Cyclic 3′:5′-Nucleotide Phosphodiesterase

INTERCONVERTIBLE MULTIPLE FORMS AND THEIR EFFECTS ON ENZYME ACTIVITY AND KINETICS*

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An extract of rat liver or human platelet displayed three cyclic 3′:5′-nucleotide phosphodiesterase activity peaks (I, II, and III) in a continuous sucrose density gradient when assayed with millimolar adenosine 3′:5′-monophosphate (cAMP) or guanosine 3′:5′-monophosphate (cGMP). The three fractions obtained from each nucleotide were not superimposable. The molecular weights corresponding to the three activity peaks of cAMP phosphodiesterase in rat liver were approximately: I, 22,000; II, 75,000; and III, 140,000. In both tissues, fraction I was barely detectable when assayed with micromolar concentrations of either nucleotide, presumably because fraction I has low affinity for cAMP and cGMP. Any one of the three forms upon recentrifugation on the gradient generated the others, indicating that they were interconvertible. The multiple forms appear to represent different aggregated states of the enzyme. The ratio of the three forms of cAMP phosphodiesterase in the platelet was shifted by dibutyryl cAMP (B,*cAMP) and by the enzyme concentration. B,*cAMP enhanced the formation of fraction I. Low enzyme concentration favored the equilibrium towards fraction I, while high enzyme concentration favored fraction III. When phosphodiesterase activities in the extract of rat liver, human platelets, or bovine brain were examined as a function of enzyme concentration, rectilinear rates were observed with micromolar, but not with millimolar cAMP or cGMP. The specific activity with millimolar cAMP was higher with low than with high protein concentrations, suggesting that the dissociated form catalyzed the hydrolysis of cAMP faster than that of the associated form. In contrast, the specific activity with millimolar cGMP was lower with low than with high protein concentrations. Supplementing the reaction mixture with bovine serum albumin to a final constant protein concentration did not affect the activity, suggesting that the concentration of the enzyme rather than that of extraneous proteins affected the enzyme activity. A change in enzyme concentration affected the kinetic properties of phosphodiesterase. A low enzyme concentration of cAMP phosphodiesterase yielded a linear Lineweaver-Burk plot, and a $K_m$ of $1.2 \times 10^{-4} \text{ M}$ (bovine), $3 \times 10^{-4} \text{ M}$ (platelet), or $5 \times 10^{-4} \text{ M}$ (liver), while a high enzyme concentration yielded a nonlinear plot, and apparent $K_m$ values of $1.4 \times 10^{-4} \text{ M}$ and $2 \times 10^{-4} \text{ M}$ (brain), $4 \times 10^{-4} \text{ M}$ and $3 \times 10^{-4} \text{ M}$ (platelet), or $4 \times 10^{-4} \text{ M}$ and $3 \times 10^{-4} \text{ M}$ (liver). Since a low enzyme concentration favored fraction I, the dissociated form, whereas a high enzyme concentration favored fraction III, the associated form, these kinetic constants suggest that the dissociated form exhibits a high $K_m$ and the associated form exhibits a low $K_m$.

In contrast, a high enzyme concentration gave a linear kinetic plot for cGMP phosphodiesterase, while a low enzyme concentration gave a nonlinear plot. A nonlinear kinetic Lineweaver-Burk plot is usually attributed to negative cooperativity of phosphodiesterase. The findings that phosphodiesterase exists as interconvertible multiple forms and that the kinetic plots could be either linear or nonlinear by a change in enzyme concentration in the reaction mixture suggest that the anomalous kinetics seen in many tissues may also be explained on the basis of the multiple forms. Further, the B,*cAMP-induced shift towards the dissociated form which is more active for cAMP may represent a self-compensating mechanism to degrade excessive cAMP quickly following stimulation of the tissue.

Cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) catalyzes the hydrolysis of cAMP* or cGMP to 5′-AMP or 5′-GMP.

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†The abbreviations used are: cAMP, adenosine 3′:5′-monophosphate; B,*cAMP, dibutyryl adenosine 3′:5′-monophosphate; cGMP, guanosine 3′:5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

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cGMP, its cellular activity is critical in governing the extent and duration of the action of these nucleotides. Thus, a study of the regulatory properties of the enzyme assumes significance.

In early works on phosphodiesterase, it was noted that during purification the enzyme invariably suffered unexplained loss of activity, and the yield was relatively low. It was later found that an endogenous protein activator was dissociated from the enzyme during the course of purification (2). The activator has been purified to homogeneity and characterized with respect to its physical chemical properties and mode of action (3-12).

The activator protein binds Ca²⁺; the active form appears to be a Ca²⁺-activator complex (3, 4, 6). In the presence of Ca²⁺, the activator combines with the enzyme to form an active enzyme-activator complex. Lowering the concentration of Ca²⁺ dissociates the complex into its component proteins, thus returning the activator to its relatively inactive state (4, 8, 9). The dissociation constant of the activator for Ca²⁺ is 4 x 10⁻⁹ M, which is the concentration giving half-maximum stimulation of phosphodiesterase (3, 6). In many tissues, the activator appears to be in excess of the enzyme (13). Recently, the activator was shown to stimulate also brain adenylyl cyclase (EC 4.6.1.1) (14, 15). Stimulation requires Ca²⁺ and the mode of action appears identical to that of phosphodiesterase (16).

The stimulation of adenylate cyclase and phosphodiesterase by the same protein activator raises a question of its efficacy in terms of physiological function. The protein activator increases the activity of the cytoplasmic but not the membrane phosphodiesterase. It is believed that the influx of Ca²⁺ through the plasma membrane or the release of membrane-bound Ca²⁺ in response to stimuli activates adenylate cyclase, leading to an increase of intracellular cAMP. Ca²⁺ subsequently arriving at the cytosol then activates the soluble phosphodiesterase, thus returning the elevated level of cAMP to its prestimulated level.

The sequential stimulation of the two enzymes would allow a transient elevation of cAMP as seen in many tissues. Alternatively, the cytoplasmic phosphodiesterase also catalyzes the hydrolysis of cGMP: in fact, at micromolar concentration of substrates, the rate of cGMP hydrolysis exceeds that of cAMP (3). The influx of Ca²⁺, therefore, could increase cAMP and concomitantly decrease cGMP (14, 15).

Another aspect of phosphodiesterase that has received extensive study is the multiple molecular forms and its complex kinetics (17-28). Phosphodiesterase from bovine brain was separated into two activity peaks in a gel filtration column (29, 30). Multiple forms of phosphodiesterase, their distribution, characterization, and kinetics have been studied in considerable detail in other tissues (27, 28).

In spite of many attempts to purify phosphodiesterase from mammalian tissues, the enzyme has not been purified sufficiently for rigorous characterization. An exception is a phosphodiesterase from frog photoreceptor rod, which has been purified to homogeneity (31). Attempts to purify the enzyme from microorganisms have been more successful (32-34), probably because it possesses a simpler structure. Unlike mammalian phosphodiesterase, the microbial enzyme does not require a protein activator or metal divalent cations for full activity. Further, it does not exhibit multiple molecular forms and anomalous kinetics.

In this communication, we report the separation of multiple forms of phosphodiesterase(s) from rat liver and human blood platelet, using sucrose density gradient centrifugation. We found that these multiple forms were interconvertible, that the equilibrium of these forms was altered by enzyme concentration and by dibutyryl cAMP, and that phosphodiesterase activity and its apparent Kₘ values were affected by enzyme concentration. A preliminary account has appeared (35).

**EXPERIMENTAL PROCEDURE**

**Materials**—[³H]cAMP (2.7 Ci/mmol) and [³H]GMP (0.5 Ci/mmol) were obtained from Schwarz/Mann. They were purified by thin layer chromatography on cellulose sheets. AG 1-X2 (a styrene polymer, 200-400 mesh; was obtained from Bio-Rad, and IRP-58 (a phenolic polymer) was gift of Rohm and Haas. The resin was washed with 0.5 M NaOH, distilled water, 0.5 M HCl, and then extensively with distilled water to a final pH between 4 and 5. IRP-58 is unstable in 0.5 M NaOH; washing in alkaline should be completed within 30 min. The resin was prepared as a slurry, resin/water, 1/2. The washed platelet was used immediately or kept at -20°.

**Preparation of Soluble Phosphodiesterase from Various Tissues**

**Rat Liver**—Male Sprague-Dawley rats were beheaded; the liver was removed and perfused with 0.9% NaCl solution at 22°. The perfused liver was cut into fresh or stored at -20°.

**Human Platelets**—Platelet-rich plasma was centrifuged at 800 x g for 2 min to remove contaminating erythrocytes and leukocytes. The supernatant fluid was centrifuged at 8,000 x g for 20 min; the pellet was saved and washed with 0.9% NaCl solution. The washing was repeated three times; all operations were carried out at 22°. The washed platelet was used immediately or kept at -20°.

**Bovine Brain**—Fresh bovine brain was obtained from a local slaughterhouse. The cortex was isolated, washed in distilled water, and stored at -20°.

All tissues were homogenized in 3 volumes of 40 mM Tris-HCl (pH 8.0) at 4°. The homogenate was centrifuged at 100,000 x g (or 30,000 x g for human platelets) for 1 h. The clear supernatant fluid was used as a source of soluble phosphodiesterase.

**Preparation of Particulate Bovine Brain Phosphodiesterase**—Bovine brain cortex was homogenized in 9 volumes of 20 mM Tris-HCl (pH 7.5). The homogenate was centrifuged at 12,000 x g for 30 min. The supernatant fluid was discarded and the pellet was washed twice with 20 mM Tris-HCl (pH 7.5). The washed pellet was extracted once with 1 M NaCl and then washed twice with 40 mM Tris-HCl, pH 8. All steps were carried out at 4°.

**Assay of Phosphodiesterase**—Phosphodiesterase was assayed by a two-stage procedure. In the first stage, 100 µl of a reaction mixture containing 40 mM Tris-HCl (pH 8.0), 5 mM MgSO₄, 50 µM CaCl₂, 2 mM or 1 µM [³H]cAMP (200,000 cpm) (or 1 mM or 0.1 µM [³H]GMP), and an appropriate amount of enzyme. The reaction was incubated at 30° for 10-180 min, and then stopped by boiling for 30 s. After thermal equilibrium to 30°, 20 µg of snake venom (Crotalus atrox, 1 mg/ml) was added and the incubation was continued for 10 min to allow complete conversion of 5'-AMP to adenosine. The data given were not corrected for the underestimation of cAMP in the reaction mixture. The pellet was removed by centrifugation and the [³H]adenosine or [³H]guanosine remaining in solution was counted in a liquid scintillation spectrometer (17). We have shown that the batch use of AG 1 X2 underestimates cAMP phosphodiesterase by 40% and cGMP phosphodiesterase by 80%; the resin apparently binds adenosine and guanosine (36), which are removed by the resin and are therefore not accounted. IRP-58, a weaker anionic exchanger than AG 1-X2, binds less of the nucleosides and therefore gives a more accurate assay of phosphodiesterase. IRP-58 underestimates CAMP phosphodiesterase by 10% and cGMP phosphodiesterase by 20% (37). CAMP phosphodiesterase was assayed with IRP-58 resin and cAMP phosphodiesterase was sometimes assayed with AG 1-X2 in the early phase of this work. The data given were not corrected for the underestimation by these resins. All the data presented herein were obtained by this procedure. Alternatively, at the end of the second stage of incubation, 1 ml of a resin slurry was added to the reaction mixture. The resin was removed by centrifugation and the [³H]adenosine or [³H]guanosine remaining in solution was counted in a liquid scintillation spectrometer (17). We have shown that the batch use of AG 1 X2 underestimates cAMP phosphodiesterase by 40% and cGMP phosphodiesterase by 80%; the resin apparently binds adenosine and guanosine (36), which are removed by the resin and are therefore not accounted. IRP-58, a weaker anionic exchanger than AG 1-X2, binds less of the nucleosides and therefore gives a more accurate assay of phosphodiesterase. IRP-58 underestimates CAMP phosphodiesterase by 10% and cGMP phosphodiesterase by 20% (37). CAMP phosphodiesterase was assayed with IRP-58 resin and cAMP phosphodiesterase was sometimes assayed with AG 1-X2 in the early phase of this work. The data given were not corrected for the underestimation by these resins. All the data presented herein were obtained by this procedure. Alternatively, at the end of the second stage of incubation, adenosine (or guanosine) in the reaction mixture was separated from cAMP (or cGMP) by paper chromatography (38). The nucleoside was localized on paper under ultraviolet illumination; the spots were cut out and counted in a liquid scintillation counter. This procedure gives an accurate measure of cAMP and cGMP phosphodiesterase. It is more time consuming.
Specific activity of phosphodiesterase is defined as nanomoles of cyclic nucleotides hydrolyzed/mg of protein/min at 30°C.

Ultracentrifugation of Phosphodiesterase on Sucrose Density Gradient—A clear supernatant fluid (0.2 ml) containing 1 mg of protein (unless otherwise indicated) was layered on 4.8 ml of a linear sucrose density gradient (5 to 20%). The gradient was prepared in 40 mM Tris- HCl, pH 7.5. After centrifugation in a Spinco SW 50.1 rotor at 38,000 rpm for 18 h at 4°C, the bottom of the tube was punctured and 0.2-ml fractions were collected manually. An aliquot, usually 50 μl, was assayed for phosphodiesterase. The crude enzyme preparation usually contained some hemoglobin, which served as a convenient internal marker for the gradient.

Determination of Protein—Protein was determined by the method of Lowry et al. (39) with bovine serum albumin as standard.

RESULTS

Multiple Forms of Phosphodiesterase(s) and Their Interconversion—Multiple forms of phosphodiesterase have been observed in many tissues using various separation techniques: gel filtration chromatography (17, 26, 30), anionic exchange chromatography (40, 41), isoelectric focusing (42), starch gel (21, 23) and acrylamide gel electrophoresis (22, 43). Fig. 1A depicts the profile of phosphodiesterase activity of a 100,000 × g rat liver extract on a continuous sucrose density gradient. Three activity peaks, I, II, and III, were observed when the gradient was assayed with 2 mM CAMP. The molecular weights of these activity peaks corresponded to 22,000, 75,000, and 140,000, using hemoglobin and cytochrome c as internal markers. These molecular weights are consistent with those of CAMP phosphodiesterase in Fig. 1, suggesting that the two enzyme activities may be associated with separate proteins.

Fig. 2, B, C, and D displays the pattern of cyclic GMP phosphodiesterase activity generated from each fraction pooled from several gradients. Although each fraction gave rise to other fractions, the pattern was not as reproducible as with CAMP phosphodiesterase, especially with fraction III, from which the generated fraction I and fraction II were hardly discernible at 1 mM cGMP (Fig. 2D). This further suggested that CAMP phosphodiesterase was probably separate from cGMP phosphodiesterase. It should be noted that other explanations are possible (see “Discussion”). However, in cell cultures of mouse fibroblasts (37) and chick embryo fibroblasts (44) the two enzymes appeared to be regulated by separate genes.

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The platelets offer a distinct advantage: they are suspended in plasma and clean, bulk quantities can be isolated easily. Interconversion of platelet phosphodiesterase on sucrose gradient was studied with cAMP and cGMP at both millimolar and micromolar concentrations. As with rat liver, there were three fractions of cAMP phosphodiesterase (Fig. 3A) and cGMP phosphodiesterase (Fig. 4A) at millimolar concentrations, but only two fractions at micromolar concentrations. In addition, when assayed with millimolar substrates, anyone of the fractions yielded the other two fractions (Fig. 3, B, C, and D and Fig. 4, B, C, and D). Although fraction I was not detected at micromolar nucleotide, it generated fractions II and III. These results indicate that multiple interconvertible forms of phosphodiesterase are also present in human blood platelets.

Rat liver and human platelet phosphodiesterases shared several properties in common. First, although the different fractions of cAMP and cGMP phosphodiesterases were interconvertible, the positions of the corresponding fractions of the two enzymes in the sucrose gradient did not necessarily coincide. Second, three activity fractions were observed with millimolar substrates but apparently only two with micromolar substrates. Third, the activity pattern with millimolar and micromolar substrates did not coincide. Fourth, the activity ratio of the different fractions varied from preparation to preparation, although fraction III appeared to be the usual predominant species under the specified conditions (see also Fig. 8).

Effect of Enzyme Concentration on Phosphodiesterase Activity—A curve depicting the activity of phosphodiesterase as a function of protein concentration is usually rectilinear. An apparent exception was observed when phosphodiesterase activity was followed in such an experiment with a titrimetric technique (29), which yielded a nonlinear curve that did not go through the origin. It was interpreted that different protein concentrations could produce different aggregated states of the enzyme.

Closer scrutiny of the activity curve of rat liver cAMP phosphodiesterase activity as a function of protein concentra-
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A HUMAN PLATELET

FIG. 4. Activity profile of cGMP phosphodiesterase of a 30,000 × g human platelet supernatant in a sucrose density gradient. The procedure was the same as that described in Fig. 1. Phosphodiesterase activity was determined with 1 mM or 1 μM cGMP. The activity recovery at 1 mM and 1 μM cGMP was 47% and 75%, respectively.

B

i 0.15

0 0.1

0 1 2

0 0.05

2 fig PROTEIN/ml

Fig. 5. Activity of rat liver phosphodiesterase as a function of protein concentration. All assay tubes contained standard reagents and varying amounts of protein. A, 2 mM or 1 μM CAMP; B, 1 mM or 0.1 μM CAMP. Numbers indicate phosphodiesterase activities either as nmol/mg/min (for millimolar CAMP or cGMP) or as pmol/mg/min (for micromolar cAMP or cGMP). Insets show the specific activity of phosphodiesterase as a function of protein concentration at 2 mM CAMP (A) or 1 mM CAMP (B).

C

D

experiments on the rates of CAMP or cGMP hydrolysis by platelet phosphodiesterase as a function of protein (enzyme) concentrations showed similar results. The rate of hydrolysis with 2 mM CAMP was higher (77.5 nmol/mg/min) at low protein concentrations (<50 μg/ml) than that (40 nmol/mg/min) at high protein concentrations (>100 μg/ml) (Fig. 6A and inset), whereas the rate with 1 mM CAMP or cGMP was lower (9 nmol/mg/min) at low protein concentrations (<100 μg/ml) than that (28.5 nmol/mg/min) at high protein concentrations (>100 μg/ml) (Fig. 6B and inset). On the other hand, the rate of hydrolysis with 1 μM CAMP or cGMP remained linear.

These studies were extended to bovine brain phosphodiesterase. Fig. 7, A and B shows an experiment with phosphodiesterase partially purified from brain cortex. Again, the rate of hydrolysis with 2 mM CAMP was higher (77.5 nmol/mg/min) at low protein concentrations (<50 μg/ml) than that (40 nmol/mg/min) at high protein concentrations (>100 μg/ml) (Fig. 5A). These data were replotted as specific activity as a function of protein concentration in the inset of Fig. 5A, which shows clearly that the activity was higher below 40 μg of protein/ml and that it decreased to a constant level when the protein concentration was higher than 80 μg/ml. The lower specific activity at a high protein concentration did not result from extensive hydrolysis of the substrate, since the amount of cAMP hydrolyzed did not exceed 20%. In fact, when 1 mM cAMP was used the reverse was true, i.e. the rate (2.7 nmol/mg/min) was lower at low protein concentrations (<80 μg/ml) than that (4.3 nmol/mg/min) at high protein concentrations (>80 μg/ml) (Fig. 5B and inset). Moreover, at 1 μM CAMP or 0.1 μM cGMP, the rates were rectilinear throughout the protein concentration range. The nonlinear curves did not result from procedural artifacts. Supplementing the reaction mixture with bovine serum albumin to make the final protein concentration constant did not change the shape of the overall curve (data not shown). This indicated that the concentration of the enzyme and not that of extraneous proteins in the reaction mixture affected the enzyme activity. Subsequently, using an independent assay such as paper chromatography (38), we found essentially the same type of curves: nonlinear with millimolar CAMP or cGMP and linear with micromolar CAMP or cGMP (data not shown).

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phosphodiesterase as a function of enzyme concentration with a 100,000 x g supernatant of bovine brain extract showed essentially similar results (data not shown).

In the three tissues studied, the rate of cAMP hydrolysis at millimolar concentration was lower at low protein concentrations than that at high protein concentrations, whereas the reverse was true with cGMP hydrolysis. On the other hand, the rates of hydrolysis for both cAMP and cGMP at micromolar concentrations were rectilinear. These experiments indicate that phosphodiesterase activity was dependent on the enzyme concentration in the incubation mixture at millimolar concentrations of substrate, but was independent of it at micromolar concentrations of substrate. One interpretation of these results is that the enzymes exist in different aggregated states which have unequal specific activities, and that these aggregated states are affected by the concentration of the enzyme and that of the substrate.

**Effect of Enzyme Concentration and B, cAMP on Ratio of Multiple Forms of cAMP Phosphodiesterase**—The effect of enzyme concentration on the activity profile of cAMP phosphodiesterase activity on the sucrose density gradient is depicted in Fig. 8. Human platelet was the source of phosphodiesterase, and two concentrations of protein were used: 0.5 mg or 0.01 mg per gradient. When the gradient contained 0.5 mg of protein, fraction III was predominant, and fractions II and I were barely detectable. However, when the protein was decreased to 0.01 mg, fraction III was greatly diminished, and fraction I and fraction II were correspondingly enhanced. In fact, fraction I now predominated over the other two fractions. This experiment showed that high enzyme concentration favored fraction III while low enzyme concentration favored fraction I, and that fraction III may be an aggregated form of fraction I.

In another experiment (Fig. 9), the sucrose gradient contained 1 mM B, cAMP, and fraction I and fraction II were increased relative to fraction III (Panel B), suggesting that B, cAMP favored the formation of the dissociated form of the enzyme. B, cAMP is not a substrate for platelet phosphodiesterase but it inhibits the hydrolysis of cAMP (45). Under the conditions for the enzymic assay of the gradient, the amount of B, cAMP carried over to the reaction mixture was low, and it decreased phosphodiesterase activity slightly. The decrease of enzymic activity was uniform in all the fractions; therefore, it would not change the activity profile qualitatively.
It is obviously important to examine the possible effects of cAMP, cGMP or other nucleotide derivatives on the equilibrium of the multiple forms of phosphodiesterase. Unfortunately, the phosphodiesterase preparation is relatively crude, and the enzyme acts on these compounds; thus, satisfactory experiments could not be performed.

The experiments in Figs. 8 and 9 suggest that the multiple forms of phosphodiesterase exist in an equilibrium and that the equilibrium may be shifted towards the dissociated or associated states by B,cAMP or by the concentration of the enzyme, respectively. The nonlinear curves showing the activity as a function of enzyme concentration at millimolar cAMP (Figs. 5, 6, and 7) may be explained on the basis of a shifted equilibrium of phosphodiesterase in the reaction system. At low enzyme concentration the equilibrium favors the dissociated form while at high enzyme concentration the equilibrium favors the associated form. Because the rate of cAMP hydrolysis was higher at low enzyme concentration, it appeared that the specific activity of the dissociated form of cAMP phosphodiesterase was higher than that of the aggregated form.

If we assume that multiple forms of cGMP phosphodiesterase also exist in an equilibrium and that this equilibrium is affected similarly by enzyme concentration, it would appear that low enzyme concentration would favor the dissociated form. cGMP phosphodiesterase activity in rat liver or human platelet was considerably lower than cAMP phosphodiesterase activity at millimolar substrates; experiments similar to the ones described in Fig. 8 and Fig. 9 could not be done satisfactorily because the activity was too low for accurate measurement. Because the rate of cGMP hydrolysis was lower at low enzyme concentration, it appears that the specific activity of the dissociated form of cGMP phosphodiesterase is lower than that of the aggregated form.

FIG. 9. Activity profile of cAMP phosphodiesterase of a 30,000 \times g human platelet supernatant in a continuous sucrose gradient. Protein (0.5 mg) was layered onto the sucrose gradient. A, no B,cAMP; B, 1 mM B,cAMP in the sucrose gradient. As a control for the effect of B,cAMP on phosphodiesterase activity, the gradient in A was assayed twice; once without added B,cAMP and once with 0.4 mM B,cAMP added to the reaction mixture. This concentration of B,cAMP was comparable to that carried over from the gradient into the reaction mixture in B. The amount of B,cAMP carried over from the sucrose gradient to the assay mixture slightly inhibited phosphodiesterase, but inhibition was uniform for all the fractions. Therefore, B,cAMP did not affect the activity profile qualitatively. A shows the assay without B,cAMP in the reaction mixture.

Lack of Effect of Protein Activator on Interconversion and Equilibrium of Multiple Forms of Phosphodiesterase—Brain soluble phosphodiesterase requires a protein activator for maximum activity. The activator binds Ca\(^{2+}\), and undergoes a change toward a more helical conformation (46), which apparently induces the interaction between the activator and the enzyme to form an enzyme-activator complex. In the presence of EGTA, which chelates Ca\(^{2+}\), the enzyme-activator complex dissociates into its components (4).

In the present studies, the high speed supernatant fluid that served as a source of phosphodiesterase contained excess activator over the enzyme. Such a preparation from bovine brain gave the same activity profile in the sucrose gradient as a partially purified phosphodiesterase from which most of the activator had been removed (2). Also, the activity profile of platelet or rat liver phosphodiesterase in a sucrose density gradient containing EGTA was qualitatively identical to the control. Moreover, the pattern of the activity curve as a function of enzyme concentration was qualitatively the same whether brain phosphodiesterase was assayed in the presence of EGTA or an exogenous activator (data not shown). These results suggested that the activator did not affect the equilibrium among the interconvertible forms of phosphodiesterase. The possibility remains, however, that the techniques used in the present studies lack the sensitivity to reveal the role of the activator.

If the nonlinear curves depicting activity as a function of enzyme concentration result from a shifted equilibrium of the multiple forms of soluble phosphodiesterase, then an enzyme associated with a particulate fraction should not display such nonlinear curves. Indeed, when a bovine brain particulate phosphodiesterase was used, the curve was rectilinear with...
millimolar concentrations of both nucleotides (Fig. 10, A and B). This experiment shows that a soluble phosphodiesterase capable of forming different aggregated states yields a nonlinear curve, whereas a particulate phosphodiesterase that lacks this capability yields a linear curve.

Kinetics of cAMP Phosphodiesterase and cGMP Phosphodiesterase—Phosphodiesterase from mammalian tissues displays anomalous kinetics: the Lineweaver-Burk plot is usually nonlinear. Extrapolation of the two apparent linear portions of the kinetic curve has been used to obtain two apparent $K_m$ values (17). The Lineweaver-Burk plot of rat liver cAMP phosphodiesterase shown in Fig. 11A is typical of the nonlinear curves seen in many tissues (97). Extrapolation of the linear regions gave two apparent $K_m$ values, one of $4 \times 10^{-5}$ M and one of $3 \times 10^{-6}$ M. However, the nonlinear curve was restored to a linear curve when the protein concentration was lowered from 450 $\mu$g/ml to 17 $\mu$g/ml of reaction mixture (Fig. 11B). The $K_m$ of $4 \times 10^{-4}$ M obtained at this lower protein concentration was essentially the same as the “apparent high $K_m$” obtained in Fig. 11A using a higher protein concentration. This experiment suggested that at a low protein concentration phosphodiesterase exhibited a low affinity for CAMP. As shown in Fig. 8, low protein concentration apparently favored the dissociated form of phosphodiesterase. The low affinity of the dissociated form (fraction I) for CAMP probably explains why this fraction was not detectable at 1 $\mu$M CAMP in the experiments shown in Figs. 1 and 3. A corollary of this finding is that the aggregated form of the enzyme yields the apparent low $K_m$, $3 \times 10^{-6}$ M.

Similar kinetic experiments were done with human platelets (Fig. 12) and bovine brain (Fig. 13). Again, the plots were nonlinear with a high enzyme concentration and linear with a low enzyme concentration; the one $K_m$ obtained with a low protein concentration corresponded to the “apparent high $K_m$” obtained with a high protein concentration. This suggested again that the dissociated form of phosphodiesterase displayed a lower affinity for cAMP than the associated form.

Anomalous kinetics observed with phosphodiesterase may be a result of multiple forms of the enzyme, each having a different $K_m$ (20). Accordingly, the kinetic hydrolysis of CAMP was examined with a particulate phosphodiesterase from bovine brain (Fig. 14A). With a protein concentration comparable to the one used in Fig. 13A, the plot was linear and the $K_m$ was estimated to be $6 \times 10^{-4}$ M, which is comparable to the apparent low $K_m$ in Fig. 13A. This experiment suggests that the enzyme in the particulate fraction has a kinetic property equivalent to the associated form of the soluble enzyme. It will be recalled that the rate of CAMP hydrolysis by the particulate brain phosphodiesterase was linear with respect to the enzyme concentration (Fig. 10A).

In contrast to CAMP phosphodiesterase, the reported Lineweaver-Burk plots of cGMP phosphodiesterase are usually linear. As shown in Fig. 15A (rat liver), Fig. 16A (human platelet) and Fig. 17A (bovine brain), the double-reciprocal plots were linear and the $K_m$ values were $5 \times 10^{-5}$ M, $2.5 \times 10^{-5}$ M and $3 \times 10^{-5}$ M, respectively. However, when similar kinetic experiments were done at lower enzyme concentrations, one-tenth of what were used in these experiments, the Lineweaver-Burk plots for the three tissues became nonlinear (Figs. 15B, 16B, and 17B). Extrapolation of the apparent two linear

![Fig. 11. Lineweaver-Burk plot of cAMP hydrolysis catalyzed by a 100,000 $\times g$ supernatant of rat liver. A, 450 $\mu$g of protein/ml of reaction mixture; B, 17 $\mu$g of protein/ml of reaction mixture. The concentration of cAMP varied from 0.5 $\mu$M to 2 mM. The incubation time was 10 min in A and 20 min in B.](http://www.jbc.org/)

![Fig. 12. Lineweaver-Burk plot of cAMP hydrolysis catalyzed by a 30,000 $\times g$ human platelet supernatant. A, 100 $\mu$g of protein/ml of reaction mixture; B, 10 $\mu$g of protein/ml of reaction mixture. The concentration of cAMP varied from 1 $\mu$M to 2 mM. The incubation time was 10 min in A and 20 min in B.](http://www.jbc.org/)
regions gave two apparent $K_a$ values, which differed by about 1 order of magnitude. The $K_a$ values obtained with a high enzyme concentration generally lie between the two apparent $K_a$ values obtained with a low enzyme concentration for the three tissues.

The double reciprocal plot of bovine brain particulate cGMP phosphodiesterase was examined. The kinetic plot was linear, whether the protein concentration was high, 83 pg/ml (not shown) or low, 8.3 pg/ml (Fig. 14B). The $K_m$ was $3 \times 10^{-5}$ M, comparable to the apparent low $K_m$ observed with a soluble brain enzyme shown in Fig. 17.

The apparent paucity of nonlinear double reciprocal plots for cGMP phosphodiesterase in the literature deserves comment. The batch use of an anionic exchange resin (AG 1-X2) has been widely used to assay phosphodiesterase activity. This technique could underestimate cGMP phosphodiesterase as much as 80% (36). Because of this, cGMP phosphodiesterase activity obtained by the resin assay could be erroneously low (37). The apparently lower cGMP phosphodiesterase activity probably prompted the use of a much higher enzyme concentration to get reasonable observable activity. As seen in Figs. 15A, 16A, and 17A, a higher enzyme concentration gave linear kinetic plots. Thus, the possibility of observing nonlinear kinetic plots for cGMP phosphodiesterase may have been missed in some studies.

**DISCUSSION**

The apparent complexities of the phosphodiesterase system have received increasing attention. The enzyme has been known to exhibit multiple forms; the experiments described here using sucrose density gradient centrifugation provide direct evidence that these forms are interconvertible, even without the intervention of a foreign agent. Chassy (47) showed that a slime mold phosphodiesterase with a low $K_m$ for cAMP was converted to one with a high $K_m$ upon aging of the enzyme; dithiothreitol prevented the conversion. Schrijder and Rickemberg (48) observed that bovine liver phosphodiesterase exhibited two molecular forms; the ionic strength of the buffer, the concentration of Mg$^{2+}$, and the presence of CAMP or cGMP affected the predominance of one or the other form. The conversion in these two instances was effected by a change in the composition of the buffer. In contrast, the interconversion of the multiple forms of phosphodiesterase in the sucrose density gradient in the present study took place without the intervention of any foreign agent.

Although the interconversion of the multiple forms of phosphodiesterase could be easily demonstrated in the sucrose density gradient, it is highly desirable to confirm this observation with another technique such as gel filtration chromatography. Multiple forms of phosphodiesterase from several tissues have been well documented with this method; however, their interconversion has not been reported. Because of the apparent complexity of the enzyme system, failure to demonstrate their
The interconvertibility of the multiple forms of cAMP phosphodiesterase allows them to exist in an equilibrium, subject to change by the enzyme concentration and by CAMP. The shift in equilibrium towards one predominant form appears to affect the enzyme activity; for example, at high concentrations of substrate, the dissociated form appears to have a higher activity than the aggregated form. The apparent difference in cAMP phosphodiesterase activity as a function of enzyme concentration at a millimolar concentration of cAMP may be related to the nonlinear double reciprocal plots (see below).

The nonlinear kinetic plot of phosphodiesterase has been explained on the basis of (a) two or more isoenzymes with different $K_m$ values, (b) a single enzyme with two or more noninteracting substrate sites having different $K_m$ values, and (c) a single enzyme with negatively cooperative sites. It has been proposed that phosphodiesterase is a negatively cooperative enzyme (19). In all instances in which nonlinear kinetic plots of phosphodiesterase have been described, probably none of the enzyme preparation is homogeneous, and the presence of multiple forms was not vigorously excluded. On the other hand, phosphodiesterase highly purified from lower forms of life including a bacterium (34), yeast (32), and silkworm (33), did not show multiple forms, and the kinetic plots were linear. Further, phosphodiesterase purified to homogeneity from visual photoreceptors did not show multiple forms and it displayed a linear kinetic plot (31). In the present study, we have shown that a nonlinear kinetic plot is not necessarily an obligatory property of soluble phosphodiesterase. The plot could be linear or nonlinear, depending on the enzyme concentration in the reaction mixture. At a low enzyme concentration the dissociated form prevails and the kinetic plot is linear, whereas at a high enzyme concentration the associated form predominates and the kinetic plot is nonlinear. Since the dissociated form appears more active than the associated form, the contribution of the dissociated form at a low enzyme concentration could outweigh that of the associated form. This could result in an apparent linear kinetic plot seen in Figs. 11B, 12B, and 13B. Although a high enzyme concentration favors the associated form, the more active dissociated form could contribute significantly toward the total enzyme activity. At a high concentration range of cAMP, which favors the dissociated form, the contribution by the dissociated form toward the total enzyme activity would be even more significant. These two factors would reinforce the contribution of the dissociated form to give a nonlinear kinetic plot seen in Figs. 11A, 12A, and 13A. On the other hand, a particulate brain enzyme shows a linear kinetic plot, irrespective of the enzyme concentration. A similar argument may apply to the linear kinetic plot of cGMP phosphodiesterase at a high enzyme concentration and the nonlinear kinetic plot at a low enzyme concentration. Since a high enzyme concentration favors the associated, more active form, its contribution towards the total enzyme activity may greatly exceed that of the dissociated, less active form, resulting in an apparent linear plot. On the other hand, a low enzyme concentration favors the dissociated form, and the contribution of the two forms towards the total activity would be more equitable, especially at the range of high substrate.
Further investigation, preferably with a pure enzyme, is necessary to ascertain if indeed it may serve as an in vivo regulatory mechanism.

The dependence of phosphodiesterase activity on enzyme concentration may not be limited to the several mammalian tissues examined here. Prasad et al. (49) noted that the activities of both cAMP and cGMP phosphodiesterases changed as a function of protein concentration. The change of phosphodiesterase activity as a function of protein (enzyme) concentration underscores the importance in defining assay conditions, especially when phosphodiesterase activity is to be compared, as in studies with ontogenetic development, and enzyme activity levels in normal and diseased states. Similarly, it is important to specify conditions for kinetic studies because the enzyme concentrations could determine the linearity of the kinetic plot and hence the apparent $K_m$ values.

In general, purification of mammalian phosphodiesterases, with the possible exception of the frog photoreceptor (31), is achieved invariably with poor recovery of activity. The interconversion of multiple forms suggests that different conditions prevailing at each purification step may affect the equilibrium of the different forms and that each step may retain preferentially one of the forms, resulting in poor recovery of enzyme activity. Thus, it becomes important in future studies to uncover conditions to prevent this phenomenon if the yield is to be improved.

In all sucrose gradients, the activity profile of phosphodiesterase assayed at millimolar substrate appears different from that at micromolar substrate. Since the enzyme activity varies with its concentration at millimolar and not at micromolar nucleotides, this could cause an apparent lack of correspondence of the activity peaks at different substrate concentrations.

On the other hand, cAMP phosphodiesterase and cGMP phosphodiesterase may be associated with separate proteins. First, the two activity profiles did not match one another in the sucrose gradient. Second, although the two activities varied as a function of protein (enzyme) concentration, the direction of change was not parallel, in fact, was opposite. Third, the kinetic plots of the two enzyme activities as a function of enzyme concentration was different: the plot for cAMP phosphodiesterase was nonlinear with a high enzyme concentration, whereas that for cGMP phosphodiesterase was nonlinear with a low enzyme concentration. However, because of the interconvertible multiple forms and the differential activities towards cAMP and cGMP, these observations do not constitute unequivocal evidence that the two activities are associated with different proteins.

Indeed, one could argue that the different activities of the multiple forms of phosphodiesterase may give rise to the apparent differences. A partially purified phosphodiesterase from many tissues catalyzes the hydrolysis of cAMP and cGMP, and the presence of one nucleotide decreases the rate of hydrolysis of the other, in a manner predictable by the absolute affinity of the enzyme towards the two substrates. The interference of one nucleotide with the hydrolysis of the other strongly indicates that the same catalytic site is involved with both nucleotides.

A word should be said about the molecular weight of cAMP phosphodiesterase, especially that for fraction I, which was estimated to be 22,000. This value is comparable to the molecular weight of 30,000 reported for cAMP phosphodiesterase of Escherichia coli (50); most other molecular weights ranged from 60,000 to over 200,000 (28). The molecular weights...
of phosphodiesterase obtained from the sucrose density gradients are at best estimates. Experiments described in Figs. 5, 6, and 7 clearly demonstrate that the enzyme activity varies as a function of enzyme concentration. The activity profile in the gradient could therefore vary. The findings that the activity peaks obtained with millimolar cyclic nucleotides did not necessarily match those obtained with micromolar substrates are in line with this argument. Until the enzyme is highly purified and rigorously characterized, the molecular weights of phosphodiesterase should perhaps be taken with reservation.

Although the studies described herein used crude phosphodiesterase, we feel that these results are important. First, these results confirm the existence of multiple forms and, more importantly, they demonstrate the direct interconversion of these forms. Second, they show that the equilibrium of the multiple forms may be shifted by enzyme concentration and by BtCAMP. The effect of BtCAMP on the equilibrium of the multiple forms may prove to be physiologically relevant. Third, the kinetic plots of phosphodiesterase could be made to be linear or nonlinear, simply by changing the enzyme concentration. The activity profile in the gradient could therefore vary. The findings that the activity peaks obtained with millimolar cyclic nucleotides did not necessarily match those obtained with micromolar substrates are at best estimates. Experiments described in Figs. 5, 6, and 7 clearly demonstrate that the enzyme activity varies as a function of enzyme concentration. The activity profile in the gradient could therefore vary. The findings that the activity peaks obtained with millimolar cyclic nucleotides did not necessarily match those obtained with micromolar substrates are in line with this argument. Until the enzyme is highly purified and rigorously characterized, the molecular weights of phosphodiesterase should perhaps be taken with reservation.

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Cyclid 3':5'-nucleotide phosphodiesterase. Interconvertible multiple forms and their effects on enzyme activity and kinetics.
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