously (8). The procedure consisted of extracting bovine brain cerebri with water, followed by pH fractionation, differential centrifugation, (NH₄)₂SO₄ fractionation, calcium phosphate gel adsorption, and DEAE-cellulose chromatography. An aliquot of each of the fractions obtained at the different stages of the purification procedure was assayed for enzymic activity according to a two-stage procedure described in the text.
The resolution of phosphodiesterase into a partially inactive enzyme and its activator shown in Fig. 1 was achieved by eluting with an exponential gradient. A similar resolution was also achieved by using a linear gradient generated from 500 ml of 20 mM Tris-chloride, pH 7.5, and 500 ml of 500 mM (NH₄)₂SO₄ in Tris-chloride. The elution pattern was essentially identical. Phosphodiesterase emerged at about 0.15 M with respect to (NH₄)₂SO₄. When a gradient with a lower ionic strength was used, for example, 500 ml of 20 mM Tris-chloride, pH 7.5, and 500 ml of 200 mM (NH₄)₂SO₄ protein as well as enzyme activity emerged as a broader peak. In this case, phosphodiesterase was eluted at 0.1 M with respect to (NH₄)₂SO₄. The enzyme eluted in the peak tubes of both gradient systems was partially inactive, similar to that obtained with the exponential gradient described in Fig. 1.

Distribution of Activator in and Its Effects on Different Fractions Obtained during Purification of Phosphodiesterase—To examine the distribution of the activator in the fractions representing different stages of purification, we studied the effect of these fractions on the activity of the purified phosphodiesterase. Table III shows the activities of the individual fractions as well as the combined fractions. The figures in the first column show the activities of the individual fractions, those in the second column combined fractions. The figures in the first column show the activities of the individual fractions, those in the second column combined fractions. The figures in the first column show the activities of the mixture of the DEAE-cellulose eluate and one of the other fractions, and those in the third column are algebraic sums of the activities of the individual assays. The difference between the observed activity and the calculated activity for any fraction is due to the stimulating effect of that fraction. The difference was most definite in the fractions obtained during the early stages of purification, i.e., the homogenate and the pH 5.9 supernatant. This was consistent with the notion that these fractions already contained abundant activator. The stimulation on the activity of the (NH₄)₂SO₄ and calcium phosphate gel fractions was small, indicating that further purification possibly caused a slight deficiency in the activator. The effect of the activator on the DEAE-cellulose eluate was pronounced, the stimulation being several fold.

These results revealed that removal of the cofactor from the enzyme took place mainly at two stages. The first stage coincided with the (NH₄)₂SO₄ fractionation. Whereas most of the enzyme was precipitated with the 30 to 50% (NH₄)₂SO₄ fraction, a large amount of the activator remained in the 50% supernatant fluid and was discarded, indicating that as purification proceeded, more activator was removed from the enzyme. The reverse experiment, showing the effect of the activator on phosphodiesterase activity in the different fractions, was presented in the last column of Table III. No significant effect was seen on the enzymic activity of the homogenate and the pH 5.9 supernatant. This was consistent with the notion that these fractions already contained abundant activator. The stimulation on the activity of the (NH₄)₂SO₄ and calcium phosphate gel fractions was small, indicating that further purification possibly caused a slight deficiency in the activator. The effect of the activator on the DEAE-cellulose eluate was pronounced, the stimulation being several fold.

The data in Table III substantiate the hypothesis that the partial inactivation of purified phosphodiesterase was due to dissociation of an activator from the enzyme during the course of purification. The crude enzyme was fully active because the activator was present in excess; the purified enzyme was partially...
Nature of brain activator

The incubation mixture of 0.4 ml contained 40 mm Tris-chloride, pH 7.5, 0.1 mM Mn++, 20 μg of activator, and where applicable, 10 μg of trypsin, 10 μg of RNase, or 10 μg DNase. Tubes f, g, and h contained no activator and served as controls for tubes c, d, and e, respectively. After incubation for 2 hours at 30°, the tubes containing trypsin received 20 μg of soybean inhibitor. Purified phosphodiesterase (50 μg) was added to all tubes and they were then assayed by the usual two-stage procedure. A boiled pH 5.9 supernatant fluid served as a source of the activator. The boiled pH 5.9 supernatant served as a source of the activator.

Additions (tubes a through h) | Activity (μmoles/mg/min)
--- | ---
(a) None | 125
(b) Activator | 665
(c) Activator (RNase treated) | 646
(d) Activator (DNase treated) | 682
(e) Activator (treated first with trypsin, then with soybean inhibitor) | 138
(f) RNase | 122
(g) DNase | 130
(h) Trypsin and soybean inhibitor | 137

Fig. 2. Elution pattern of brain activator on Sephadex G-100. A Sephadex G-100 column, 2.5 X 64.5 cm was prepared according to the method of Andrews (15). The column was equilibrated at 22° with 20 mm Tris-chloride, pH 7.0, containing 100 mm NaCl. A boiled pH 5.9 supernatant fluid served as a source of the activator. One milliliter of the activator with 5 mg of protein was dissolved in the Tris-NaCl buffer, fortified with 10 mg of sucrose. The column was eluted with the same buffer. Each tube collected 3.1 ml. Protein was followed spectrophotometrically at 230 μm. An aliquot was assayed for its ability to activate a purified phosphodiesterase. The stimulated activity in arbitrary units is the activity over and above that given by the purified phosphodiesterase. The void volume of the column was 117 ml and the peak stimulated activity was eluted at 178 ml. Calibration of the column with cytochrome c, trypsin inhibitor, ovalbumin, and bovine serum albumin as markers indicated that this elution volume corresponded to a molecular weight of 40,000.

Nature of Activator—The activator was exposed to the action of trypsin, RNase, or DNase before assay for its stimulatory activity (Table IV). The untreated activator increased phosphodiesterase activity 5-fold (compare tube b with tube a). Exposure of the activator to either RNase (tube c) or DNase (tube d) did not affect its stimulatory activity. After treatment with trypsin, however, the stimulatory activity was lost (tube e). Control tubes with RNase (tube f), DNase (tube g), or trypsin plus its inhibitor (tube h), did not affect phosphodiesterase activity. This experiment demonstrated that the stimulatory activity was associated with a polypeptide, and not with a nucleic acid.

Estimation of Molecular Weight by Gel Filtration—Preparation of the activator from the DEAE-cellulose column during the initial phase of this work involved dialysis in a cellulose tubing. Since the activator was retained inside the membrane, it could not be a small polypeptide. Fig. 2 depicts the elution profile of a boiled pH 5.9 supernatant fluid from a Sephadex G-100 column. The protein was heterogeneous and a substantial amount of inactive protein came out with the void volume. The peak stimulated activity was eluted at 178 ml. Calibration of this column with cytochrome c, trypsin inhibitor, ovalbumin, and bovine serum albumin as markers indicated that this elution volume corresponded to a molecular weight of 40,000.

In a separate experiment, the molecular weight of an unboiled activator in a high speed supernatant fluid of the brain homogenate was also determined. The peak stimulatory activity was eluted in a volume similar to the boiled pH 5.9 preparation, indicating that their molecular weights were comparable. This suggested that the heat treatment in the isolation procedure probably did not cause a gross change on the activator molecule.

Thermal and pH Stability of Activator—A supernatant fluid of a crude homogenate was used as a source of the activator to test its stability. As shown in Table V, the activator survived exposure to pH 1.7, to boiling, and to boiling at pH 1.7. Exposure to pH 1.7, to boiling, and to boiling at pH 1.7.
also activates purified phosphodiesterase, contains an activating
agent, the stability of which is comparable under these treat-
ments. Snake venom, which was incubated in 8 M urea for 60 min at 22°C prior to assay. Separate experiments showed that this concentration of urea did not affect the activity of the purified enzyme significantly. Assay was performed according to the two-stage procedure.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Activity (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Purified phosphodiesterase</td>
<td>125</td>
</tr>
</tbody>
</table>
| (b) Purified phosphodiesterase after incuba-
  tion with urea                           | 1.5                            |
| (c) Purified phosphodiesterase plus activa-
  tor                                      | 785                            |
| (d) Purified phosphodiesterase plus activa-
  tor after incubation with urea            | 799                            |

The molecular weight of the brain activator was estimated from the literature (18).

<table>
<thead>
<tr>
<th>Additions (tubes a through k)</th>
<th>Activity (nmol/tube/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) None</td>
<td>48</td>
</tr>
<tr>
<td>(b) Cytochrome c (mol wt 12,400; 66 µg)</td>
<td>54.7</td>
</tr>
<tr>
<td>(c) RNase (mol wt 12,700; 54 µg)</td>
<td>62.3</td>
</tr>
<tr>
<td>(d) Myoglobin (mol wt 17,800; 54 µg)</td>
<td>49.5</td>
</tr>
<tr>
<td>(e) Trypsin inhibitor (mol wt 21,500; 45 µg)</td>
<td>52</td>
</tr>
<tr>
<td>(f) Brain activator (mol wt 40,000; 51 µg)</td>
<td>307</td>
</tr>
<tr>
<td>(g) Horseradish peroxidase (mol wt 40,000; 48 µg)</td>
<td>64</td>
</tr>
<tr>
<td>(h) Ovalbumin (mol wt 45,000; 52 µg)</td>
<td>49</td>
</tr>
<tr>
<td>(i) DNase (mol wt 63,000; 47 µg)</td>
<td>60.7</td>
</tr>
<tr>
<td>(j) Bovine serum albumin (mol wt 67,000; 65 µg)</td>
<td>36.5</td>
</tr>
<tr>
<td>(k) Thyroglobulin (mol wt 670,000; 47 µg)</td>
<td>48</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of activator concentration on phosphodiesterase activity. The stimulatory activity was assayed as described in the text. A boiled pH 5.9 supernatant was used as a source of the activator. Purified phosphodiesterase was 50 µg. The amount of activator varied from 1 to 100 µg of protein.

### Table VI

**Effect of 8 M urea on activity of brain activator**

The purified phosphodiesterase in tube b and the activator in tube d were incubated in 8 M urea for 60 min at 22°C prior to assay. In the assay system, tubes b and d contained some residual urea (about 0.25 M) from their previous incubation. Separate experiments showed that this concentration of urea did not affect the activity of the purified enzyme significantly. Assay was performed according to the two-stage procedure.

### Table VII

**Specificity of brain activator**

The stimulatory activity was assayed as described in the text. The amount of protein in all these reagents was comparable. The molecular weight of the brain activator was estimated from their previous incubation. Separate experiments showed that this concentration of urea did not affect the activity of the purified enzyme significantly. Assay was performed according to the two-stage procedure.

The fact that the activator was resistant to boiling and the fact that the boiled as well as the unboiled activator exhibited a similar molecular weight suggested that the boiled activator was probably not a degradation product of the native species.

**Effect of Urea on Potency of Activator**—The effect of exposing the activator to urea was examined in Table VI. The activator was incubated in 8 M urea at 22°C for 60 min, and then assayed for its stimulatory activity. As a result of this treatment, no adverse effect was noted (tube d). On the other hand, phosphodiesterase activity was totally lost under this condition (tube b). The resistance of the activator to boiling in an acidic medium and to 8 M urea indicated that the tertiary structure necessary for activity was either minimal or very resistant to changes in its environment.

**Specificity of Activator**—In the initial stage of this work, the activator was prepared from a DEAE-cellulose column by elution with a high ionic strength solution, indicating that it was acidic. Preliminary experiments with an electrofocusing column estimated the isoelectric point to be pH 4.2. In Table VII, a number of proteins with various sizes and charge properties were tested for their ability to stimulate purified phosphodiesterase. Three of these, ovalbumin, bovine serum albumin, and thyroglobulin are acidic proteins, with isoelectric points around pH 4.6 (18). Of special interest is ovalbumin, which has a molecular weight (45,000) close to that of the brain activator (40,000), and appears to possess size and charge properties comparable to the activator. Note that the amount of each protein used in this experiment was nearly identical with the activator.

Table VII shows that none of these proteins caused a significant change in phosphodiesterase activity over the control (tube a) under conditions in which the activator produced a 7-fold stimulation (tube f). The ineffectiveness of these proteins to augment phosphodiesterase activity indicates that the action of the activator was specific.

**Effect of Activator Concentration on Phosphodiesterase Activity**—The relation between the degree of stimulation and the concentration of activator is depicted in Fig. 3. The curve is sigmoidal. At low concentrations of the activator, i.e. 2 µg of protein or less per 37 µg of phosphodiesterase, the stimulation was minimal.

As the concentration of activator increased, the extent of stimulation (tube f). The ineffectiveness of these proteins to augment phosphodiesterase activity indicates that the action of the activator was specific.

**Effect of Preliminary Incubation on Phosphodiesterase Activity**—A purified phosphodiesterase was previously incubated with suboptimal concentrations of the activator for various lengths of time before assay. In the absence of the activator, phospho-

---

Fig. 4. Effect of the time of preliminary incubation with the activator on phosphodiesterase activity. The time of preliminary incubation varied from 0 to 60 min. A boiled pH 5.9 supernatant served as a source of the activator. The purified phosphodiesterase was 50 μg. Assay was performed as described in the text.

diesterase activity was 50 μmoles per min, as in Fig. 4. In the presence of 5 μg and 10 μg of activator, phosphodiesterase activity was 150 μmoles per min and 250 moles per min, respectively. These activities were independent of the time of preliminary incubation, indicating that activation was not a catalytic process.

The fact that activation of phosphodiesterase is dependent on the concentration of the activator and not on the time of its exposure to the enzyme is more clearly demonstrated with a continuous titrimetric technique. Fig. 5 reproduces a titrimetric trace showing the effect of the activator on the rate of hydrolysis of cyclic AMP. No detectable lag was observed after the addition of the activator. The rate was linear and was maintained until another addition of the activator was made, whereby a new rate was established and maintained thereafter. Note the constant increase after each addition of the activator. As a comparison, a trace showing the catalytic activation of phosphodiesterase by trypsin is reproduced in Fig. 6. Note the increasing rate of hydrolysis with time after the addition of trypsin.

Although the process of activation by the two agents appears different mechanistically, the final effects are comparable. Either the activator or trypsin was capable of causing maximal stimulation, after which no further effect was observed by the addition of the other agent. It should be added that chymotrypsin, pronase, and snake venom stimulated the purified enzyme in a manner similar to trypsin (5).

Mechanism of Stimulation of Phosphodiesterase by Activator—
The mechanism of activation of the purified enzyme by the brain activator was examined by the double reciprocal plot shown in Fig. 7. In the absence of the activator, the K_m for the purified enzyme was 2 mM, and was decreased to 1.25 and 0.65 mM in the presence of 7 and 55 μg of brain activator, respectively. The

![Graph showing the effect of time of preincubation on phosphodiesterase activity.](image-url)

**Fig. 5.** Reproduction of a titrimetric trace showing the effect of brain activator on the activity of a purified phosphodiesterase. A micro version of the Metrohm Combi-Titrator 3D equipped with a 0.2-ml syringe was used. Assay was carried out at 30° in a final volume of 1.5 ml containing 40 mM NaCl, 0.1 mM MnCl_2, 240 μg of purified phosphodiesterase, and 1 mM cyclic AMP. The substrate (15 μl of 0.1 M cyclic AMP, pH 8.0) was added to initiate the reaction, and the rate of cyclic AMP (cAMP) hydrolysis was followed by the consumption of the titrant (2 mM NaOH). The reaction mixture was maintained at pH 8.0. The ordinate represents the time axis and the abscissa shows the volume of titrant. The full length of the abscissa corresponds to 0.2 ml. The scales of the trace are as shown. Where indicated, 3.8 μg of activator was added. The figures in parentheses indicate the rate of cyclic AMP hydrolysis expressed in millimicromoles per min. Before addition of the activator, the rate of hydrolysis was 6.2 μmoles per min. Note the constant increase of the rate of hydrolysis after each addition of the activator.

V_max under those conditions was increased from 210 to 500 and 670 μmoles per mg of protein per min, respectively.

In another experiment, trypsin was used as the stimulatory agent (Fig. 8). The K_m was decreased and the V_max increased.
Fig. 6. Reproduction of a titrimetric trace showing the effect of trypsin on the activity of a purified phosphodiesterase. Assay was carried out as in Fig. 4, except that the purified enzyme was 95 μg. The scale of the trace was also similar. At the point indicated, 0.1 μg of trypsin was added. Note that the rate of hydrolysis increased with time. The rate of hydrolysis was 7 μmoles per min prior to the addition of trypsin, and increased to 32 μmoles per min 39 min after the addition. cAMP, cyclic AMP.

in a manner similar to that in Fig. 7. Thus, the consequence of the stimulation of the purified enzyme by the activator appears to be identical to that by trypsin even though the mechanisms of stimulation by them may be different.

It can be recalled that the crude phosphodiesterase was fully active, and that only the purified enzyme was stimulated by the activator. Likewise, trypsin stimulated the activity of the purified, but not the crude enzyme. Although prolonged incubation of the enzyme with trypsin led to a loss of phosphodiesterase activity, no such effect was seen with the activator.

The activator was assayed for possible proteolytic activity with three substrates: p-toluenesulphenyl-L-arginine methyl ester, N-benzoyl-L-tyrosine ethyl ester, and casein. Various amounts of the activator (up to 200 μg), which was prepared from a DEAE-cellulose column as described in the legend to Fig. 1, were incubated with these substrates for times up to 120 min. No reaction was noted in any of them. It was concluded that the activator exhibited no proteolytic activity.

Reconstitution of Phosphodiesterase Activity—Table VIII documents an experiment in which the activity of the purified phosphodiesterase was reconstituted with the activator or with the homogenate. As expected, the activity of the purified enzyme stimulated by the activator in tube e was much more than the nonstimulated activity in tube b. The reconstituted activity by the activator in tube e compares favorably with that by the homogenate in tube g. In this experiment, the activity obtained in the presence of the cofactor (tube e) was about 80% that obtained in the presence of the homogenate (tube g). In other experiments, the activity reconstituted was better than 90% of that by the homogenate.

Demonstration of Residual Activator in Purified Phosphodiesterase—Although the purified enzyme is relatively inactive, it is not absolutely dependent on an exogenous factor for activity. The residual activity may indicate one of two possibilities. One possibility is that there are two species of phosphodiesterase; one species is independent of the cofactor for activity, the other is dependent on it for activity. The “independent” species gives the residual activity, and the “dependent” species the stimulated activity by the cofactor. Attempts to resolve the “dependent” from the “independent” species have not been successful.

The other possibility is that there is only one species of phosphodiesterase and that the purified preparation still retains some residual cofactor not removable from the enzyme under our experimental conditions. The residual cofactor gives the “independent” activity. Since the cofactor, but not the enzyme, is resistant to boiling, we took advantage of this fact to demonstrate the presence of the residual cofactor in the purified preparation. Table IX shows such an experiment, in which a boiled purified preparation itself exhibits no phosphodiesterase activity (tube b), yet definitely augments the activity of an unboiled preparation when assayed in a mixture (tube e). This experiment suggests that the activity of the purified preparation is due to some residual activator. However, it does not exclude the possibility
that the purified preparation also contains the "independent" species, since the latter would have been inactivated by heat treatment. Definite proof must await further purification of the enzyme with a complete removal of the activator.

Phosphodiesterase and Activator from Different Tissues—To determine whether phosphodiesterase from other tissues also loses activity upon purification, we prepared phosphodiesterase from the human brain, porcine brain, rat brain, and bovine heart, according to the procedure developed for the bovine brain. As with bovine brain, the crude enzyme of these tissues was fully active, and the purified enzyme was relatively inactive unless supplemented with an exogenous activator isolated from the DEAE-cellulose column. Table X shows the activity of these phosphodiesterases, in the absence or presence of their corresponding activators. In all cases, the activity of the purified phosphodiesterase from one tissue was augmented by the activator from the same tissue.

Cross Activation between Phosphodiesterase and Activator from Different Tissues—To determine whether an activator from one tissue cross-activated a phosphodiesterase from another tissue, we examined all the paired combinations of phosphodiesterase and its activator from bovine brain, human brain, porcine brain, rat brain, and bovine heart. Twenty-five paired combinations of phosphodiesterase and activator were possible, as shown in Table XI. A plus sign in the table indicates that a definite stimulation of phosphodiesterase activity was observed. A question mark means that stimulation was marginal. This experiment was performed over an extended period. During this time, the purified enzyme from human brain, rat brain, and bovine heart was stored at -20°C at a dilute protein concentration and lost activity. Because of the low activity of these preparations, definite stimulation was not demonstrated in three instances indicated in Table XI. It should be pointed out that in these instances the activator involved was active towards other purified enzymes. It thus appeared that the failure of stimulation was due to the loss of the activity of the enzyme and not to tissue incompatibility. The ability of an activator from one tissue to cross-activate a purified enzyme from another tissue demonstrated that the activator lacked tissue specificity.

Stability of Phosphodiesterase—Evidence has been presented to show that the loss of phosphodiesterase activity upon purification is due to dissociation of the activator from the enzyme. However, the possibility that the loss of enzymic activity upon purification was due to instability of the purified enzyme was

![Diagram](http://example.com/diagram.png)
Phosphodiesterase activity upon purification is due to the instability of the purified enzyme. During the process of purification the activator was gradually removed from the enzyme. The crude enzyme was fully active because it contained abundant activator; the purified enzyme was partially inactive because it lacked a stoichiometric amount of the activator. Stimulation of the purified enzyme was brought about by the addition of an exogenous activator.

Although the molecular weight of the activator was estimated by gel filtration to be 40,000, it was not established whether the protein might be composed of subunit structures. It is feasible that an active small polypeptide may be bound to a protein by hydrogen bonds and the complex then gives an apparent molecular weight of 40,000. The estimation of molecular weight in Fig. 2 was performed under conditions that would not normally rupture hydrogen bonds.

The fact that an activator was isolated from several tissues examined may mean that the removal of the activator from the enzyme during the course of purification and its subsequent requirement for the protein cofactor for activity is a feature common to phosphodiesterase of other sources.

The work of Monard, Janecek, and Rickenberg (19) on the enzymic degradation of cyclic AMP in *Escherichia coli* is of interest. They found that the bacterial extract contained three components, all of which were required for maximal degradative activity.

The decreased *K₅* of phosphodiesterase for cyclic AMP in the presence of the activator suggests that the activator increases the affinity of the enzyme for the substrate. We have recently demonstrated in bovine brain a protein with a binding site specific for cyclic AMP (20). When we examined possible binding of cyclic AMP to the activator, we could not detect any binding. It thus appears that the activator does not stimulate phosphodiesterase activity through a direct binding of the substrate. Rather, the activator is believed to bring about a conformational change on the enzyme, resulting in an increased affinity for the substrate.

The possibility that stimulation of the purified phosphodiesterase was due to stabilization of an unstable enzyme by a protein of the right size and charge properties was considered unlikely because, first, phosphodiesterase was a stable enzyme. Neither the crude nor the purified enzyme lost activity upon storage at -20° or at 0-4° over an extended period of time. Secondly, a number of proteins with various sizes did not activate the purified enzyme. In view of the evidence for a specific activator, we conclude that the increased activity of the purified enzyme was due to stimulation of a relatively inactive enzyme rather than stabilization of an unstable enzyme.

The molecular weight of phosphodiesterase may vary over a range between 130,000 to 750,000, depending on the experimental conditions (6). The variability of the molecular weight may indicate the existence of subunit structures; and the cofactor removed from the purified enzyme during the process of purification may be a subunit of the quaternary enzyme complex. This possibility seems an attractive one, except for the fact that the cofactor is present in excess in the crude enzyme. An excess of an enzyme subunit seems paradoxical, and we are not aware of a similar instance. However, until further purification is achieved, the possibility that the cofactor is a subunit of phosphodiesterase cannot be dismissed.

The demonstration of the presence of activator in the purified enzyme strongly suggests that the residual activity of the puri-

### TABLE X

<table>
<thead>
<tr>
<th>Phosphodiesterase or activator (tubes a through I)</th>
<th>Activity (mumole/tube/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Human brain phosphodiesterase</td>
<td>8.8</td>
</tr>
<tr>
<td>(b) Human brain activator</td>
<td>0.8</td>
</tr>
<tr>
<td>(c) Phosphodiesterase and activator</td>
<td>85.0</td>
</tr>
<tr>
<td>(d) Porcine brain phosphodiesterase</td>
<td>38.0</td>
</tr>
<tr>
<td>(e) Porcine brain activator</td>
<td>0.0</td>
</tr>
<tr>
<td>(f) Phosphodiesterase and activator</td>
<td>235.0</td>
</tr>
<tr>
<td>(g) Rat brain phosphodiesterase</td>
<td>11.7</td>
</tr>
<tr>
<td>(h) Rat brain activator</td>
<td>0.4</td>
</tr>
<tr>
<td>(i) Phosphodiesterase and activator</td>
<td>34.4</td>
</tr>
<tr>
<td>(j) Bovine heart phosphodiesterase</td>
<td>7.2</td>
</tr>
<tr>
<td>(k) Bovine heart activator</td>
<td>2.4</td>
</tr>
<tr>
<td>(l) Phosphodiesterase and activator</td>
<td>19.6</td>
</tr>
</tbody>
</table>

### TABLE XI

Cross activation between phosphodiesterase and activator from different tissues

The purified phosphodiesterase and the activator were the same samples used in Table X. Assay was performed as described in the text.

<table>
<thead>
<tr>
<th>Phosphodiesterase</th>
<th>Activator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine brain</td>
</tr>
<tr>
<td>Bovine brain</td>
<td>+</td>
</tr>
<tr>
<td>Human brain</td>
<td>+</td>
</tr>
<tr>
<td>Porcine brain</td>
<td>+</td>
</tr>
<tr>
<td>Rat brain</td>
<td>+</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>+</td>
</tr>
</tbody>
</table>

Not excluded. In our experience, both the crude and purified bovine brain enzyme were stable. Different preparations of the purified enzyme stored at -20° for several months showed no appreciable change in enzymic activity. A purified enzyme at a concentration of 1.5 mg of protein per ml of 20 mM Tris-chloride, pH 7.5, was kept at 0-4° for 22 days. During this time, enzymic activity was checked periodically, and no significant loss of activity was observed, indicating again that the purified enzyme was stable. Thus, the possibility that the decrease of the phosphodiesterase activity upon purification is due to the instability of the purified enzyme appears unlikely.

DISCUSSION

We have presented evidence to show that a protein activator of phosphodiesterase is present in excess in the crude enzyme and is deficient in the purified enzyme. During the process of purification the activator was gradually removed from the enzyme. The crude enzyme was fully active because it contained abundant activator; the purified enzyme was partially inactive because it lacked a stoichiometric amount of the activator. Stimulation of the purified enzyme was brought about by the addition of an exogenous activator.

Although the molecular weight of the activator was estimated by gel filtration to be 40,000, it was not established whether the protein might be composed of subunit structures. It is feasible that an active small polypeptide may be bound to a protein by hydrogen bonds and the complex then gives an apparent molecular weight of 40,000. The estimation of molecular weight in Fig. 2 was performed under conditions that would not normally rupture hydrogen bonds.

The fact that an activator was isolated from several tissues examined may mean that the removal of the activator from the enzyme during the course of purification and its subsequent requirement for the protein cofactor for activity is a feature common to phosphodiesterase of other sources.

The work of Monard, Janecek, and Rickenberg (19) on the enzymic degradation of cyclic AMP in *Escherichia coli* is of interest. They found that the bacterial extract contained three components, all of which were required for maximal degradative activity.

The decreased *K₅* of phosphodiesterase for cyclic AMP in the presence of the activator suggests that the activator increases the affinity of the enzyme for the substrate. We have recently demonstrated in bovine brain a protein with a binding site specific for cyclic AMP (20). When we examined possible binding of cyclic AMP to the activator, we could not detect any binding. It thus appears that the activator does not stimulate phosphodiesterase activity through a direct binding of the substrate. Rather, the activator is believed to bring about a conformational change on the enzyme, resulting in an increased affinity for the substrate.

The possibility that stimulation of the purified phosphodiesterase was due to stabilization of an unstable enzyme by a protein of the right size and charge properties was considered unlikely because, first, phosphodiesterase was a stable enzyme. Neither the crude nor the purified enzyme lost activity upon storage at -20° or at 0-4° over an extended period of time. Secondly, a number of proteins with various sizes did not activate the purified enzyme. In view of the evidence for a specific activator, we conclude that the increased activity of the purified enzyme was due to stimulation of a relatively inactive enzyme rather than stabilization of an unstable enzyme.

The molecular weight of phosphodiesterase may vary over a range between 130,000 to 750,000, depending on the experimental conditions (6). The variability of the molecular weight may indicate the existence of subunit structures; and the cofactor removed from the purified enzyme during the process of purification may be a subunit of the quaternary enzyme complex. This possibility seems an attractive one, except for the fact that the cofactor is present in excess in the crude enzyme. An excess of an enzyme subunit seems paradoxical, and we are not aware of a similar instance. However, until further purification is achieved, the possibility that the cofactor is a subunit of phosphodiesterase cannot be dismissed.

The demonstration of the presence of activator in the purified enzyme strongly suggests that the residual activity of the puri-
fied preparation is due to the activator. Extrapolation of this interpretation would mean that complete removal of the cofactor would produce an inactive phosphodiesterase. Experiments described here do not exclude the possibility that the activator and phosphodiesterase may exist as separate entities in a tissue. Association of the enzyme and the activator may be a consequence of homogenization of the tissue. This association, once formed, seems to be not readily reversible, as indicated by the presence of some residual activator which is retained by the purified enzyme. Were the activator and the enzyme present as distinct entities, it is conceivable that the enzyme may be completely inactive in situ.

Attempts to prepare a phosphodiesterase free of the activator have been unsuccessful. The main problem has been that under conditions whereby further purification of the phosphodiesterase appeared feasible, for example, the resolution of the purified enzyme into multiple bands in preparative acrylamide gel electrophoresis, irreversible loss of enzymic activity resulted. It may be that the activity of phosphodiesterase becomes highly labile once the residual activator is removed.

The potent inhibition of phosphodiesterase by ATP and by other nucleoside triphosphates has been noted (4). It remains to be elucidated whether these compounds exert their effects directly on the enzyme or indirectly through the activator. An understanding of the underlying mechanism should be of great interest physiologically as well as pharmacologically. The methyl xanthines and several other pharmacologically active agents are known to inhibit the activity of phosphodiesterase (1).

A knowledge of the exact mode of action of these drugs relative to the activator will be helpful in the designed synthesis of potential pharmacological inhibitors for phosphodiesterase.

Acknowledgments—It is a pleasure to acknowledge the skillful technical assistance of Ann Jenkins and Sandra Patrick.

REFERENCES
Cyclic 3',5'-Nucleotide Phosphodiesterase: EVIDENCE FOR AND PROPERTIES
OF A PROTEIN ACTIVATOR
Wai Yiu Cheung


Access the most updated version of this article at http://www.jbc.org/content/246/9/2859

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/246/9/2859.full.html#ref-list-1