Purification and Properties of 3′,5′-Cyclic Nucleotide Phosphodiesterase from Dog Heart*

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ABSTRACT: The procedure for the isolation and purification of 3′,5′-cyclic nucleotide phosphodiesterase from dog heart involves ammonium sulfate and ethanol fractionations of the homogenate followed by DEAE-cellulose chromatography. Using these methods, a 173-fold purification was obtained.

Nucleoside 3′,5′-cyclic phosphates were synthesized chemically (including 3′,5′-cyclic deoxyadenosine monophosphate) and the substrate specificity of the enzyme was investigated. A preference for nucleoside 3′,5′-cyclic phosphates with purine bases was observed. The data referring to K_m and V_{max} and enzyme inhibition studies with caffeine are presented.

In 1957 Sutherland and Rall discovered that the stimulation of phosphorylase activity in liver and heart muscle homogenates by epinephrine was mediated by a heat-stable factor. This factor has now been established to be adenosine 3′,5′-phosphoric acid (Lipkin *et al.*, 1959). There are several reports on the role of adenosine 3′,5′-cyclic phosphoric acid (3′,5′-cyclic AMP)[1] as an intermediate factor in the action of certain hormones such as epinephrine, glucagon (Sutherland and Rall, 1957, 1960; Rall *et al.*, 1957), and vasopressin (Orloff and Handler, 1961, 1962).

Adenosine 3′,5′-cyclic phosphoric acid appears to be synthesized by an enzyme found in the particulate fraction of tissue homogenates sedimented at 600–1000*g*. Conversely, 3′,5′-cyclic AMP is broken down by a phosphodiesterase which is widely distributed in animal tissues. This enzyme has been partially purified by Sutherland and Rall (1958), Butcher and Sutherland (1962), Drummond and Perrott-Yee (1961), and Nair (1962). This report deals with the purification and properties of 3′,5′-cyclic nucleotide phosphodiesterase isolated from dog ventricular myocardium.

Experimental Procedure

Materials. Adenosine 3′,5′-cyclic phosphoric acid was obtained from Sigma Laboratories, St. Louis, Mo. 3′,5′-Cyclic AMP and other 3′,5′-cyclic purine and pyrimidine nucleotides were also synthesized by the method of Smith *et al.* (1961) (see under Chemical Methods). *Crotalus adamanteus* venom was purchased from Ross Allen's Reptile Institute, Silver Springs, Fla. DEAE-cellulose was obtained from Schleicher & Schuell, Keene, N. H. Selectacel brand standard DEAE-cellulose was washed thoroughly with 1 N NaOH until the yellowish color disappeared. The cellulose was then washed with water until the pH of the effluent was 7.0. Conversion to the carbonate form was achieved by treating the cellulose with ten column volumes of 2 M $(NH_4)_2CO_3$. The column was finally washed well with distilled ion-free water. Each column was 25 × 3 cm in size and packed under 5–6 psi of nitrogen. Enzyme grade ammonium sulfate was obtained from Mann Biochemical Corp.

Chemical Methods

Nucleoside 3′,5′-cyclic phosphates were synthesized by the method of Smith *et al.* (1961). The following procedures were found to be especially helpful. Reagent grade pyridine was maintained in an anhydrous state by drying it over calcium hydride mesh 4–40 for several days. Redistilled benzoyl chloride was used in the preparation of the tetrabenzoyl compounds of cytidine 5′-monophosphate (CMP) and guanosine 5′-monophosphate (GMP). Nucleotides and their derivatives were separated by ion-exchange chromatography using DEAE carbonate. Higher yields of nucleoside 3′,5′-cyclic phosphates were invariably obtained by using dicyclohexylcarbodiimide (DCC) in fivefold excess of the stoichiometric amounts required for the formation of the DCC phosphomonoester complex.

Using the general procedures outlined above nucleoside 3′,5′-cyclic phosphates were obtained in the following yield: adenosine 3′,5′-cyclic phosphoric acid (60%); deoxyadenosine 3′,5′-cyclic phosphoric acid (56%); uridine 3′,5′-cyclic phosphoric acid (3′,5′-cyclic UMP) (40%); cytidine 3′,5′-cyclic phosphoric

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[1] Abbreviations used: AMP, CMP, GMP, UMP, and IMP, adenosine, cytidine, guanosine, uridine, and inosine monophosphates or phosphoric acids; DCC, dicyclohexylcarbodiimide; TCA, trichloroacetic acid; dAMP, deoxy-AMP.

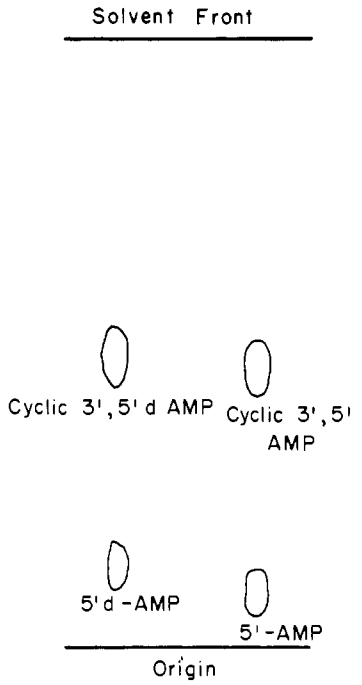

FIGURE 1: Descending paper chromatogram of 5'-deoxy-AMP and 3',5'-cyclic deoxy-AMP compared with 5'-AMP and 3',5'-cyclic AMP in solvent system I.

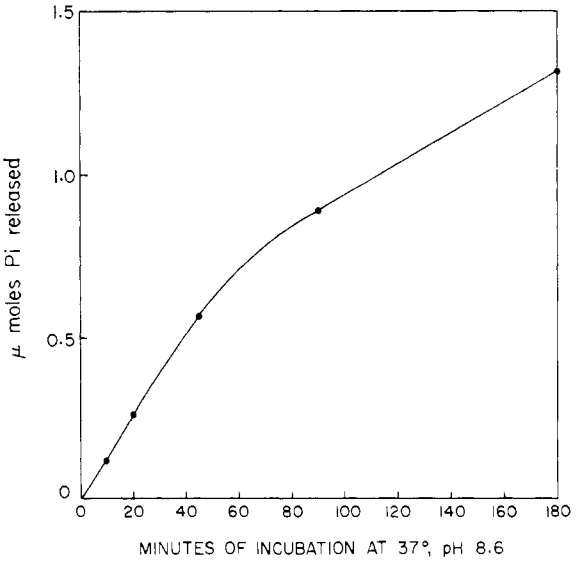

FIGURE 2: Progress curve of enzymatic breakdown of 3',5'-cyclic AMP by cyclic nucleotide phosphodiesterase.

acid (3',5'-cyclic CMP) (20%); guanosine 3',5'-cyclic phosphoric acid (3',5'-cyclic GMP) (20%); inosine 3',5' cyclic phosphoric acid (3',5'-cyclic IMP) (80%).

Paper Chromatographic Procedures. Descending paper chromatography was utilized to identify the nucleotides that were synthesized. Whatman 3MM paper was employed and the compounds were detected by viewing the spots under an ultraviolet lamp. The following solvent systems were used: solvent I, isopropyl alcohol–concentrated ammonia–water (7:1:2 v/v); solvent II, *n*-butyl alcohol–glacial acetic acid–water (5:2:3 v/v); solvent III, isobutyric acid–1 M concentrated ammonia–0.1 M EDTA (100:60:1.6 v/v); solvent IV, ethanol–1 M ammonium acetate, pH 7.5 (5:2 v/v).

The new compound 3',5'-cyclic deoxy-AMP, hitherto unreported, had the same spectrum as 3',5'-cyclic AMP in neutral, acid, and alkaline pH ranges; however, when examined by paper chromatography using solvent system I the following R_F values were obtained: 5'-AMP, 0.09; 3',5'-cyclic AMP, 0.47; 5'-dAMP, 0.14; 3',5'-cyclic dAMP, 0.50 (Figure 1).

Standard Assays. PRINCIPLE. 3',5'-Cyclic nucleotide diesterase hydrolyzes 3',5'-cyclic AMP completely to 5'-AMP. Since both compounds have the same spectral characteristics, breakdown to 5'-AMP cannot be followed spectrophotometrically. The 5'-AMP produced as a result of the action of the diesterase may be treated with 5'-nucleotidase obtained from snake venom and the released inorganic phosphate (P_i) was measured colorimetrically. In a 20-min incubation period the hydrolysis of 3',5'-cyclic AMP by the phosphodiesterase present in snake venom is negligible.

PROCEDURE. The assay mixture consisted of the following: 25 μmoles of Tris–HCl buffer pH 8.6, 2.5 μmoles of $MgCl_2$, 10 μmoles of $(NH_4)_2SO_4$, 0.050 ml of *Crotalus adamanteus* venom 5 mg/ml, 0.75 μmole of 3',5'-cyclic nucleotide (neutralized), 3',5'-cyclic nucleotide diesterase, and H_2O in a final volume of 0.350 ml. Controls contained boiled 3',5'-cyclic nucleotide diesterase added at zero time. Blanks were also run without the substrate. The mixture was incubated at 37° for 20 min. The reaction was stopped by boiling the reaction mixture for 2 min. Then 0.05 ml of 10% trichloroacetic acid (TCA) was added to ensure complete precipitation of proteins. The reaction tubes were then centrifuged and the supernatant fraction was used for estimation of P_i release.

In certain studies this assay was performed in two stages. These consisted of a preliminary incubation of the cyclic nucleotide with the dog heart diesterase for 20 min; the enzyme was then inactivated by boiling the mixture for 2 min and a second incubation was carried out in the presence of snake venom for 20 min at 37°. The two-stage assay was used to study the effect of heat, pH, and ions on the diesterase. A typical progress curve of the one-stage assay method is shown in Figure 2. Enzyme activity measured by this method is a linear function of protein concentration (Figure 3).

Spectrophotometric Determination of P_i Release. An adaptation of the Fiske and Subbarow method (1925) was used. To a 0.2-ml aliquot of the TCA supernatant were added 0.1 ml of a fresh solution of 1-amino-2-naphthol-4-sulfonic acid (Eiconogen), 0.1 ml of ammonium molybdate, and 0.6 ml of H_2O. Eiconogen was prepared according to the directions of Fiske and

TABLE I: Summary of Purification of 3′,5′-Cyclic Nucleotide Diesterase.[a]

No.	Sample	Specific Activity	Total Activity	Purification	Yield (%)
1	4000*g* supernatant	0.45	4959		
2a	0.37–0.62 $(NH_4)_2SO_4$ fraction	2.05	3054	4.5	61.6
2b	Supernatant after freezing and thawing	3.50	1470	7.8	29.6
3	0.17–0.38 ethanol fraction	5.55	1035	12.3	20.9
4	0.40–0.60 $(NH_4)_2SO_4$ fraction	6.66	725	14.8	14.6
5	DEAE carbonate chromatography	19.00	400	42.2[b]	8.0

[a] The total activities of the 4000*g* supernatant (starting point) and various fractions are shown. The crude homogenate was made from 110 g of dog ventricular myocardium. The specific activity of the homogenate was 0.11. Definition of activity, μmoles of P_i/20 min. [b] If the purification is considered from the homogenate step, 173-fold.

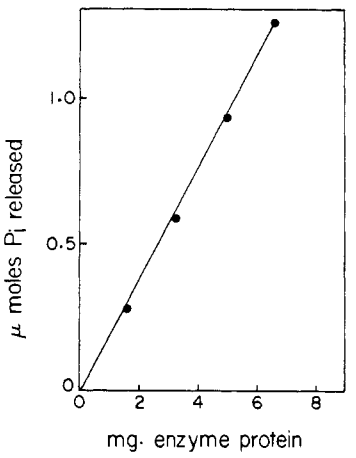

FIGURE 3: Enzyme activity as a linear function of protein concentration. The assay system contained in a final volume of 1 ml: 50 μmoles of Tris–HCl buffer pH 8.6, 5 μmoles of $MgCl_2$, 2.25 μmoles of 3′,5′-cyclic AMP (sodium salt), 0.1 ml of *Crotalus adamanteus* venom (5 mg/ml), varying amounts of crude enzyme (4000*g* supernatant) as shown above, and water. The one-stage assay was used. Control tubes consisted of the complete system minus the snake venom.

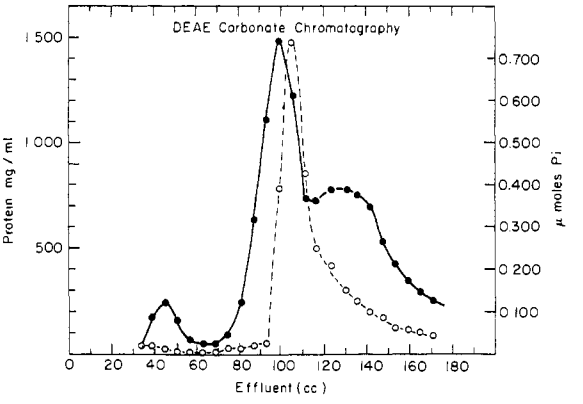

FIGURE 4: DEAE-cellulose chromatography of 3′,5′-cyclic nucleotide phosphodiesterase. An exponential gradient elution system is used with a 125-ml mixing chamber containing 0.05 M Tris–HCl buffer, pH 7.5, and an upper reservoir containing 0.45 M $(NH_4)_2CO_3$ in 0.05 M Tris–HCl buffer, pH 8.6. ●—●—●, protein (mg/ml); O—O—O, μmoles of P_i.

Subbarow and diluted to a 40% solution in water. The blue color was allowed to develop for 10 min and the optical density of the sample and blank were recorded spectrophotometrically at 660 mμ. A standard curve was run with each assay using a phosphate solution containing 1% TCA.

The specific activity of the enzyme is defined as the μmoles of inorganic phosphate released per mg of enzyme protein in a 20-min incubation period. Enzyme protein was estimated by the TCA turbidity method (Stadtman *et al.*, 1951) and from 280/260 mμ ratios (Warburg and Christian, 1942).

Purification of 3′,5′-Cyclic Nucleotide Phosphodiesterase

Dog ventricular myocardium was utilized as a source for the enzyme, which is very active in this tissue. Mature dogs were bled rapidly by cannulation of the femoral artery. Immediately after total exsanguination, the heart was removed and placed in ice. For the sake of convenience the hearts were frozen at −18° and used at varying intervals; they were rewarmed to 0° by gentle thawing, and all subsequent procedures were done at 4°.

Homogenization. The ventricular myocardium was carefully dissected; all fibrous tissue, heart valves, and clotted blood were removed. The myocardial tissue (100–120 g) was minced and homogenized in a Waring Blendor (3 min top speed) using five volumes of 0.25 M sucrose solution containing 0.001 M $MgCl_2$. The crude homogenate was filtered through cheesecloth. Syste-

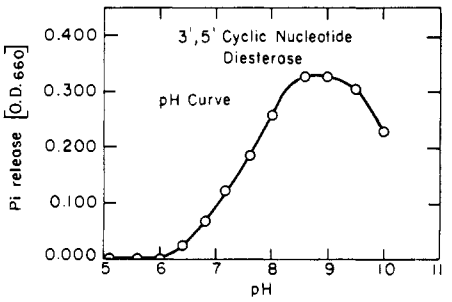

FIGURE 5: pH curve of 3',5'-cyclic nucleotide diesterase.

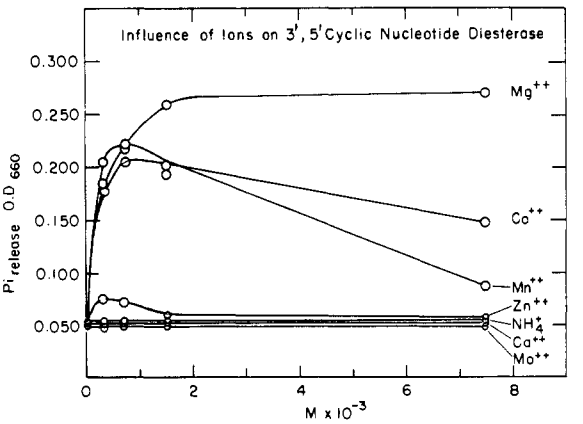

FIGURE 6: Influence of ions on 3',5'-cyclic nucleotide phosphodiesterase activity.

matic fractionation of the homogenate by the method of Hogeboom (1955) showed that the diesterase activity was present in the supernatant fluid (see Table I).

First Ammonium Sulfate Fractionation. The homogenate was centrifuged for 10 min at 4000*g*. The supernatant fluid may be stored in plastic bottles at −20° for several months without significant loss of activity. The 4000*g* supernatant fluid was fractionated with enzyme grade $(NH_4)_2SO_4$. To each 100 ml, 20.7 g of $(NH_4)SO_4$ was added to obtain the initial 0–0.37 fraction. The precipitate was discarded and 15.0 g of $(NH_4)_2SO_4$ was added/100 ml of supernatant fluid. The 0.37–0.62 fraction thus obtained was collected by centrifugation and suspended in 0.05 M Tris–HCl buffer, pH 7.5. The protein concentration of this fraction was maintained between 15 and 20 mg/ml. It was then dialyzed against 20 volumes of Tris–HCl buffer, pH 7.5, at 6° for 15 hr. Any precipitate which formed after dialysis was discarded.

Ethanol Fractionation. The dialyzed 0.37–0.62 $(NH_4)_2SO_4$ fraction was stored at −20° for 24 hr. After thawing, the precipitate which formed was removed by centrifugation and the supernatant fluid, adjusted to a protein concentration of 10–15 mg/ml, was subjected to ethanol fractionation. Aliquots not larger than 150 ml were used because of difficulty in maintaining proper temperature control with larger volumes. Ethanol was added to a concentration of 0.17, the temperature being maintained at −4°. The precipitate was discarded and ethanol was added to 0.42 concentration, temperature being gradually lowered to −15°. The 0.17–0.42 fraction was collected by centrifugation at −15° and dissolved in 0.05 M Tris–HCl buffer, pH 7.5, the protein concentration being adjusted to 15–20 mg/ml. It was stored at −20° for 24 hr. On thawing, a small amount of precipitate usually formed. The precipitate tended to absorb 10–15% of the total activity; recovery of active enzyme absorbed on the precipitate could be achieved by elution with two volumes of 1 M $(NH_4)_2SO_4$. The eluate was then added to the 0.40–0.60 $(NH_4)_2SO_4$ fraction obtained in the next step.

Second Ammonium Sulfate Fractionation. To each 100 ml of the 0.17–0.42 ethanol fraction 22.6 g of $(NH_4)_2SO_4$ was added. The precipitate (0–0.40) collected by centrifugation was discarded and 12.0 g of $(NH_4)SO_4$/100-ml volume was added to the supernatant fluid. The 0.40–0.60 fraction so obtained was suspended in 0.05 M Tris–HCl buffer, pH 7.5, the protein concentration being kept at 20–30 mg/ml. It was then dialyzed against 20 volumes of Tris–HCl buffer, pH 7.5, for 15 hr.

DEAE-Cellulose Chromatography. The dialyzed enzyme was absorbed on a DEAE carbonate column which was previously equilibrated with ten volumes of 0.05 M Tris–HCl, pH 7.5. A column 25 × 1.5 cm is suitable for 200–250 mg of protein. Gradient elution was carried out using a 125-ml mixing chamber containing 0.05 M Tris–HCl buffer, pH 7.5, and an upper reservoir containing 0.45 M $(NH_4)_2CO_3$ in 0.05 M Tris–HCl buffer, pH 8.6. The flow rate was 0.2–0.3 ml/min and 3-ml fractions were collected. The optical density of the fractions was followed at 280 and 260 mμ.

A small initial protein peak is obtained between fractions 10 and 20. A sharp second peak follows within the next 20 tubes; diesterase activity is associated with its descending limb (Figure 4), generally between fractions 30–40. A third protein peak containing no diesterase activity may be present.

The fractions with the most activity were combined and concentrated by osmotic means. The pooled enzyme was placed inside a Visking cellophane casing, and the bag was immersed in 20 volumes of 60% sucrose solution. The bag was observed to shrink rapidly, the volume being reduced to one-fourth the original in 4–5 hr. Attempts to concentrate the enzyme by lyophilization or by use of other osmotically active substances such as Carbowax or Dextran are inferior to this simple technique, inasmuch as the former causes loss of activity and the latter produces interference with the assay for inorganic phosphate by the Fiske and Subbarow method.

The enzyme solution thus concentrated by sucrose has a protein concentration of 1–1.5 mg/ml. Fifty grams of $(NH_4)_2SO_4$ was added/100 ml. This solution and the mixture were stirred overnight. The precipitated protein was collected by centrifugation and dissolved in 0.05 M Tris–HCl, pH 7.5, to a concentration of 15–20 mg/ml. The enzyme was then dialyzed against the same buffer for 15 hr at 6°, after which it was ready for use. It can be

stored at −20° for 1–2 months without appreciable loss of activity. Dilute solutions, however, deteriorate rapidly in the cold.

Results

General Properties

The distribution of 3′,5′-cyclic nucleotide phosphodiesterase activity in fractions from dog heart differs markedly if frozen heart tissue is used instead of fresh material (Table II). With fresh heart considerable activity is found in the particulate fraction (600*g*); this is in accord with the findings of Butcher and Sutherland (1962). However, when frozen heart tissue is used, most of the enzyme activity is recovered in the 105,000*g* supernatant.

TABLE II: Distribution of 3′,5′-Cyclic Nucleotide Phosphodiesterase Activity in Fractions Obtained from Fresh *vs.* Frozen Heart Tissue.

Fraction	Total Activity (%)[a]	
	Fresh	Frozen
Homogenate	100.0	100.0
"Nuclei" (600*g*)	40.2	5.2
Mitochondria (10,500*g*)	0.0	0.4
Microsomes (105,000*g*)	1.7	0.5
105,000*g* supernatant	57.1	96.4

[a] Total activity is expressed as the per cent activity of each fraction compared with the total activity of the homogenate.

Influence of pH. The influence of pH upon enzyme activity was investigated using Tris–maleate–glycine buffer (pH 5.2–9.0), Tris–HCl buffer (pH 7.5–9.5), and carbonate–bicarbonate buffer (pH 7.5–10.0). No difference in activity was noted with these buffers at any given pH. The assay was done by the two-stage method outlined earlier. In the first stage, the incubation was carried out at 37° for 20 min with 6.6×10^{-2} M buffer at pH values ranging from 5 to 10. The reaction was stopped by boiling the incubation mixtures for 2 min. The tubes were cooled and the pH was adjusted to 8.6 with 0.1 M Tris–HCl buffer. A solution (0.05 ml) containing 5 mg/ml of snake venom was added in a second incubation for 20 min at 37°. It was previously determined that the 5′-nucleotidase of snake venom was not inhibited by the high ionic strength of the buffer used. The optimal pH range is between 8.5 and 9.2 (Figure 5).

Ion Effects. 3′,5′-Cyclic nucleotide diesterase is dependent on Mg^{2+} for expression of activity (Figure 6). Substantial activity was observed in the concentration range $1–10 \times 10^{-3}$ M Mg^{2+} with a maximum effect between 5 and 7×10^{-3} M Mg^{2+}. Using a crude enzyme system from rabbit brain homogenates, Drummond and Perrott-Yee (1961) obtained maximal diesterase activity with 0.8×10^{-3} M $MgCl_2$; higher amounts produced inhibition. The following salts were studied in concentration ranges varying from 3×10^{-4} to 7.5×10^{-3} M: (1) magnesium chloride, (2) manganous chloride, (3) cobalt acetate, (4) zinc chloride, (5) calcium chloride, (6) ammonium sulfate, and (7) sodium molybdate.

The two-stage assay was performed. The first incubation was carried out with each of the above ions in the absence of Mg^{2+} and snake venom. The second incubation was carried out in the presence of excess snake venom and Mg^{2+}. It was determined earlier that the salts used in the above concentration range did not inhibit snake venom.

In the concentration range $5–10 \times 10^{-4}$ M, Mn^{2+} and Co^{2+} can substitute for Mg^{2+}. Higher concentrations are inhibitory. Ca^{2+}, Zn^{2+}, Mo^{2+}, and NH_4^+ were ineffective in the range in which they were studied.

Stimulation by NH_4^+ and Imidazole. In the presence of optimal levels of Mg^{2+}, ammonium salts in the concentration range 10^{-2} to 10^{-1} M produced a 1.5-fold stimulation. The salts used were: $(NH_4)_2CO_3$, $(NH_4)_2SO_4$, and NH_4Cl with the pH adjusted to 8.0 in the case of the sulfate and chloride salts. The stimulatory effect was noted in both crude and relatively pure preparations.

Imidazole in a similar concentration range, 10^{-2} to 10^{-1} M, produced the same order of stimulation. Under conditions of maximal stimulation with imidazole, addition of ammonium salts did not produce any further effect. L-Histidine, pH 8.0, used in the same high concentration range as imidazole produced no stimulation. That the stimulatory effect was not merely due to high ionic strength is shown by the fact that comparable concentrations of Na_2SO_4 were ineffective.

Ammonium salts did not stimulate the phosphodiesterase present in snake venom so that the two-stage assay method using boiled dog heart diesterase for the blank was not invalid.

Substrate Specificity. The relative rates of hydrolysis of 3′,5′-cyclic ribonucleoside phosphates were investigated. P_i release was obtained from the corresponding 5′-mononucleotide produced by using excess snake venom 5′-nucleotidase in a second incubation. The deoxy analog of 3′,5′-cyclic AMP was also investigated. Comparison of the initial velocities gave the rates shown in Table III. It is immediately apparent that the diesterase has a preference for 3′,5′-cyclic nucleotides with purine bases. The fact that 3′,5′-cyclic deoxy-AMP was broken down at a faster rate than 3′,5′-cyclic AMP is of considerable interest. It remains to be established whether the deoxy analogs of 3′,5′-cyclic ribonucleoside phosphates are natural substrates for the diesterase.

Nucleoside 2′,3′-Cyclic Phosphates. Adenosine 2′,3′-cyclic phosphate (2.7×10^{-3} M), 3.1×10^{-3} M uridine 2′,3′-cyclic phosphate, and 2.0×10^{-3} M cytidine 2′,3′-

TABLE III: Relative Rates of Hydrolysis of Purine and Pyrimidine 3′,5′-Cyclic Nucleotides.[a]

Compound	Relative Rate of Hydrolysis (%)		
	This Study	Sutherland and Rall Preliminary Estimate (1960)	Drummond and Perrott-Yee (1961)
	Purine Bases		
3′,5′-Cyclic AMP	100	100	100
3′,5′-Cyclic deoxy AMP	130		
3′,5′-Cyclic IMP	55–65	100	
3′,5′-Cyclic GMP	33		33
	Pyrimidine Bases		
3′,5′-Cyclic UMP	12–15	60	11
3′,5′-Cyclic CMP	0		0

[a] The one-stage assay was used in the presence of a 173-fold pure enzyme. The percentages given for the breakdown of the different substrates are relative to those of 3′,5′-cyclic AMP under the conditions of the assay.

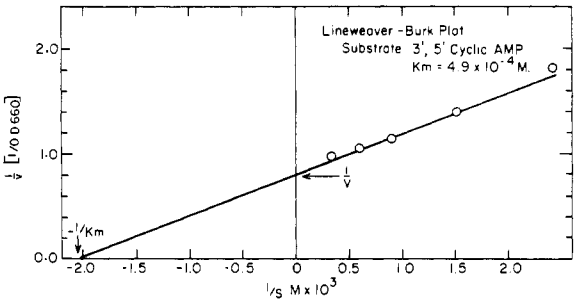

FIGURE 7: Lineweaver–Burk plot using 3′,5′-cyclic AMP as substrate. The K_m value is 4.9 × 10^{-4} M and the V_{max} is 1.25 OD units (1.25 OD units is equal to 0.026 μmole min^{-1}).

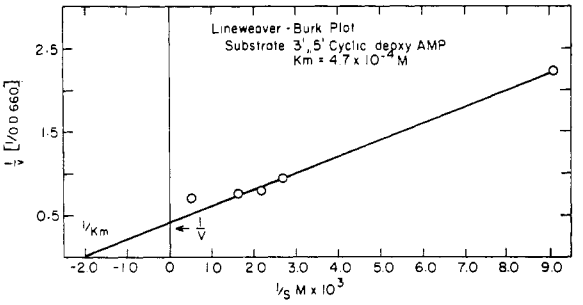

FIGURE 8: Lineweaver–Burk plot using 3′,5′-cyclic deoxy-AMP as substrate. The K_m is 4.7 × 10^{-4} M and the V_{max} is 2.36 OD units (2.36 OD units is equal to 0.049 μmole min^{-1}).

cyclic phosphate were incubated with excess diesterase in an assay mixture containing 2 × 10^{-2} M $(NH_4)_2SO_4$, 2.5 × 10^{-2} M Tris buffer pH 8.6, and 2.5 × 10^{-3} M $MgCl_2$ in a final volume of 0.300 ml.

Paper chromatography of the heat-deproteinized supernatant fluid in solvent system I using Whatman 3MM paper revealed no additional ultraviolet absorbing areas when compared to zero-time control, thus proving that the 2′,3′-cyclic nucleotides were not broken down by the diesterase. In contrast, the relatively crude preparation of the diesterase from rabbit brain by Drummond and Perrott-Yee (1961) seemed to break down 2′,3′-cyclic nucleotides probably because of contamination by a separate enzyme.

Polyadenylic and Polyuridylic Acids. Polyadenylic acid (poly-A) (1.15 × 10^{-3} M) and polyuridylic acid (poly-U) (2.9 × 10^{-3} M) were incubated with excess enzyme using the same assay system as above, and the reaction was stopped with TCA. An aliquot of the TCA supernatant layer was applied to Whatman 3MM paper and developed in solvent system II. 5′-AMP and 5′-UMP were used as markers. No breakdown was observed.

DNA and RNA. Employing the same methods as used for the polymers, 4 mg of yeast RNA and 1.5 mg of calf thymus DNA were incubated with excess enzyme for 24 hr. No breakdown products were observed on chromatographic examination of the reaction mixture in solvent I.

Mononucleotides. 3′,5′-Cyclic nucleotide phosphodiesterase did not release inorganic phosphate from 5′-AMP.

Oligonucleotides. Experiments were carried out to see if 3′,5′-cyclic nucleotide diesterase could catalyze the synthesis of oligonucleotides from 3′,5′-cyclic mononucleotides. Uridine, adenosine, guanosine, cytidine, or inosine (1.25 × 10^{-2} M in each case) was incubated with excess enzyme in the presence of 5 × 10^{-3} M $MgCl_2$, 2 × 10^{-2} M $(NH_4)_2SO_4$, and 5 × 10^{-3} M 3′,5′-cyclic AMP. The mixture was incubated for 10, 20, and 30

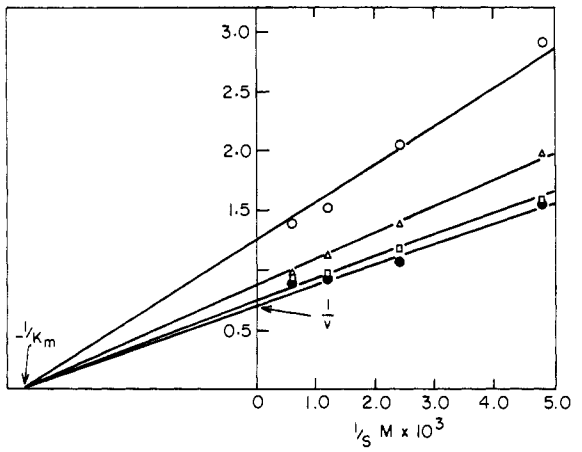

FIGURE 9: Inhibition of 3′,5′-cyclic nucleotide phosphodiesterase by caffeine. The K_i value for caffeine is 5.0×10^{-2} M. ○, 3.33×10^{-2} M caffeine; □, 0.6×10^{-2} M caffeine; △, 1.66×10^{-2} M caffeine; ●, no inhibitor.

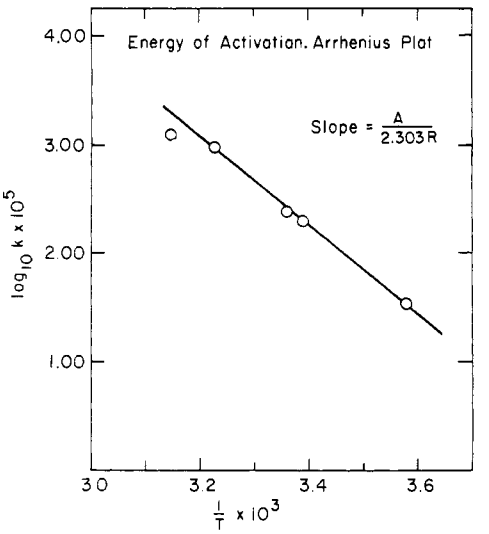

FIGURE 10: Activation energy curve.

min. The reaction was stopped by the addition of 0.05 ml of 10% TCA. Aliquots (0.10 ml) of the supernatant were chromatographed on Whatman 3MM paper in solvent system I. Similar experiments were carried out with 5′-AMP and 5′-UMP instead of adenosine and uridine. In no instance was oligonucleotide synthesis observed.

Determination of the Michaelis Constant (K_m). The K_m values (Figures 7 and 8) for adenosine 3′,5′-cyclic phosphate and deoxyadenosine 3′,5′-cyclic phosphate were obtained by incubating serial dilutions of the substrate with a 190-fold pure preparation of the diesterase at 37°, pH 8.6, for 10 min. A second incubation was then carried out in the presence of excess snake venom in order to determine the amount of 5′-nucleotide formed. All determinations were carried out in the presence of 2×10^{-2} M $(NH_4)_2SO_4$ in the reaction mixture.

Double reciprocal plots were obtained by the method of Lineweaver and Burk (1934), as seen in Figures 7 and 8. The results (K_m, V_{max}) were as follows: adenosine 3′,5′-cyclic phosphoric acid, 4.9×10^{-4} M, 1.25; deoxyadenosine 3′,5′-cyclic phosphoric acid, 4.7×10^{-4} M, 2.36.

The K_m values for both compounds are similar, *i.e.*, approximately 4.8×10^{-4} M. The range of values varies from 2.5 to 5×10^{-4} M. The V_{max} for the deoxy analog appears to be 1.8 times higher.

Inhibition Studies. Enzyme inhibition studies were carried out using caffeine as an inhibitor in concentrations varying from 0.6×10^{-2} to 3.33×10^{-2} M with 3′,5′-cyclic AMP as substrate. Ammonium sulfate was omitted from the incubation mixture. Caffeine produced a noncompetitive inhibition of 3′,5′-cyclic nucleotide diesterase (Figure 9). The K_i value for caffeine was 5.0×10^{-2} M.

Since 3′,5′-cyclic CMP was not hydrolyzed by the diesterase from dog heart, experiments were carried out to see if this compound was an inhibitor of the enzyme when 3′,5′-cyclic AMP was used as the substrate. No inhibition was noted using 3′,5′-cyclic CMP in concentrations varying from 3.3×10^{-4} to 4.98×10^{-3} M.

The energy of activation (Figure 10) was calculated by means of the integrated form of the Arrhenius equation.

$$A = \frac{2.303(\log K_2 - \log K_1)R}{(1/T_1) - (1/T_2)}$$

The calculated value of the energy of activation was 19,000 cal/mole.

Discussion

Although the participation of 3′,5′-cyclic nucleotides in hormonal control mechanisms remains to be proven, much evidence has been presented in support of this view. However, both 3′,5′-cyclic adenylic acid and the enzymes that lead to its synthesis and breakdown are widely distributed in the tissues of several species, and the cyclic nucleotide has been shown to accumulate in liver homogenates in response to epinephrine or glucagon and in heart muscle under the influence of epinephrine (Rall and Sutherland, 1958; Murad *et al.*, 1960). In addition to stimulation of phosphorylase *b* kinase, 3′,5′-cyclic AMP has been shown to stimulate phosphofructokinase (Mansour *et al.*, 1961). Other effects include a vasopressin-like effect on the toad bladder (Orloff and Handler, 1961) and augmented steroid production in beef adrenals (Haynes *et al.*, 1959).

Butcher and Sutherland (1959, 1962) have shown that 3′,5′-cyclic nucleotide phosphodiesterase is widely distributed in the tissues of dog. It appears that this diesterase is specific for the 3′,5′-diester linkage in

cyclic nucleotide phosphates. DNA, RNA, polyadenylic, and uridylic acids are not attacked at all. Nucleotide 2′,3′-cyclic phosphates are also not hydrolyzed. Although the effects of this enzyme on oligonucleotides have not been studied, experimental evidence indicates that this enzyme is not involved in oligonucleotide synthesis. It appears, therefore, that 3′,5′-cyclic nucleotide diesterase is the only mechanism for the destruction of adenosine 3′,5′-cyclic phosphate and thereby exerts an important control on the concentration of this nucleotide in tissues.

Of considerable interest is the specificity of this enzyme for 3′,5′-cyclic nucleotides with purine bases. A separate diesterase for similar nucleotides with pyrimidine bases has not yet been described. It is likely that adenosine 3′,5′-cyclic phosphate is the only nucleotide of physiological significance. Sutherland and Rall (1960) have tested 3′,5′-cyclic UMP and 3′,5′-cyclic IMP in liver and muscle homogenates and found these nucleotides to be inactive with regard to the stimulation of phosphorylase *b* kinase.

Unlike snake venom phosphodiesterase which attacks 3′,5′-cyclic AMP very slowly and produces both 5′-AMP and 3′-AMP, the phosphodiesterase described above hydrolyzes 3′,5′-cyclic AMP rapidly with the production of 5′-AMP alone (Sutherland and Rall, 1958).

Enzyme inhibition studies using caffeine in varying concentrations indicate a noncompetitive inhibition with a high K_i value of 5.0×10^{-2} M. The enzyme is stimulated by imidazole, an observation first made by Butcher and Sutherland (1959). Ammonium salts likewise produce a 1.5-fold stimulation in both crude and relatively pure preparations. The mechanism of stimulation is unknown.

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