Stahl, W. L., Smith, J. C., Napolitano, L. M., and Basford, R. E. (1963), *J. Cell Biol. 19*, 293.

Utter, M. F., Wood, H. G., and Renier, J. M. (1945), *J. Biol. Chem. 161*, 197.

Properties of Cyclic 3′,5′-Nucleotide Phosphodiesterase from Rat Brain*

Wai Yiu Cheung

ABSTRACT: A cyclic 3′,5′-nucleotide phosphodiesterase from a 30,000*g* supernatant of rat brain cortex showed maximal activity around pH 8 with a K_m of 0.1–0.3 mM. The energy of activation was 7.5 kcal/mole between 25 and 35° and 3.5 kcal/mole between 38 and 45°. Optimal activity was observed at 45°. The enzyme required Mg^{2+} or Mn^{2+} for full activity. In the absence of added divalent cations, it exhibited about one-third of maximal activity. When brains were homogenized in water or isotonic sucrose solution, one-half of the potential enzymic activity was latent, which could be unmasked by the addition of Triton X-100 The enzyme was strongly inhibited by inorganic polyphosphates and nucleoside triphosphates. Citrate and methylxanthines were also inhibitory, but less potent than the nucleoside triphosphates. The inhibition by adenosine triphosphate was pH dependent, but that by inorganic pyrophosphate was not. It is believed that the active form of phosphodiesterase is a metal–enzyme complex and that the mechanism of inhibition by inorganic polyphosphate, nucleoside triphosphates, and citrate is *via* chelating the metal ion in the enzyme.

Adenosine 3′,5′-phosphate (cyclic AMP)[1] exerts its action in a variety of ways at the level of cellular metabolism. As a mediator of catecholamines, it triggers glycogenolysis by activating phosphorylase *b* kinase, which in turn converts phosphorylase *b* to *a* (Posner *et al.*, 1962). It stimulates phosphofructokinase (Mansour and Mansour, 1962), usually the rate-limiting step of glycolysis, thus allowing increased glycolytic flux. On the other hand, glycogen synthesis is probably kept low as cyclic AMP increases the conversion of glycogen synthetase I to D (Rosell-Perez and Larner, 1964). Cyclic AMP also affects pigmentation of the frog skin (Bitensky and Burnstein, 1965), hydroxylation of steroid hormones (Roberts *et al.*, 1964), permeability of the toad bladder (Orloff and Handler, 1962), and the oscillations of NADH in a cell-free extract of *Saccharomyces carlsbergensis* (Cheung, 1966a). Although tissue levels of cyclic AMP are generally low, marked increases have been noted in *Escherichia coli* changing from a glucose medium to one containing no glucose (Makman and Sutherland, 1965), in perfused rat heart following administration of epinephrine (Cheung and Williamson, 1965; Robison *et al.*, 1965), and in the brain cortex after decapitation (Rall and Kakiuchi, 1965). The physiological significance of the cyclic AMP change observed in the brain is not known.

Sutherland and Rall (1958) first described a cyclic 3′,5′-nucleotide phosphodiesterase from beef heart. This enzyme hydrolyzes cyclic AMP into 5′-adenosine monophosphate. Studies on tissue distribution show that it is widely distributed and brain cortex is the richest source (Butcher and Sutherland, 1962). Using a subcellular fractionation technique according to De Robertis *et al.* (1962, 1963), we have established that phosphodiesterase of rat brain is mostly microsomal and that considerable soluble activity is concentrated inside the nerve endings (Cheung and Salganicoff, 1966). We have also found that both adenosine 5′-triphosphate and inorganic pyrophosphate are potent inhibitors of phosphodiesterase, suggesting to us that the enzyme might exist in a greatly inhibited state *in vivo* (Cheung, 1966b). This communication describes further some of the properties of phosphodiesterase from the rat brain.

Materials and Methods

Cyclic 3′,5′-Nucleotide Phosphodiesterase. Brain cortices from young male rats of Wistar strain were homogenized in five volumes of chilled glass-distilled water. The homogenate with about pH 6.8 was centrifuged for 30 min at 30,000*g* and the supernatant fluid

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[1] Abbreviations used: cyclic AMP, adenosine 3′,5′-phosphate; TCA, trichloroacetic acid; GMP, guanosine monophosphate; NADH, reduced nicotinamide–adenine dinucleotide; ATP, CTP, UTP, GTP, TTP, and ITP, adenosine, cytidine, uridine, guanosine, thymidine, and inosine triphosphates, respectively.

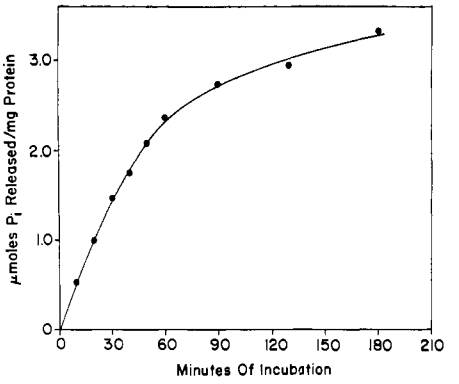

FIGURE 1: Progress curve of cyclic AMP breakdown by cyclic 3′,5′-nucleotide phosphodiesterase. Ten pairs of tubes were used. Each tube contained in 1 ml 40 mM Tris-HCl (pH 7.5), 1.8 mM $MgSO_4$, and 2 mM cyclic AMP. Reaction was initiated by the addition of substrate. At times 0, 10, and 20.....180 min as indicated in Figure 1, each pair was transferred to a boiling water bath and kept there for 3 min. The tubes were cooled to 25° and 0.1 mg of snake venom dissolved in 0.1 ml of 10 mM Tris-HCl (pH 7.5) was added for another 10-min incubation. The reaction was terminated by 0.1 ml of 55% TCA. Denatured protein was removed by centrifugation and inorganic phosphate was assayed in the supernatant by the procedure of Fiske and Subbarow (1925). Protein concentration was 0.16 mg/tube with a specific activity of 1.5 units/mg of protein. Values shown have been corrected for trace hydrolysis of cyclic AMP due to snake venom.

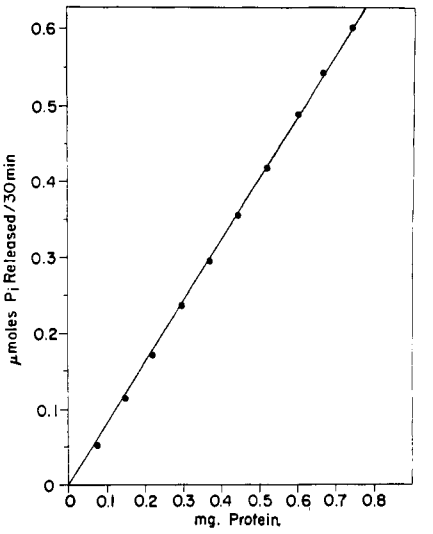

FIGURE 2: Activity of cyclic 3′,5′-nucleotide phosphodiesterase as a function of protein concentration. All tubes were of standard composition except the amount of proteins which varied as shown in Figure 2. P_i released was assayed by the Fiske–Subbarow method. The specific activity of the enzyme was 0.8 unit/mg of protein.

was dialyzed overnight against 200–400 volumes of 20 mM Tris-HCl, pH 7.5, at 4°. Glass-distilled water was used throughout. The dialyzed extract, containing about 10 units/ml,[2] could be used as such or kept at −20° until use. When an extract had been stored at −20° for 1 week or longer, microsomal particles that were present in the dialyzed extract formed flocculents, which could be removed by a low-speed centrifugation to yield a clear supernatant. Removal of the sediment was accompanied by a decrease of enzymic activity, due to loss of enzyme associated with the microsomes. This clear supernatant could then be kept at −20° for at 'east a month with no further reduction of activity upon thawing. Using a combination of calcium phosphate gel adsorption, pH, and ammonium sulfate fractionation, we have achieved purification of phosphodiesterase to no more than 20 units/mg of protein, because of difficulties to be pointed out later.

Enzymic and Chemical Assays. The assay of phosphodiesterase was usually carried out in a reaction mixture of 1 ml containing 40 mM Tris-HCl (pH 7.5), 1.8 mM $MgSO_4$, 2 mM cyclic AMP, crude phosphodiesterase, and other additions as described in the legends. The reaction was initiated by the addition of substrate after thermal equilibration for 3 min. Incubabation was at 25° for 30 min. At 20 min, 0.1 mg of *Crotalus atrox* venom (for 5′-nucleotidase activity) dissolved in 0.1 ml of 10 mM Tris-HCl (pH 7.5) was added and incubated for another 10 min. Since snake venom slowly released inorganic phosphate (P_i) from cyclic AMP, a control without phosphodiesterase was included to correct for such trace hydrolysis. The reaction was terminated by addition of 0.1 ml of 55% trichloroacetic acid (TCA) or heating in a boiling water bath for 3 min. Denatured proteins were removed by low-speed centrifugation and the supernatant fluid was assayed for P_i by the procedure of Fiske and Subbarow (1925). In principle, this procedure is essentially that described by Butcher and Sutherland (1962). In experiments involving Triton X-100, P_i was measured by the method of Lowry and Lopez (1946). For studies using inhibitors, which interfered with either the venom 5′-nucleotidase or the chemical measurement of P_i, the reaction was usually followed by the determination of 5′-adenosine monophosphate. In this case, the incubation was terminated by 0.1 ml of 3 N $HClO_4$ at the end of 30 min and 5′-AMP was determined using the coupled reaction of myokinase, pyruvate kinase, and lactate dehydrogenase as described previously (Cheung, 1966b). Protein was determined by the phenol reagent (Lowry *et al.*, 1951) or by the Buiret reagent in the presence of deoxycholate using bovine serum albumin as standard. All assays were done in duplicate.

[2] One unit of enyzme hydrolyzes 1 μmole of cyclic AMP in 30 min at 25° under standard conditions.

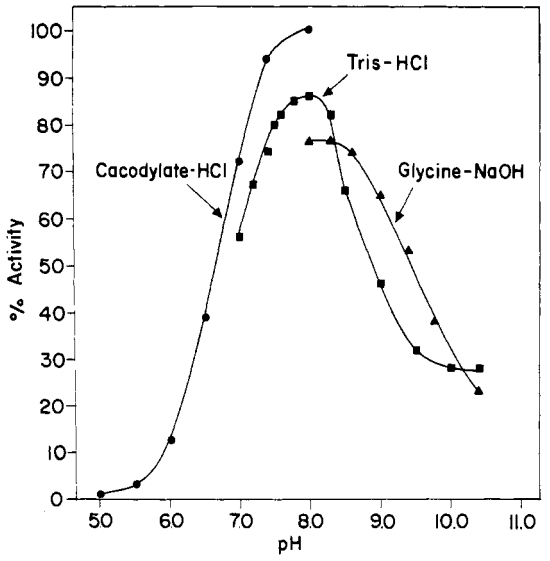

FIGURE 3: Effect of pH and buffer on the activity of cyclic 3′,5′-nucleotide phosphodiesterase. Reaction mixture of 1 ml contained 40 mM buffer, 1.8 mM $MgSO_4$, and 2 mM substrate; cacodylate-HCl (pH 5.0–8.0), 0.4 mg of protein/tube with sp act. 1.3 unit/mg; Tris-HCl (pH 7.0–10.4), 0.25 mg of protein/tube with sp act. 0.7 unit/mg; glycine-NaOH (pH 8.0–10.4), 0.27 mg of protein/tube with sp act. 1.4 unit/mg. P_i was assayed according to Fiske and Subbarow.

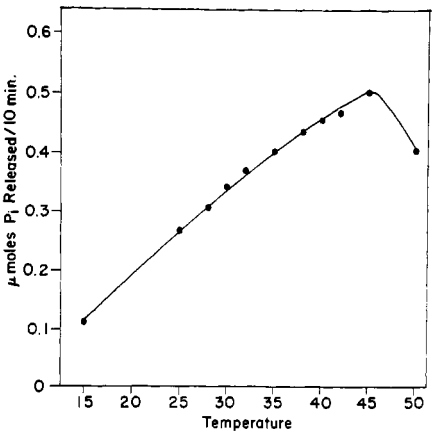

FIGURE 4: Effect of temperature on the activity of cyclic 3′,5′-nucleotide phosphodiesterase. Reaction system of 1 ml contained 40 mM Tris-HCl (pH 7.5), 1.8 mM $MgSO_4$, crude phosphodiesterase (0.16 mg of protein with sp act. 1.5 units/mg), and 0.1 mg of snake venom was equilibrated at each temperature for 2 min before addition of 2 mM cyclic AMP. After a 10-min incubation, the enzymatic reaction was stopped by the addition of 0.1 ml of 55% TCA. P_i was assayed according to Fiske and Subbarow. Proper controls established that snake venom was not a limiting step throughout the temperature range.

Chemicals and Reagents. *C. atrox* venom was obtained from Sigma Chemical Co., St. Louis, Mo.; cyclic AMP and nucleoside triphosphates were purchased from Sigma or Schwartz BioResearch Co., Orangeburg, N. Y. Triton X-100 was a product of Rohm and Haas Co., Philadelphia, Pa. Myokinase and pyruvate kinase were ordered from Boehringer Mannheim Corp., New York, N. Y., and lactate dehydrogenase from Worthington Biochemical Corp., Freehold, N. J. Norit A from Pfanstiehl Laboratories, Inc., Waukegan, Ill., was washed with alkali and acid before use. Uridine 3′,5′-phosphate (triethylammonium salt) and guanosine 3′,5′-phosphate (Ca salt) were gifts from Dr. Th. Pasternak, Geneva, Switzerland; inorganic tripolyphosphate and tetrapolyphosphate were gifts from Dr. John Jose, St. Louis, Mo. Other reagents were of highest commercial grade. All reagents were adjusted to pH 7 with dilute HCl or NaOH.

Results

General Properties. Cyclic 3′,5′-nucleotide phosphodiesterase has a K_m between 0.1 and 0.3 mM at pH 7.5 Tris-HCl buffer. The progress curve of cyclic AMP breakdown by phosphodiesterase in Figure 1 shows that the amount of P_i released maintained a linear rate up to 30 min. Figure 2 shows that the amount of cyclic AMP hydrolyzed was directly proportional to a wide range of enzyme concentration in the incubation system.

Descending paper chromatography with a solvent system of seven volumes of isopropyl alcohol, one volume of concentrated NH_4OH, and two volumes of 0.1 M boric acid (Drummond and Perrott-Yee, 1961)

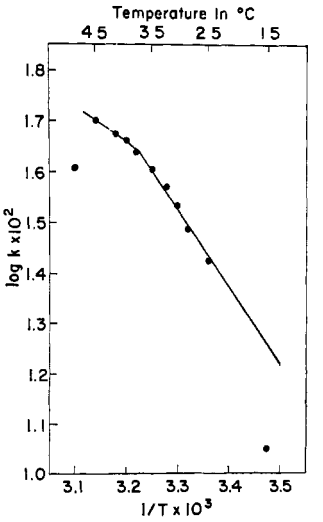

FIGURE 5: Arrhenius plot, energy of activation. Data are taken from Figure 4 and experimental conditions are described therein.

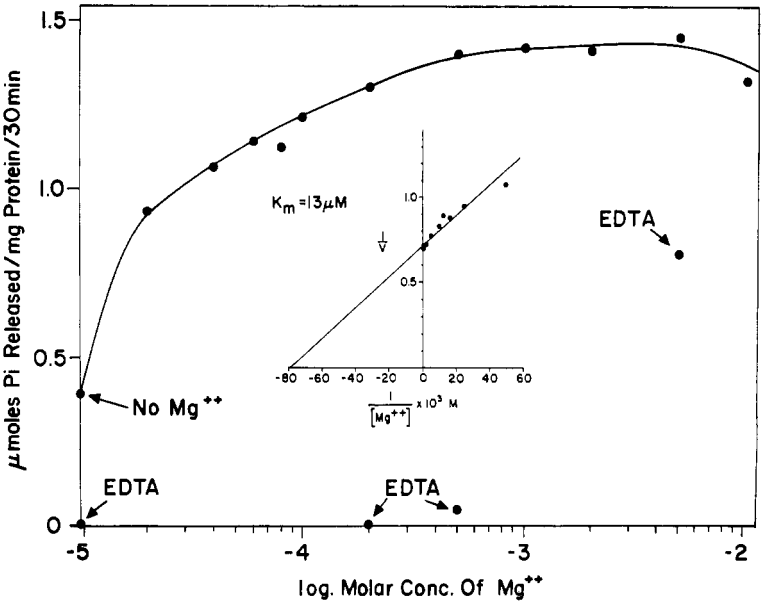

FIGURE 6: Effect of Mg^{2+} on the activity of cyclic 3′,5′-nucleotide phosphodiesterase. Reaction mixture of 1 ml contained 40 mM Tris–HCl (pH 7.5), 2 mM cyclic AMP, and $MgSO_4$ (varied from 0 to 10 mM) as shown in Figure 6. Where indicated, EDTA was 1 mM. K_m for Mg^{2+} was 13 μM as calculated from Lineweaver–Burk plot inserted in Figure 6. Protein concentration 0.26 mg/tube, sp act. 1.4 unit/mg. P_i was measured according to Fiske and Subbarow.

established that the product of hydrolysis was exclusively 5′-AMP. No 3′-adenosine monophosphate could be detected on the paper chromatogram under ultraviolet illumination. This solvent system separates 3′-AMP, 5′-AMP, and cyclic AMP.

Effect of pH and Buffer. Figure 3 shows the activity of phosphodiesterase as a function of pH using three different buffers: cacodylate–HCl, Tris–HCl, and glycine–NaOH. Maximal activity was observed at about pH 8. Between pH 7 and 8, phosphodiesterase was more active in cacodylate–HCl than in Tris–HCl. Studies on a similar enzyme from beef heart by Butcher and Sutherland (1962) indicated that imidazole caused a marked stimulation and shifted the pH maximum from 7.9 to 7.4. We confirmed the stimulation by imidazole, but failed to observe significant changes in pH optimum. Nair (1966) also noted a stimulation by imidazole as well as by NH_4^+ on a cyclic nucleotide phosphodiesterase prepared from dog heart. This enzyme exhibited optimal activity at higher pH, between pH 8.5 and 9.2. In our hands NH_4^+ (as $(NH_4)_2SO_4$) up to 0.1 M gave about 40% stimulation on phosphodiesterase prepared from rat brain.

Temperature Dependence. Phosphodiesterase could be stored at −20° for weeks with no apparent loss of activity. However, exposure of a dilute solution of phosphodiesterase (0.1 mg of protein/ml in pH 7.5 Tris–HCl) to 55° for 5 min in the absence of substrate caused complete loss of activity. The enzyme displayed an optimal temperature at 45° under the conditions described in Figure 4. It is noted that heat inactivation began appreciably at 50°. Figure 5 shows that the Arrhenius plot of data taken from Figure 4 displays a change in slope at 38°. Using the integrated form of the Arrhenius equation we calculated the activation energy to be 7.5 kcal/mole between the temperature range of 25 and 35° and 3.5 kcal/mole between 38 and 45°. The energy of activation as found by Nair (1966) on the dog heart phosphodiesterase was 19 kcal/mole.

Effect of Divalent Ions. Phosphodiesterase required Mg^{2+} or Mn^{2+} to express full activity. In the absence of added divalent cations, the enzyme retained about one-third of its maximal activity (Table I, Figure 6). The residual activity could not be removed by exhaustive dialysis against glass-distilled water or 20 mM Tris–HCl (pH 7.5), but it could be completely abolished when the enzyme was assayed in a reaction system containing 1 mM EDTA and no added divalent cations. Figure 6 shows that 2 mM Mg^{2+} gave full activity and that essentially there was no inhibition up to 10 mM. K_m for Mg^{2+} was 13 μM. Contrary to our finding and that of Nair (1966), Drummond and Perrott-Yee (1961) reported that a cyclic nucleotide phosphodiesterase from rabbit brain was highly inhibited by Mg^{2+} at concentrations above 0.8 mM. The inhibition of phosphodiesterase by EDTA could be reversed by Mg^{2+}. As can be seen in Figure 6, 5 mM Mg^{2+} relieved about 50% of the inhibition caused by 1 mM EDTA. Table I shows that Mn^{2+} was as effective as Mg^{2+} in satisfying the requirement for divalent cations. Co^{2+}, Ni^{2+}, and Ba^{2+} were much less effective and Ca^{2+}, Cu^{2+}, and Zn^{2+} at 2 mM were inhibitory.

Effect of Triton on Unmasking of Latent Activity. Brain tissues homogenized in water or isotonic sucrose solution did not exhibit full potential activity of phosphodiesterase. About one-half of the activity was

TABLE I: Effect of Divalent Cations on the Activity of Cyclic 3′,5′-Nucleotide Phosphodiesterase.[a]

Addn (mM)	% of Control
$MgSO_4$ (2)	100
$MnCl_2$ (2)	104
$CoCl_2$ (2)	50
$NiCl_2$ (2)	46
$BaCl_2$ (2)	36
$CaCl_2$ (2)	12
$CuCl_2$ (2)	6
$ZnSO_4$ (2)	6
None	30
EDTA (1)	0

[a] Reaction mixture of 1 ml contained 40 mM Tris-HCl (pH 7.5), 2 mM cyclic AMP, 2 mM divalent cations or 1 mM EDTA, and 0.17 mg of protein with sp act. 2.0 units/mg. At the end of a 30-min incubation, reaction was stopped by immersing in a boiling water bath for 3 min. Tubes were allowed to cool and 2 mM EDTA and 0.1 mg of snake venom were added, incubated for another 10 min, and stopped by 0.1 ml of 55% trichloracetic acid. EDTA was added to the venom step to relieve inhibition otherwise imposed by Co^{2+}, Cu^{2+}, and Zn^{2+} on the 5′-nucleotidase activity. Parallel controls established that EDTA virtually had no effect on venom activity or on P_i assay by Fiske and Subbarow (1925). Activity in the presence of 2 mM Mg^{2+} was taken as 100%.

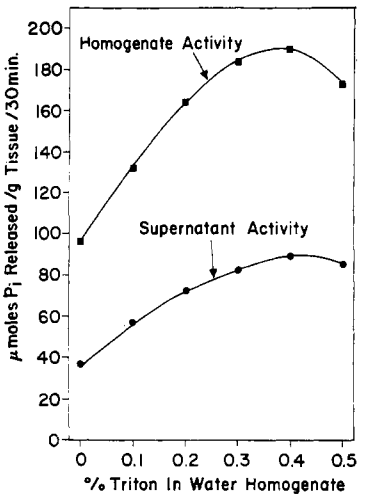

FIGURE 7: Effect of Triton X-100 on unmasking of latent activity on cyclic 3′,5′-nucleotide phosphodiesterase as prepared in water homogenate of rat brain. Each brain cortex was homogenized in five volumes of chilled glass-distilled water containing from 0 to 0.5% Triton (v/v, final concentration). The homogenate was subject to high-speed centrifugation (100,000*g* for 60 min) at 0° to give a clear supernatant. The protein concentration of the homogenate was about 20 mg/ml. Enzyme activity, expressed as units per gram of wet tissue, was assayed both in the homogenate and in the 100,000*g* supernatant. Separate experiments showed that the recovery of phosphodiesterase activity from the 100,000*g* supernatant and sediment was usually better than 90%. The activity associated with the 100,000*g* pellet (not shown in Figure 7) could thus be estimated from the difference between the curve showing homogenate activity and that showing supernatant activity. P_i was measured according to Lowry and Lopez (1946). Each point in the figure represents one separate brain. This experiment was repeated once with results virtually identical with the one described here.

latent, which could be unmasked by adding to the homogenate a nonionic detergent, Triton X-100. Figure 7 represents a typical experiment and shows that 0.4% Triton exposed all latent activity in a water homogenate containing 20 mg of protein/ml. The activity was about 90 units/g of tissue in the absence of Triton X-100 and increased to 190 units in its presence. Triton X-100 also solubilized part of the particulate enzyme into the 100,000*g* supernatant. There were 80 units/g of tissue in the supernatant containing 0.5% Triton and 40 units in the supernatant of the control. However, the distribution of activity between the pellet and the supernatant remained unchanged. About 60% of the total assayable activity was associated with the sediment in samples with or without Triton X-100. Table II shows the effect of Triton X-100 on phosphodiesterase activity associated with different fractions of rat brain. The detergent was added to each fraction and the system was incubated for 30 min at 0° prior to enzymic assay. With the exception of the 100,000*g* supernatant, all the fractions in Table II show an increase of activity. The increase in the microsomes was most pronounced, being more than 300%. The increase in the "nuclei" and mitochondria could be attributed to the expected contamination by microsomes. The fact that the 100,000*g* supernatant (which was devoid of microsomes) did not show any increase of activity suggests that Triton X-100 did not activate the enzyme *per se*. It has been established elsewhere (Cheung and Salganicoff, 1967) that the latent activity in the homogenate as revealed by Triton X-100 was associated with the microsomes (also see Table II) and that the action of the detergent was to expose the enzyme, probably embedded in a lipoprotein matrix, which otherwise would have been inaccessible to the substrate under our assay conditions. Preliminary experiments with pH optimum, caffeine inhibition, Mg^{2+} requirement, and cold stability have not revealed differences between the soluble and the particulate enzyme.

Inhibition by Methylxanthines. Butcher and Sutherland (1962) first noted the inhibition of 3′,5′-nucleotide phosphodiesterase by methylxanthines. They found that theophylline was more potent than theobromine or caffeine, the latter two being almost equal. We have

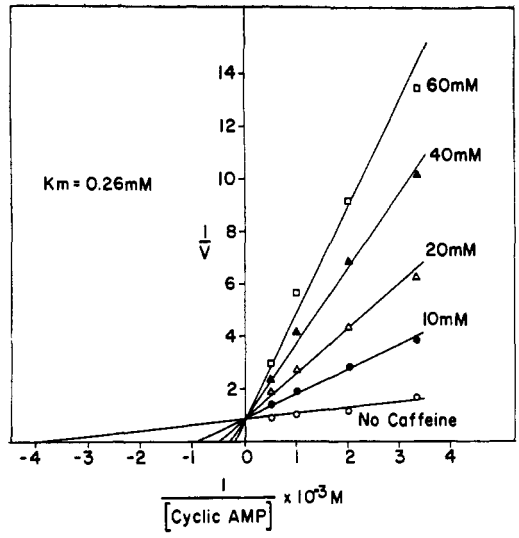

FIGURE 8: Inhibition of cyclic 3′,5′-nucleotide phosphodiesterase by caffeine. Reaction mixture of standard composition containing 0.1 mg of protein (sp act. 2 units/mg) was preincubated for 10 min in the presence of inhibitor. Venom was added as usual at 20 min and the reaction was stopped by TCA at 30 min. Norit A (100 mg) suspended in 1.4 ml of water was added to the mixture to remove caffeine, which otherwise interfered with the P_i measurement by Fiske and Subbarow. After 20 min at room temperature, Norit A was removed by centrifugation and the supernatant, now free of caffeine, was determined for P_i.

TABLE II: Effect of Triton X-100 on Phosphodiesterase Activity in Fractions Obtained from Isotonic Sucrose Homogenates of Rat Brain.[a]

Fraction	Activity (units/ml)	
	Control	Triton X-100
Homogenate	8.8	14.1 (160)
"Nuclei" (900*g* for 10 min)	2.2	3.6 (160)
Mitochondria (11,500*g* for 20 min)	11.8	19.2 (160)
Microsomes (100,000*g* for 30 min)	11.6	37.4 (320)
Supernatant (100,000*g* for 30 min)	2.1	2.1 (100)

[a] Rat brains were homogenized in chilled isotonic sucrose solution and fractions were prepared by differential centrifugation in the usual manner. They were diluted with isotonic sucrose solution to a protein concentration of about 5 mg/ml. Triton X-100 was added to a final concentration of 0.2% (v/v) and incubated at 0° for 30 min. The control received no Triton X-100. P_i was measured according to Lowry and Lopez (1946) with slight modifications (see Cheung and Salganicoff, 1967). Figures in parentheses indicate per cent over the corresponding controls.

used both theophylline and caffeine and found that their potency was comparable. As shown in Figure 8, caffeine competitively inhibits phosphodiesterase. The K_m was 0.3 mM in the absence of inhibitor and was increased to 2 mM at 20 mM inhibitor. Caffeine at 20 mM caused a 50% inhibition at 2 mM substrate, and is thus much less potent than the nucleoside triphosphates listed in Table III. The K_i for caffeine as calculated from Figure 8 was 3 mM as compared with 50 mM for phosphodiesterase from dog heart (Nair, 1966). The inhibition of the latter enzyme by caffeine was found to be noncompetitive.

TABLE III: Inhibition of Cyclic 3′,5′-Nucleotide Phosphodiesterase by Nucleoside Triphosphates.[a]

Addn (3 mM)	% Inhibn
ATP	75
GTP	42
UPT	61
TTP	41
ITP	57
CTP	62

[a] Reaction mixture of 1 ml contained 40 mM Tris-HCl (pH 7.5), 1.8 mM $MgSO_4$, 2 mM cyclic AMP, and 3 mM nucleoside triphosphates. Protein concentration was 0.2 mg of protein/tube with sp act. 1.7 units/mg. With the exception of the control, which contained no inhibitor, all was preincubated in the presence of inhibitors for 10 min and the reaction was then started with substrate. At 30 min, 0.1 ml of 3 N $HClO_4$ was added to terminate the reaction and 5′-AMP was measured as described under Materials and Methods.

Inhibition by Citrate. Figure 9 shows the inhibition of phosphodiesterase by citrate. The curve tends to be sigmoid, similar to the inhibition caused by inorganic pyrophosphate reported earlier (Cheung, 1966b). Inhibition (50%) was obtained at 12 mM citrate, whose potency thus falls between the nucleoside triphosphates and the methylxanthines. The nature of citrate inhibition seemed complex as Lineweaver–Burk plots did not give straight lines. Other organic acids tested at 10 mM included isocitrate (10% inhibition), pyruvate (10%), α-ketoglutarate, and β-hydroxybutyrate (both 0); and at 20 mM included oxalate (18%), 1-malate (9%), tartrate (6%), malonate, succinate, and fumarate (all 0). Also there was little or no inhibition with the following compounds at 10 mM: histamine, acetylcholine, thiamine pyrophosphate, nicotinamide–adenine dinucleotide, and nicotinamide–adenine dinucleotide phosphate.

Inhibition by Nucleotides. As described previously (Cheung, 1966b), 3′,5′-nucleotide phosphodiesterase was inhibited by adenosine triphosphate and inorganic

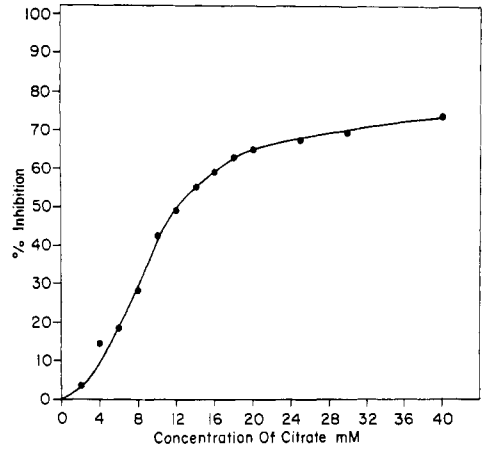

FIGURE 9: Inhibition of cyclic 3′,5′-nucleotide phosphodiesterase by citrate. Standard composition plus various concentrations of citrate and protein (0.2 mg/tube, sp act. 1.1 units/mg of protein). After a 10-min preincubation, the reaction was started with substrate and incubated for 30 min. The reaction was terminated by the addition of 0.1 ml of 3 N $HClO_4$. 5′-AMP was assayed in the supernatant as described under Materials and Methods. Proper controls established that the concentrations of citrate used did not interfere with the 5′-AMP assays.

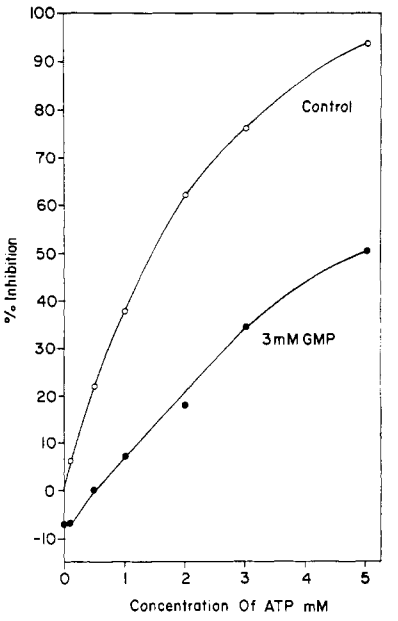

FIGURE 10: Relief of ATP inhibition by GMP. Reaction mixture of 0.5 ml containing 40 mM Tris-HCl (pH 7.5), 1.8 mM $MgSO_4$, 2 mM cyclic AMP, protein (0.1 mg, with sp act. 2 units/mg), 3 mM 5′-GMP, and various concentrations of ATP as shown in figure. Control tubes received no 5′-GMP. Preincubation was for 10 min. At 30 min, reaction was terminated by 0.05 ml of 3 N $HClO_4$ and the pH was brought back to neutrality with solid $KHCO_3$. An aliquot of the supernatant fluid was assayed for 5′-AMP as described in Table II.

pyrophosphate with 50% inhibition at about 1 and 4 mM, respectively. Competition by both compounds was of a mixed type. Table III shows that other nucleoside triphosphates were equally potent inhibitors. At 3 mM, ATP, CTP, UTP, and ITP all caused more than 50% inhibition while GTP and TTP gave about 40% inhibition. Not shown is the inhibition by inorganic tripolyphosphate and tetrapolyphosphate which at 4 mM gave inhibition better than 90%. Of considerable interest was the effect of 5′-guanosine monophosphate which at 3 mM protected quite effectively the enzyme from ATP inhibition. Figure 10 shows the concentrations of ATP to give 50% inhibition with or without 5′-GMP are 1.4 and 4 mM, respectively. Under similar conditions, 5′-GMP was rather ineffective in relieving inhibition by PP_i. It should be added that 3 mM 5′-GMP alone might slightly increase or decrease phosphodiesterase activity, seemingly dependent on the enzyme preparation. In Figure 10, 3 mM 5′-GMP in the absence of ATP gave slight stimulation over the control.

Effect of pH on ATP Inhibition. Studies on the inhibition of phosphodiesterase by ATP at different pH in the presence of identical concentrations of substrate showed that the inhibition was highly dependent on pH. While the extent of inhibition was not easily reproduced, inhibition at pH above neutrality was generally less severe. As shown in Figure 11, 1 mM ATP at pH 7.0 caused 60% inhibition while at pH 10.4 it gave 20%. Unlike the inhibition by ATP, that by PP_i was essentially unaffected by pH. In the pres-

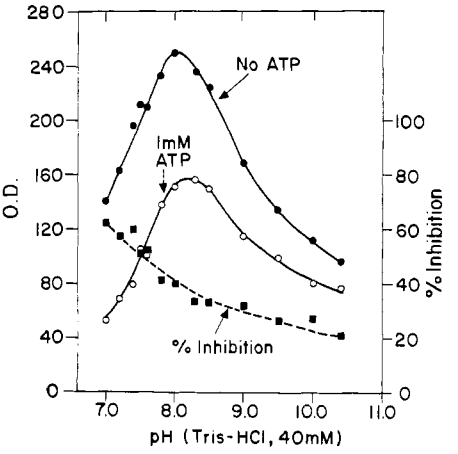

FIGURE 11: Effect of pH on the ATP inhibition of cyclic 3′,5′-nucleotide phosphodiesterase. Reaction mixture of 0.5 ml containing 1.8 mM $MgSO_4$, 2 mM cyclic AMP, 40 mM Tris-HCl, ranging from pH 7.0 to 10.4, protein (0.1 mg with sp act. 2 units/mg), and 1 mM ATP. Control tubes received no ATP. Preincubation was for 10 min. At 30 min, reaction was terminated by addition of $HClO_4$ and 5′-AMP was assayed as in Table II. The extent of inhibition at each pH was calculated with activity in 1 mM ATP expressed as per cent of the corresponding control.

TABLE IV: Comparison of Cyclic 3′,5′-Nucleotide Phosphodiesterase from Various Tissues.

Ref	Source of Enzyme	Localization	Sp Act.[a]	Optimal pH	K_m (M)
b	Beef heart	Mostly particulate	26 (30)	7.9	6×10^{-5}–1×10^{-4}
c	Rabbit brain	100,000*g* supernatant	1.2 (25)	7.5 or above	
d	Dog heart	Mostly in 100,000*g* supernatant	28 (37)	8.5–9.2	4.9×10^{-4}
e	Rat brain	Mostly microsomal[f]	2 (25)	8	1–3×10^{-4}

[a] Micromoles of cyclic AMP hydrolyzed in 30 min per milligram of protein at the temperature (in degrees centrigrade) indicated. [b] Butcher and Sutherland (1962). [c] Drummond and Perrott-Yee (1961); specific activity estimated from the original paper. [d] Nair (1966). [e] This study. [f] See Cheung and Salganicoff (1967).

ence of 5 mM PP_i, the inhibition was about 70% whether at pH 7.0 or 10.4.

Discussion

The inhibition of phosphodiesterase from the rat brain by a variety of compounds, namely all nucleoside triphosphates, inorganic polyphosphates, citrate, and methylxanthines, suggests that it is highly susceptible to regulation. With the exception of the methylxanthines, all these compounds are known to be metal-chelating agents. Preliminary experiments indicate that the active form of phosphodiesterase might be a metal–enzyme complex. As shown in Figure 6 and Table I, the enzyme required added divalent cations, preferably Mg^{2+} and Mn^{2+}, to express full activity. The inhibition by nucleoside triphosphates, inorganic polyphosphates, and citrate could be due to formation of Mg^{2+} complexes with the chelating reagents, thereby reducing Mg^{2+} ions available to the enzyme. The complete suppression of residual activity in the presence of 1 mM EDTA is presumably due to complete chelation of the metal ion in the enzyme molecule.

Of the organic acids studied, citrate proved to be the most effective inhibitor. Isocitrate, malate, oxalate, pyruvate, and tartrate were only mildly inhibitory and α-ketoglutarate, fumarate, succinate, and malonate were without effect, suggesting that the enzyme is not indiscriminate toward the chelating agents. The protection of 5′-GMP against ATP inhibition might mean that phosphodiesterase has affinity for both guanosine and adenosine moieties. Guanosine competes successfully with adenosine for an allosteric site adjacent to the active site, thus reducing the chances for the polyphosphate chain of ATP to chelate with the metal ion of the enzyme. In the case of PP_i inhibition, even though 5′-GMP competes for the same site on the enzyme molecule, the metal component might still be unobstructed toward PP_i, leaving it fully susceptible to chelation. This mechanism would be compatible with the mixed-type inhibition noted previously (Cheung, 1966b). It is not fully understood why the inhibition by ATP was dependent on pH and that by PP_i was not. One possibility would be that the change in pH alters the tertiary structure of the enzyme molecule in such a way that it either limits the access of the allosteric site to the adenosine moiety of ATP or it decreases the affinity of that site for adenosine. The implicit assumption is that the geometry of the active site itself is relatively unaffected by such a change in pH.

The enzyme as studied under our assayed conditions also hydrolyzed guanosine 3′,5′-phosphate (cyclic GMP) and uridine 3′,5′-phosphate (cyclic UMP) at 70 and 10%, respectively, of the rate of cyclic AMP. According to Drummond and Perrott-Yee (1961), the phosphodiesterase from rabbit brain hydrolyzed cyclic GMP at only one-third the rate of cyclic AMP. It is possible that the relative high rate of the hydrolysis of cyclic GMP by our preparation is due to a separate enzyme. Hardman and Sutherland (1966) recently described a cyclic 3′,5′-nucleotide phosphodiesterase with specificity for cyclic UMP. Two other points are of interest in comparing the enzyme from the rabbit brain and the rat brain. The rabbit enzyme is inhibited by Mg^{2+} ions above 0.8 mM while the enzyme from the rat requires 2 mM Mg^{2+} for maximal activity. The former is localized entirely in a 100,000*g* supernatant while the latter is mostly microsomal (see Table IV).

In Table IV, some of the salient features of cyclic 3′,5′-nucleotide phosphodiesterase from different sources are presented for comparison. It is readily seen that no satisfactory purification has been achieved on any of these enzymes. One of the difficulties involved in the purification of phosphodiesterase is the low enzyme level in the tissues. Although the cortex is a rich source, it has a high lipid content and the majority of phosphodiesterase is firmly associated with the microsomes. The finding that phosphodiesterase of rabbit brain is completely soluble in the 100,000*g* supernatant is thus of interest.

Because of the strong inhibition by ATP and PP_i at concentrations approximating physiological levels, we have been led to believe that phosphodiesterase might exist *in vivo* in a greatly inhibited state (Cheung, 1966b). The present finding that the enzyme is inhibited

by all the nucleoside triphosphates, inorganic polyphosphates, and citrate strengthens our belief. Furthermore, if phosphodiesterase is studied in the absence of added Mg^{2+}, it is even more potently inhibited by ATP or citrate. Under this condition, half-maximal inhibition is given by ATP at 80 μM and by citrate at 300 μM (W. Y. Cheung, unpublished data).

From the work of Butcher and Sutherland (1962) and Sutherland *et al.* (1962), it could be noted that phosphodiesterase is present in much greater excess over adenyl cyclase. Since cyclic AMP is involved in many aspects of cellular metabolism, its tissue level could have far-reaching repercussions. The availability of a potential excess of phosphodiesterase might be a warranty against prolonged elevated levels of the cyclic nucleotide after its influence(s) has been felt. The susceptibility of phosphodiesterase to inhibition by physiological compounds such as nucleoside triphosphates, inorganic pyrophosphate, and citrate might prove to be a useful *in vivo* control mechanism, although the exact nature of this has yet to be elucidated.

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References

Bitensky, M. W., and Burnstein, S. R. (1965), *Nature 208*, 1283.

Butcher, R. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237*, 1244.

Cheung, W. Y. (1966a), *Biochim. Biophys. Acta 111*, 235.

Cheung, W. Y. (1966b), *Biochem. Biophys. Res. Commun. 23*, 214.

Cheung, W. Y., and Salganicoff, L. (1966), *Federation Proc. 25*, 714.

Cheung, W. Y., and Salganicoff, L. (1967), *Nature* (in press).

Cheung, W. Y., and Williamson, J. R. (1965), *Nature 207*, 979.

De Robertis, E., Pellegrino de Iraldi, A., Rodriguez de Lores Arnaiz, G., and Salganicoff, L. (1962), *J. Neurochem. 9*, 23.

De Robertis, E., Salganicoff, L., Zieher, L. M., Rodriguez de Lores Arnaiz, G. (1963), *J. Neurochem. 10*, 255.

Drummond, G. I., and Perrott-Yee, S. (1961), *J. Biol. Chem. 236*, 1126.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Hardman, J. G., and Sutherland, E. W. (1966), *J. Biol. Chem. 240*, PC3704.

Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Makman, R. S., and Sutherland, E. W. (1965), *J. Biol Chem. 240*, 1309.

Mansour, T. E., and Mansour, J. M. (1962), *J. Biol. Chem. 237*, 629.

Nair, K. G. (1966), *Biochemistry 5*, 150.

Orloff, J., and Handler, J. S. (1962), *J. Clin. Invest. 41*, 702.

Posner, J. B., Stern, R., and Krebs, E. G. (1962), *Biochem. Biophys. Res. Commun. 9*, 293.

Rall, T. W., and Kakiuchi, S. (1965), Abstracts, 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept, p 68C.

Roberts, S., Creange, J. E., and Fowler, D. D. (1964), *Nature 203*, 759.

Robison, G. A., Butcher, R. W., Øye, I., Morgan, H. E., and Sutherland, E. W. (1965), *Mol. Pharmacol. 1*, 168.

Rosell-Perez, M., and Larnar, J. (1964), *Biochemistry 3*, 81.

Sutherland, E. W., and Rall, T. W. (1958), *J. Biol. Chem. 232*, 1077.

Sutherland, E. W., Rall, T. W., and Menon, T. (1962), *J. Biol. Chem. 237*, 1220.