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CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN PEA SEEDLINGS*

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SUMMARY

An enzyme from pea seedlings that hydrolyzes both 2',3'-cyclic nucleoside monophosphate and 3',5'-cyclic nucleoside monophosphate has been partially purified from pea seedlings. It has a molecular weight of 350 000 and has optimal activity at pH 5.4–6.0. It is insensitive to methylxanthines and imidazole. It catalyzes the formation of 3'-AMP exclusively from 2',3'-cyclic AMP and the formation of 3'-AMP and 5'-AMP from 3',5'-cyclic AMP. The ratio of 3'-AMP to 5'-AMP in the latter case is 7 to 1. The activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP are quite similarly affected by pH, metal ions, sulfhydryl reagents, temperature, and urea. Furthermore, the two activities have identical physical properties. It is suggested, therefore, that a single enzyme molecule is responsible for both activities.

INTRODUCTION

Since the discovery of 3',5'-cyclic AMP in biological tissues[1], it has been implicated as a second messenger in the action of a variety of animal hormones[2]. It is also known to be a mediator of catabolite repression or the so-called "glucose-effect" in bacteria[3]. Although extensive studies have been made on the distribution and function of this nucleotide in animal and unicellular organisms[2], information concerning 3',5'-cyclic AMP in higher plants is meager.

Preliminary attempts to detect adenylate cyclase and to incorporate radioactive adenine and adenosine into 3',5'-cyclic nucleoside monophosphate in pea and barley tissues were unsuccessful. However, an enzymatic system for the degradation of 3',5'-cyclic AMP in both pea and barley tissues has been found. It seemed necessary to study such a 3',5'-cyclic nucleotide phosphodiesterase in more detail in order to develop a better assay for adenylate cyclase or endogeneous cyclic nucleoside mono-

Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; BIS-acrylamide, *N,N'*-methylenebisacrylamide.

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phosphates in higher plants. The partially purified phosphodiesterase hydrolyzed not only 3′,5′-cyclic nucleoside monophosphate but also 2′,3′-cyclic nucleoside monophosphate. It has been suggested that RNA degradation in higher plants is catalyzed by a ribonuclease that hydrolyzes both RNA and 2′,3′-cyclic nucleoside monophosphate[4,5]. Although a specific enzyme for the hydrolysis of 2′,3′-cyclic nucleoside monophosphate, but not RNA, has been found in both animal and bacterial systems[6–9], it has not up to now been reported in higher plants.

This paper presents the detailed procedures for isolation and purification of cyclic nucleotide phosphodiesterase, together with a description of the general chemical and physical properties of the enzyme with respect to its action on the substrate of 2′,3′-cyclic AMP and 3′,5′-cyclic AMP. The biological significance of cyclic nucleotide phosphodiesterase and its possible role in the degradation of RNA in higher plants are discussed.

EXPERIMENTAL PROCEDURES

Materials

Early Alaska peas (*Pisum sativum*, Var.) used for all enzyme preparations were obtained from the Vaughan's Seed Co., Chicago, Ill.. Nucleotide and nucleoside derivatives, glutathione, dithiothreitol, cysteine, Coomassie blue and Tris were obtained from Sigma; calf thymus DNA from Worthington; Sephadex products and blue dextran from Pharmacia; sucrose and $(NH_4)_2SO_4$ (special enzyme grade) from Mann Research Laboratory; polyadenylic acid and [8-^{14}C]polyadenylic acid (0.154 μCi/mg) from Miles Laboratory; cellulose powder MN 300 (Brinkmann) from Macherey and Nagel Co.; BioRad Ag 1-X2, 400 mesh, chloride form ion exchanger from BioRad Laboratory; and acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), *N,N′*-methylenebisacrylamide (BIS-acrylamide) from Eastman Kodak.

Acrylamide and BIS-acrylamide were recrystallized twice from chloroform and acetone prior to use. High molecular weight ribosomal RNA was prepared from commercial yeast by the method of Crestfield *et al.*[10]. The 3′,5′-cyclic [^{3}H]AMP with a specific activity of 16.3 Ci/μmole and about 2% impurity was purchased from Schwarz BioResearch. All standard chemicals were reagent grade and were used without further purification with the exception of 3′,5′-cyclic [^{3}H]AMP which was purified according to the following procedures. Thin-layer chromatography showed that most of the 2% impurity was located in the area of authentic adenosine, adenine, and 3′-AMP of 5′-AMP. The method used for purification was similar to that described for the assay of adenylate cyclase in animal system by Krishna *et al.*[11]. A commercial sample of cyclic AMP was applied to a Dowex 50 (H^+, 200–400 mesh) column (1.5 cm × 8 cm) and eluted with deionized water in 2 ml fractions.

The third and fourth fractions containing 95% of 3′,5′-cyclic AMP were combined and freeze-dried. Deionized water was used for dissolving the purified material. With thin-layer chromatography in a two-dimensional separation system (Solvent system I and water) as described under Methods, purified 3′,5′-cyclic [^{3}H]AMP was shown to be free of bases, nucleosides, and other nucleotides. About 80–85% recovery of 3′,5′-cyclic [^{3}H]AMP was achieved in these procedures.

Methods

Growth of pea seedlings. Alaska peas were surface-sterilized for 20 min in 1% sodium hypochlorite, rinsed with sterile distilled water several times, and planted in a 4-l Erlenmeyer flask containing moist, sterile vermiculite. After germination at 23 °C in the dark for about one week, the seedlings were removed and rinsed with distilled water.

Assay of 3'-nucleotidase. The assay measured the release of P_i from nucleoside monophosphate. The standard reaction mixture in a total volume of 0.5 ml contained 50 μmoles of potassium acetate buffer, pH 5.4, or Tris–acetate buffer, pH 8.0, 2 μmoles of nucleoside monophosphate, and 2–4 units of the enzyme preparation. The reaction mixture was incubated at 37 °C for 30 min, and the reaction terminated by the addition of 0.05 ml of cold 55% trichloroacetic acid. After standing in an ice bath for 15 min, the precipitate formed was removed by centrifugation at 2000 $\times$ *g* for 10 min. The resulting supernatant was analyzed for P_i by the method of Fiske and SubbaRow[12]. One unit of nucleotidase activity is defined as that amount of enzyme which causes the release of 0.1 μmole of P_i per 30 min under the assay conditions described above.

Assays of ribonuclease and deoxyribonuclease. Ribonuclease was assayed according to the procedures of McDonald[13] and Ibuki *et al.*[14]. One unit of ribonuclease activity is defined as that amount of enzyme which causes an increase in the absorbance at 260 nm of 0.1 unit per 30 min incubation under the assay conditions. Assay of deoxyribonuclease activity was essentially the same as that described for the ribonuclease activity with the exception that denatured calf thymus DNA (heated 10 min at 100 °C, followed by quick cooling) was used instead of ribosomal RNA as substrate. One unit of deoxyribonuclease is defined as previously described for ribonuclease.

Assay of cyclic nucleotide phosphodiesterase. With the presence of an excess of pea 3'-nucleotidase I (EC 3.1.3.6) (P. P-C. Lin and J. E. Varner, in preparation) in the reaction mixture, this assay measures the release of P_i from cyclic nucleoside monophosphate. In the routine assay, the incubation mixture (0.50 ml) contained 50 μmoles of potassium acetate buffer (pH 5.4); 2 μmoles of cyclic nucleoside monophosphate; and 2–4 units of enzyme. The reaction mixture was incubated for 1 h at 37 °C with the addition (in 0.1 ml) of 20 units of pea 3'-nucleotidase I at 45 min. The reaction was terminated by the addition of 0.05 ml of cold 70% trichloroacetic acid solution and the reaction tubes were placed in an ice bath for 15 min. The precipitate formed was removed by centrifugation at 2000 $\times$ *g* for 10 min. The resulting supernatant was analyzed for P_i by the method described for the assay of 3'-nucleotidase. Whenever 3',5'-cyclic [^{3}H]AMP was used as substrate, the standard reaction mixture (0.50 ml) contained 50 μmoles of potassium acetate buffer (pH 5.4); 2 μmoles of 3',5'-cyclic [^{3}H]AMP ($7 \cdot 10^5$ cpm/μmole); and 2 units of enzyme. The reaction mixture was incubated at 37 °C for 1 h and terminated by heating in a boiling water bath for 3 min. An aliquot of 0.01 ml of the reaction mixture was then spotted on a cellulose precoated thin-layer plate, along with 3 μg each of 5'-AMP, 3'-AMP and adenosine as carriers. The chromatogram was developed with the solvent system of 2-propanol–0.03 M NH_4HCO_3 (pH 8.6) (3:1, v/v) at room temperature for 4 h. The carriers were located with ultraviolet light, scraped off and eluted from the cellulose with 0.5 ml of distilled water in a boiling water bath for 20 min. After centrifugation at 2000 $\times$ *g* for 10 min, the supernatant was poured directly into a scintillation vial to which 15 ml of Bray's solution[15] was added. The radioactivity was determined in a Beckman liquid scintilla-

tion spectrometer. One unit of enzyme activity is defined as 0.1 μmole of P_i released per h of incubation.

Determination of protein content. Protein concentration was determined according to the method of Lowry *et al.*[16] with crystalline bovine serum albumin as a standard. Colorimetric readings were made at 660 nm. Specific activity of the enzyme is defined as units per mg of protein.

Polyacrylamide disc-gel electrophoresis. The apparatus used for gel electrophoresis was similar to that described by Ornstein[17] and Davis[18]. Electrophoresis was performed at pH 8.5, 4 °C for 45 min with a constant current of 2 mA per tube. The protein bands were located by a method similar to that described by Chrambach *et al.*[19]. The gel was stained for a minimum of 2 h in 0.05% Coomassie blue (prepared in 12.5% trichloroacetic acid) and destained by diffusion in either distilled water or 5% trichloroacetic acid.

Sucrose density gradient centrifugation. The linear sucrose density gradient was prepared according to the method of Martin and Ames[20]. Centrifugation was routinely performed at 2 °C in a swinging bucket rotor, SW39 (Beckman) in a L-2 65B ultracentrifuge (Beckman). Upon completion of the run, 10-drop fractions were collected after needle puncture of the bottom of the tube. Enzyme assays were made on alternate fractions with two different substrates for each gradient.

Electrofocusing column chromatography. An LKB model 8101 electrofocusing column with a total capacity of 110 ml was used. Electrofocusing was done according to the methods described in the LKB manual.

A potential of 350 V (kept constant throughout the procedure) was applied to the column for a period of 48 h. The working temperature was maintained at 2 °C. After completion of the run, 80-drop fractions were collected.

Because ampholine was found to form a precipitate with the ammonium molybdate used in the P_i assay, the ampholine was removed from the fractions before the enzyme assays were performed. After the determination of pH, fractions within the pH range from 3.0 to 6.0 were dialyzed against 1 M NaCl at 4 °C overnight, then against deionized water for 6 h. The resulting dialyzed fractions were free of ampholine and were assayed for enzyme activity.

Dowex ion exchange chromatography. For the separation of 2′-AMP, 3′-AMP and 2′,3′-cyclic AMP, a column of BioRad Ag 1-X2, chloride form, 400 mesh, 0.5 cm × 5 cm, was equilibrated with 2 mM HCl and calibrated by chromatographing a mixture of authentic 2′ and 3′ isomers of adenylic acid and 2′,3′-cyclic AMP. 2 mM HCl was used as the eluting solvent. Fractions of 60 drops (approx. 3.4 ml) were collected with a flow rate of 12 drops per min and the absorbance measured at 260 nm.

EXPERIMENTAL RESULTS

Purification of enzyme

All the procedures were carried out in ice bath or at 4 °C unless otherwise stated.

Preparation of crude extract of cyclic nucleotide phosphodiesterase

Routinely, 300 g of 9- to 10-day-old pea seedlings, germinated in sterile vermiculite in the dark, was homogenized with 300 ml of deionized water for 1–2 min in a Waring Blender. The homogenate was then squeezed through a double-layer of

cheese-cloth to remove the bulk of insoluble material. The resulting filtrate (375 ml) was taken as the crude extract for purification of the enzyme.

$(NH_4)_2SO_4$ fractionation and pH 5 treatment

The crude extract was centrifuged at 10 000 × *g* for 10 min. The supernatant (360 ml) was brought to 50% saturation with $(NH_4)_2SO_4$ by slowly adding solid $(NH_4)_2SO_4$ (29.5 g per 100 ml of supernatant). The solution was stirred for 30 min and the precipitate was removed and discarded by centrifugation at 10 000 × *g* for 20 min. The resulting supernatant (380 ml) was decanted and then brought to 80% saturation with the addition of 19.7 g of solid $(NH_4)_2SO_4$ per 100 ml of the extracted solution. After stirring for 2 h, the precipitate was collected by centrifugation at 10 000 × *g* for 30 min and dissolved in 30 ml of 2 mM Tris–acetate buffer, pH 7.5. The resulting solution was dialyzed against 20 vol. of 2 mM Tris–acetate buffer, pH 7.5, with constant agitation for 24 h, with 3 changes. After dialysis, the solution (32 ml) was centrifuged at 10 000 × *g* for 10 min to remove a small amount of precipitate which formed during the dialysis. The pH of the resulting supernatant (32 ml) was adjusted to 5.0 with 0.1 M acetic acid. The precipitate formed from the pH 5.0 treatment was removed and discarded by centrifugation at 10 000 × *g* for 10 min. The supernatant (30 ml) containing 50% of the original activity with a 7- to 10-fold increase in specific activity was adjusted to pH 7.5 with 0.1 M KOH.

The enzyme up to this step still contained an appreciable amount of 3'-nucleotidase.

Chromatography of cyclic nucleotide phosphodiesterase on Sephadex G-200 column

After pH 5.0 treatment, a 5-ml portion of the enzyme preparation containing 6 mg of protein was applied to a Sephadex G-200 column (1.5 cm × 120 cm) which had been equilibrated with 0.01 M Tris–acetate buffer, pH 7.5. The enzyme was eluted with the same buffer and collected in 3-ml fractions at a flow rate of 9 ml/h.

The elution profile of protein and enzyme activities is shown in Fig. 1. Although only 28.2% of the total activity toward 2',3'-cyclic AMP and 41.5% of that toward

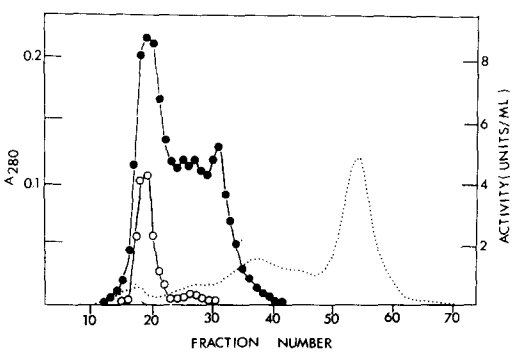


Fig. 1. Elution profile of cyclic nucleotide phosphodiesterase from Sephadex G-200 column chromatography. The enzyme preparation from the pH 5.0 treatment (5 ml, containing 6 mg protein, 120 units toward 3',5'-cyclic AMP and 380 units toward 2',3'-cyclic AMP) was applied to a column (1.5 cm × 120 cm) which had been equilibrated with 0.01 M Tris–acetate buffer, pH 7.5. Elution was carried out with the same buffer at a flow rate of 9 ml/h at 4 °C. 3-ml fractions were collected. Enzyme activities (in units/ml) on 2',3'-cyclic AMP (●—●) and on 3',5'-cyclic AMP (○—○) were determined as described under *Methods*. Protein concentration was measured as the absorbance at 280 nm (·····).

3',5'-cyclic AMP was recovered, about 95% of the protein originally applied to the column was removed from the major enzyme fractions. This resulted in a 5-fold increase in the specific activity toward 2',3'-cyclic AMP and 8-fold increase toward 3',5'-cyclic AMP. The summary of the purification of cyclic nucleotide phosphodiesterase from 300 g of pea seedlings is shown in Table I. Three peaks of enzyme activity toward 2',3'-cyclic AMP and one having activity toward 3',5'-cyclic AMP were consistently observed. Fractions 17–26 were combined as the enzyme preparation for the remaining studies. The elution peak for blue dextran was in Fraction 16.

Enzyme preparation from this step was concentrated from 10 ml to 2 ml with a Centriflo membrane cone (Amicon filter) at 1900 rev./min for 20 min in an International portable refrigerated centrifuge (Model PR-2). The specific activity of the enzyme toward cyclic nucleoside monophosphate was maintained constant with 95% recovery in total activity after concentration. The concentrated enzyme preparation was then stored at −20 °C for further use. At this temperature the enzyme was stable for at least 4 months.

Although the profile of enzyme activities in Sephadex G-200 chromatogram (Fractions 17–26) suggested that the enzymatic hydrolysis of 2',3'-cyclic AMP and 3',5'-cyclic AMP might be due to the same protein molecule, further attempts were made to separate the two activities.

Further attempts to separate the activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP

Sucrose density gradient centrifugation

Three peaks of enzyme activity toward 2',3'-cyclic AMP were observed with sucrose density gradient prepared in 0.1 M Tris–acetate buffer, pH 7.5, while only one of them showed appreciable activity toward 3',5'-cyclic AMP (Fig. 2A). The major peak represented 50% and 80% of the total activity toward 2',3'-cyclic AMP and 3',5'-cyclic AMP, respectively. About 60–70% of enzyme activity was recovered after centrifugation. Compared to bovine liver catalase (sedimentation constant, 11.3 S; mol. wt, 247 500), the major cyclic nucleotide phosphodiesterase had a sedimentation constant of 14.3 S. If an acidic sucrose density gradient (prepared in 0.1 M potassium acetate buffer, pH 5.4) was used, only one peak of enzyme activity was obtained for either 2',3'-cyclic AMP or 3',5'-cyclic AMP (Fig. 2B). Again, the two activities sedimented in an identical pattern with maximal activity in the same fraction. The loss of the two minor peaks of enzyme activity (which appeared in the alkaline sucrose density gradient) may be due to their inactivation by the acidic pH rather than by enzyme association since 60–70% of the activity was recovered. This result also suggested that the dissociation may not occur in alkaline pH as does the experiment shown in Fig. 2A. The elution profiles of enzyme activity toward 3',5'-cyclic AMP and 3',5'-cyclic GMP were essentially the same (Fig. 2C). An experiment using 2',3'-cyclic UMP as substrate gave the same distribution pattern of enzyme activity as for 2',3'-cyclic AMP in Figs 2A and 2B.

Polyacrylamide disc-gel electrophoresis and isoelectric focusing

The enzyme activity of cyclic nucleotide phosphodiesterase was separated from the contaminating enzymes such as 3'-nucleotidases, ribonuclease and most of the acidic phosphatase by polyacrylamide gel electrophoresis (Fig. 3). However, enzyme

TABLE I

SUMMARY OF PURIFICATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM 300 g OF PEA SEEDLINGS

Fraction	*Total protein (mg)*	*2',3'-Cyclic AMP as substrate*				*3',5'-Cyclic AMP as substrate*				*Ratio of spec. act. 2',3' cyclic AMP / 3',5' cyclic AMP*
		Total act. (units)	*Spec. act. (units/mg)*	*Purification (-fold)*	*Yield (%)*	*Total act. (units)*	*Spec. act. (units/mg)*	*Purification (-fold)*	*Yield (%)*	
Crude extract	5510	8816	1.6	1.0	100	1874	0.3	1.0	100	5.3
10 000 × *g* supernatant	4672	7195	1.5	1.0	81.6	2196	0.5	1.7	117	3.0
50–80% $(NH_4)_2SO_4$ (dialyzed)	612	5508	9.0	5.6	62.5	1426	2.3	7.7	76.1	3.9
pH 5 treated	41.6	2704	65	40.6	30.7	842	20.2	67.3	44.9	3.2
Sephadex G-200 fraction (Nos 17–26)	2.2	762	346	217	8.7	349	159	539	18.6	2.2

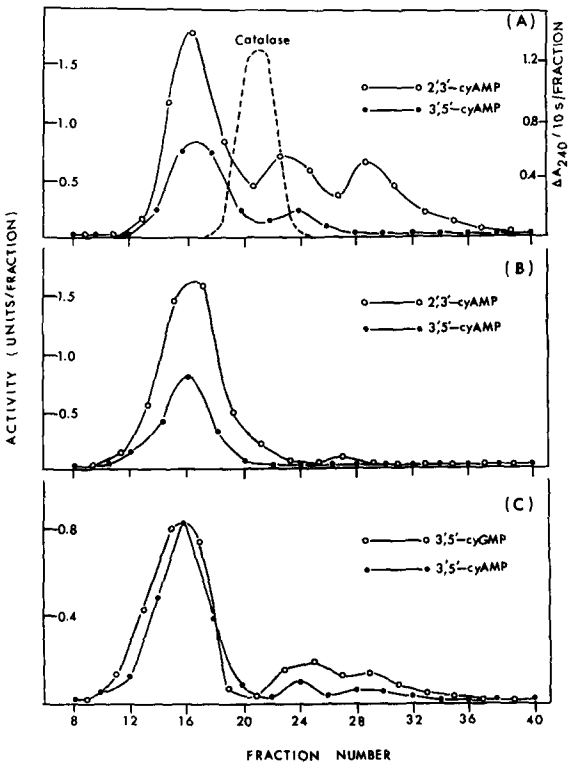


Fig. 2. The elution profiles of cyclic nucleotide phosphodiesterase activities from sucrose density gradient centrifugation. Partially purified enzyme preparation (from the pH 5.0 treatment, 0.3 ml, containing 0.39 mg protein (24 units toward 2',3'-cyclic AMP) was layered over a 5–20% sucrose density gradient. Centrifugation was performed with a swinging bucket rotor, SW39 (Beckman), in a Beckman L-265B ultracentrifuge at 34 000 rev./min for 12 h. The temperature was maintained at 2 °C. 10-drop fractions, total of 40 fractions, were collected after centrifugation. Enzyme assays were carried out in the alternate fractions with two different substrates for each gradient. The 3'-nucleotidase purified from pea was used for the coupled assay system as described under *Methods*. Beef liver catalase (mol. wt, 247 500) was used as the marker for estimation of the relative molecular weight of cyclic nucleotide phosphodiesterase. (A) Elution profile of enzyme activities toward 2',3'-cyclic AMP (○—○) and 3',5'-cyclic AMP (●—●) in the sucrose density gradient prepared in 0.1 M Tris–acetate buffer, pH 7.5. Catalase activity was assayed according to the method of Chance *et al.*[31]. (B) Same conditions as described in (A) except the sucrose density gradient was prepared in 0.1 M potassium acetate buffer, pH 5.4. (C) Elution profile of enzyme activities toward 3',5'-cyclic AMP (●—●) and 3',5'-cyclic GMP (○—○). Same conditions as described in (A).

activities toward both 2',3'-cyclic AMP and 3',5'-cyclic AMP were still found to be associated with each other. An electrofocusing experiment indicated that the activity toward 3',5'-cyclic AMP had an isoelectric point at pH 4.8 while there were 3 isoelectric points (pH 4.8, 4.6 and 4.3) for the activity toward 2',3'-cyclic AMP.

Characterization of the reaction products

Action on 2',3'-cyclic nucleoside monophosphates

Qualitative evidence that 3'-AMP and 3'-UMP were the immediate products formed by the hydrolysis of 2',3'-cyclic AMP and 2',3'-cyclic UMP, respectively, was provided by the coupled assays with 3'-nucleotidase (Table II).

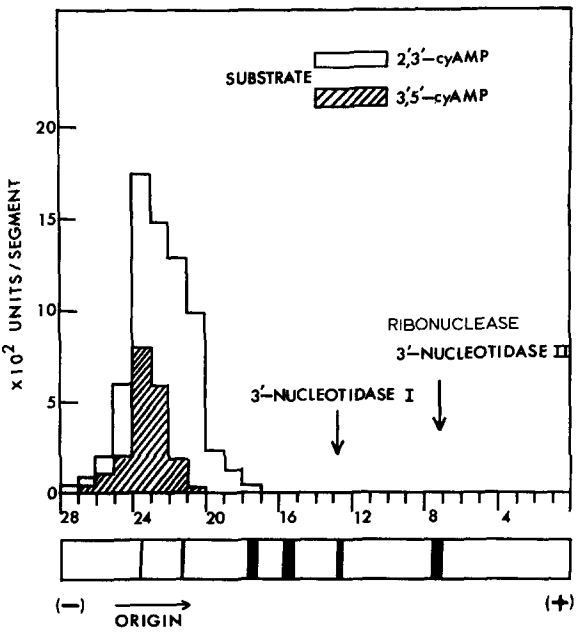


Fig. 3. Staining pattern of protein and the distribution of enzyme activities within a polyacrylamide gel after electrophoresis. 0.3 ml of cyclic nucleotide phosphodiesterase preparation containing 10 μg of protein was applied to a 7% polyacrylamide gel as described under *Methods*. Electrophoresis was conducted at pH 8.5 at 4 °C for 45 min with an applied current of 2 mA per tube. The gel was cut in half; one half was stained with Coomassie blue dye, and the other half was cut in 3 mm segments. Enzyme assays for hydrolysis of 2',3'-cyclic AMP and 3',5'-cyclic AMP were performed in alternate segments as described in Experimental Procedures.

Pea 3'-nucleotidase I and II were rather specific for 3'-nucleoside monophosphates with activities toward both 3'-AMP and 3'-UMP (P. P-C. Lin and J. E. Varner, in preparation). However, 3'-nucleotidase from rye grass (Sigma) has been shown to be without activities toward pyrimidine 3'-nucleoside monophosphates. The enzyme activity found in the control reaction may be due to the presence of a small amount of

TABLE II

ENZYMATIC ANALYSIS OF THE HYDROLYSIS PRODUCT FORMED FROM 2',3'-CYCLIC NUCLEOSIDE MONOPHOSPHATE

Activity was determined in a 0.5-ml reaction mixture containing 2 μmoles substrate, 0.1 ml enzyme solution (concentrated Fraction 18 from Sephadex G-200 column chromatography, 0.01 mg protein/ml) and 50 μmoles of potassium-acetate buffer, pH 5.4. Incubation was performed at 37 °C for 1 h. After incubation, the reaction mixture was heated in boiling water for 3 min to terminate the reaction. Then 3'-nucleotidase in excess amount was added and incubated at its optimal pH for 15 min. P_i released was determined as described in *Methods*.

Addition	*μmoles P_i released*	
	Substrate assayed:	
	2',3'-Cyclic AMP	*2',3'-Cyclic UMP*
None	0.11	0.15
3'-Nucleotidase I (pea)	0.38	0.43
3'-Nucleotidase II (pea)	0.36	0.42
3'-Nucleotidase (rye grass)	0.36	0.16

3'-nucleotidase in the preparation of cyclic nucleoside monophosphate from the Sephadex G-200 chromatogram.

Further evidence for the reaction product being exclusively 3'-AMP was obtained from an ion exchange chromatogram. The amount of 3'-AMP formed from 2',3'-cyclic AMP increased with time while there was no apparent formation of 2'-AMP (Fig. 4). Another experiment, using 2',3'-cyclic [^{14}C]AMP prepared from [^{14}C]polyadenylic acid by the action of purified pea ribonuclease (P. P-C. Lin and J. E. Varner, in preparation), gave the same result as shown above without any detectable formation of 2'-[^{14}C]AMP.

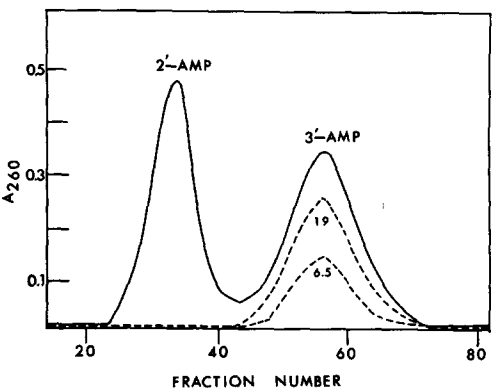


Fig. 4. Ion exchange chromatography of the hydrolysis product of 2',3'-cyclic AMP. The enzyme preparation from the sucrose density gradient (Fraction 16 in Fig. 2) was incubated with 0.1 ml of 0.01 M 2',3'-cyclic AMP and 0.05 ml of 0.2 M potassium acetate buffer, pH 5.4, in a final volume of 0.3 ml for 6.5 and 19 h at 37 °C. After termination by heating the reaction solution in a boiling water bath for 3 min, the whole reaction mixture was applied to an ion exchange column as described in *Methods*. The elution pattern of the hydrolysis product of 2',3'-cyclic AMP is represented by the dashed line (— — —). The time (h) for incubation is indicated within the figure.

Action on 3',5'-cyclic nucleoside monophosphates

Purified 3',5'-cyclic [^{3}H]AMP was used as a substrate. The preparation of cyclic nucleotide phosphodiesterase was freed of 3'-nucleotidase by sedimenting 0.3 ml of the concentrated enzyme preparation from the Sephadex G-200 column, containing 11 units toward 3',5'-cyclic AMP, in a sucrose density gradient. This separated the major cyclic nucleotide phosphodiesterase fractions from pea 3'-nucleotidases. The enzyme of Fraction 14 from the sucrose gradient as shown in Fig. 2 catalyzed the formation not only of 3'-[^{3}H]AMP but also of 5'-[^{3}H]AMP from 3',5'-cyclic [^{3}H]AMP with a ratio of 3'-AMP/5'-AMP of 6.8:1 (Fig. 5a). The enzyme preparation in this fraction was essentially free of 3'-nucleotidases, because there was no detectable [^{3}H]adenosine. The fact that the incubation of Fraction 10 of the sucrose gradient with the substrate gave no detectable 5'-[^{3}H]AMP or [^{3}H]adenosine (Fig. 5b) indicated that the formation of 5'-AMP was due to enzymatic hydrolysis rather than nonenzymatic degradation of 3',5'-cyclic [^{3}H]AMP. The minor amount of 3'-[^{3}H]AMP formed was due to the presence of a small amount of cyclic nucleotide phosphodiesterase.

In order to check the result shown above with a different enzyme preparation and to make sure of the location of the end products in the thin-layer chromatograph,

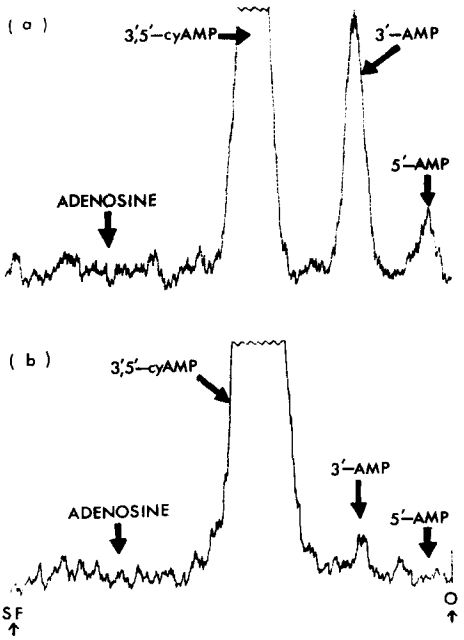


Fig. 5. Thin-layer chromatography of the hydrolysis products obtained from the action of enzyme on 3',5'-cyclic [^{3}H]AMP. The enzyme of Fraction 14 from Fig. 2B was incubated with a reaction mixture (0.5 ml) containing 2 μmoles of 3',5'-cyclic [^{3}H]AMP ($7 \cdot 10^5$ cpm/μmole) at 37 °C for 5 h. After incubation, a 0.02-ml aliquot was streaked on a cellulose thin-layer plate (5 cm × 20 cm). The chromatogram was developed as described under *Methods*. The resulting chromatogram was then scanned with a Packard Strip scanner. Enzyme from Fraction 10 of Fig. 2B was used as the control enzyme preparation. O, origin of chromatogram; SF, solvent front. The full scale was 300 cpm. (a) Radiochromatogram of the hydrolysis products obtained from Fraction 14. The locations of authentic 3',5'-cyclic AMP, 3'-AMP, 5'-AMP, and adenosine are indicated by the arrows. (b) Radiochromatogram of the hydrolysis products obtained from Fraction 10 (control).

the enzyme preparation from gel electrophoresis (Segment 24 in Fig. 3) was used. This fraction contains some 3'-nucleotidase. As shown in Fig. 6a, 3'-[^{3}H]AMP, 5'-[^{3}H]-AMP, and [^{3}H]adenosine were formed from 3',5'-cyclic [^{3}H]AMP. With the presence of excess amounts of 5'-nucleotidase, 5'-AMP but not 3'-AMP was converted to adenosine (Fig. 6b). However, 3'-AMP and some of the 5'-AMP was converted to adenosine in the presence of an excess of pea 3'-nucleotidase I (Fig. 6c). The ratio of the formation of 3'-AMP/5'-AMP was 6.8–7.0:1. These results show that both 3'-AMP and 5'-AMP were products of the enzymatic hydrolysis of 3',5'-cyclic AMP. A subsequent experiment utilizing the enzyme preparation from Fractions 16 and 17 of Sephadex G-200 chromatogram as shown in Fig. 1 gave essentially the same result as that reported in Fig. 6.

Sucrose density gradient study and time course of the formation of 3'-AMP and 5'-AMP from 3',5'-cyclic AMP

Although both 3'-AMP and 5'-AMP appeared to be products of the hydrolysis of 3',5'-cyclic AMP, it was desirable to know whether this was due to the presence of two different enzymes. As shown in Fig. 7A, both 3'-AMP and 5'-AMP increased gradually with time in the reaction mixture containing enzyme from Fraction 16 of

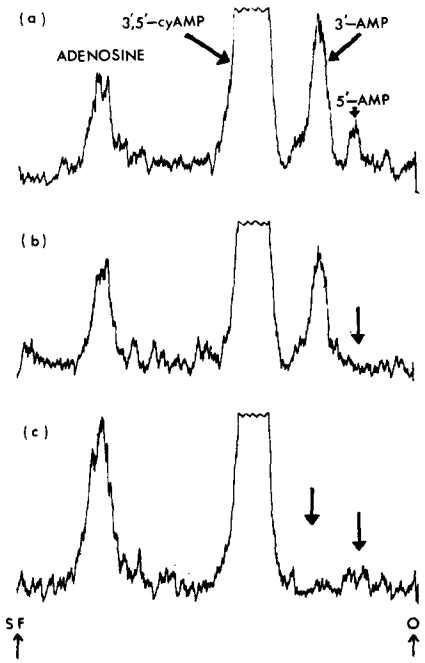


Fig. 6. Thin-layer chromatography of the hydrolysis products obtained from the action of enzyme on 3',5'-cyclic [^{3}H]AMP. The reaction mixture (1.0 ml) contained 2 μmoles of 3',5'-cyclic [^{3}H]-AMP ($7 \cdot 10^5$ cpm/μmole), 50 μmoles of potassium acetate buffer (pH 5.4) and Segment 24 from Fig. 3. After 24 h incubation at 37 °C, the reaction mixture was separated into three equal parts, 0.5 ml in each part, and incubated separately with an excess of 5'-nucleotidase (snake venom from Sigma) or pea 3'-nucleotidase (P. P-C. Lin and J. E. Varner, in preparation) or with an equal amount of buffer (0.2 M potassium acetate, pH 5.4) for an additional 1 h. At the end of the incubation, 0.01-ml aliquots from each incubation were separately subjected to thin-layer chromatography with the addition of authentic compounds. O, origin of chromatogram; SF, solvent front. The full scale was 300 cpm. (a) Radiochromatogram of the hydrolysis products from 3',5'-cyclic [^{3}H]AMP without further treatment. (b) Radiochromatogram of the hydrolysis products treated with excess 5'-nucleotidase. (c) Radiochromatogram of the hydrolysis products treated with excess 3'-nucleotidase I.

Fig. 2 while there was no detectable formation of these two nucleotides in the control reaction mixture. The ratio of 3'-AMP/5'-AMP was about 6.8:1 throughout the whole time course. This indicates that both 3'-AMP and 5'-AMP are enzymatic products from 3',5'-cyclic AMP. However, this result does not rule out the possibility of the presence of two enzymes. In an attempt to demonstrate two activities, enzyme from Fractions 12–28 from sucrose density gradient centrifugation as shown in Fig. 2A were assayed for cyclic nucleotide phosphodiesterase with the standard reaction mixture.

The radioactivity profile of reaction products is shown in Fig. 7B. It is evident that 3'-AMP and 5'-AMP are produced in identical ratios by Fractions 12–20. The ratio of 3'-AMP/5'-AMP was 6.8–7.0:1. The drop in the ratio after Fraction 20 was due to the presence of 3'-nucleotidase which converted 3'-AMP and 5'-AMP at different rates to adenosine.

Although there was no contaminating 3'-nucleotidase in Fractions 12–20 (Fig.

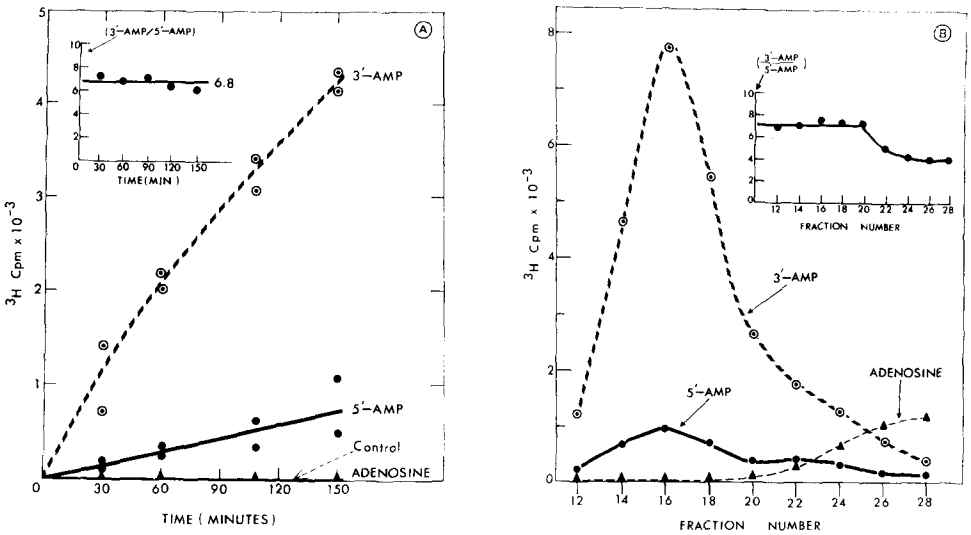


Fig. 7. Sucrose density gradient study and analysis of end product formation from enzymatic hydrolysis of 3',5'-cyclic [^{3}H]AMP as a function of time. (A) Enzyme from Fraction 16 in Fig. 2 was incubated at 37 °C with the standard reaction mixture containing 2 μmoles 3',5'-cyclic [^{3}H]-AMP ($7 \cdot 10^5$ cpm/μmole). Fraction 8 from the same enzyme preparation was used as a control. During the time courses, 0.01-ml aliquots were taken for thin-layer chromatography and developed, eluted, and counted as described in *Methods*. Duplicate aliquots were taken. The value of 3'-[^{3}H]AMP/5'-[^{3}H]AMP is shown on the left corner of the figure. ○---○, 3'-[^{3}H]AMP; ●—●, 5'-[^{3}H]AMP; ▲—▲, [^{3}H]adenosine. The control fraction shows no detectable amounts of tritiated products formed. (B) After incubation the standard reaction mixture (containing 2 μmoles 3',5'-cyclic [^{3}H]AMP with specific activity of $7 \cdot 10^5$ cpm/μmole) for 3.5 h at 37 °C. 0.01-ml aliquots were taken for thin-layer chromatography analysis. The ratio of 3'-[^{3}H]AMP/5'-[^{3}H]AMP is shown on the right corner of the figure. ○---○, 3'-[^{3}H]AMP; ●—●, 5'-[^{3}H]AMP; ▲---▲, [^{3}H]adenosine.

7B), it was still possible that 5'-AMP (or 3'-AMP) might be formed from 3'-AMP (or 5'-AMP) by the transferase enzyme which has been reported in *Escherichia coli*[21] and carrot leaves[22]. Purified 3'-[^{3}H]AMP from the enzymatic hydrolysis of 3',5'-cyclic [^{3}H]AMP as described under *Methods* was incubated in a reaction mixture containing the enzyme from Fraction 16 (in Fig. 2), 20 μmoles of potassium acetate buffer (pH 5.4), 1 μmole of 3',5'-cyclic AMP, and 0.5 μmole of 3'-[^{3}H]AMP ($4 \cdot 10^4$ cpm/μmole) in a total volume of 0.5 ml. During the time courses, 0.05-ml aliquots were streaked on a cellulose thin-layer plate with the authentic compounds (3'-AMP and 5'-AMP and adenosine) and developed as described in *Methods*. There was no formation of 5'-[^{3}H]-AMP even after 3 h incubation. In a further experiment with 1 μmole of 5'-[^{3}H]AMP ($5 \cdot 10^4$ cpm/μmole, prepared from the action of rat brain cyclic nucleotide phosphodiesterase on 3',5'-cyclic [^{3}H]AMP)instead of 3'-[^{3}H]AMP in the above reaction mixture, there also was no detectable 3'-[^{3}H]AMP after 3 h incubation. It is, therefore, suggested that both 3'-AMP and 5'-AMP are direct products from the enzymatic hydrolysis of 3',5'-cyclic AMP. Apparently, the enzyme is able to cleave both ester linkages.

Properties of cyclic nucleotide phosphodiesterase

Reaction rate as a function of time and enzyme concentration

With the standard assay procedure and 10 μg of protein of the enzyme prepara-

tion, the progress curve of cyclic AMP hydrolysis was linear up to 2 h with either 2',3'-cyclic AMP or 3',5'-cyclic AMP as substrate (Fig. 8A). In addition, the amount of cyclic AMP hydrolyzed was a linear function of protein concentration under standard incubation conditions (Fig. 8B). The rate of hydrolysis of 2',3'-cyclic AMP was about twice that of 3',5'-cyclic AMP.

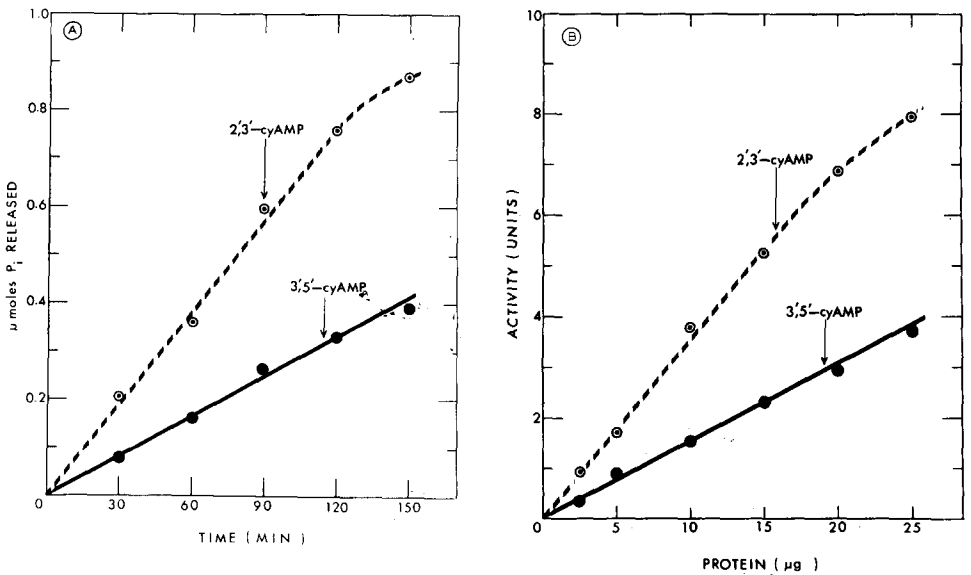


Fig. 8. (A) Time course of cyclic nucleoside monophosphate breakdown by cyclic nucleotide phosphodiesterase. The reaction mixture containing 60 μg of protein, 12 μmoles of substrate, 300 μmoles potassium acetate buffer, pH 5.4, and distilled water in a final volume of 3.0 ml was incubated at 37 °C. Every 30 min, 0.5-ml aliquots were taken for assay of inorganic phosphate as described under *Methods*. Purified 3'-nucleotidase was added at zero time. Substrate used: ○---○, 2',3'-cyclic AMP; ●—●, 3',5'-cyclic AMP. (B) Enzyme activity as a function of protein concentration. The reaction mixture and experimental conditions were as described for the standard assay except the amount of protein was varied as shown. Substrate used: ○---○, 2',3'-cyclic AMP; ●—●, 3',5'-cyclic AMP.

Effect of pH on enzyme activity and stability

As shown in Fig. 9, cyclic nucleotide phosphodiesterase had a pH optimum around 5.4–6.0 with either 2',3'-cyclic AMP or 3',5'-cyclic AMP as substrate. These enzyme activities were quite stable under pH 5.4 at 4 °C or −20 °C. The optimal pH of pea cyclic nucleotide phosphodiesterase was different from that of similar enzymes in microorganisms[23,24] and animal systems[2,25] for which the enzyme has been shown to have a maximal activity at pH 7.5 to 8.0.

Influence of various metal ions and sulfhydryl compounds

It is evident that both activities behaved similarly in response to the presence of various metal ions. A slight increase in both enzyme activities was observed in the presence of Mn^{2+}, Co^{2+}, and Zn^{2+} at concentrations of 0.1–1.0 mM. NaF at 1 mM showed a 45% inhibition of activity toward 3',5'-cyclic AMP with no apparent effect on the activity toward 2',3'-cyclic AMP. There was no significant effect on the enzyme activity with the presence of Mg^{2+}, NH_4^+, K^+ or Na^+ at concentrations of 0.1 or 1.0 mM. EDTA at concentrations of 0.1 mM and 1.0 mM had no effect on either activity.

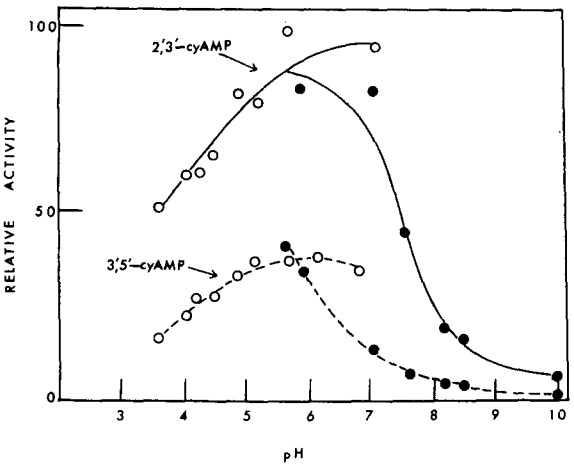


Fig. 9. Effect of pH on the activity of pea cyclic nucleotide phosphodiesterase. The reaction mixture and experimental procedures were as described for the standard assay. Buffer used: ●, 0.1 M potassium acetate; ○, 0.1 M Tris–acetate. Substrate used: ———, 2',3'-cyclic AMP; — — —, 3',5'-cyclic AMP.

Cysteine and dithiothreitol at concentrations from 0.04 mM to 4.0 mM had similar effects on the two activities with a maximum enhancement of 35–40%.

Effect of urea on enzyme activity

Both activities decreased in the presence of increasing concentrations of urea (Fig. 10). However, points of 50% activity were at 6 M urea for activity toward 3',5'-cyclic AMP, and 8 M urea for the hydrolysis of 2',3'-cyclic AMP. Both activities also decreased in parallel during various times of preincubation in 6.5 M urea.

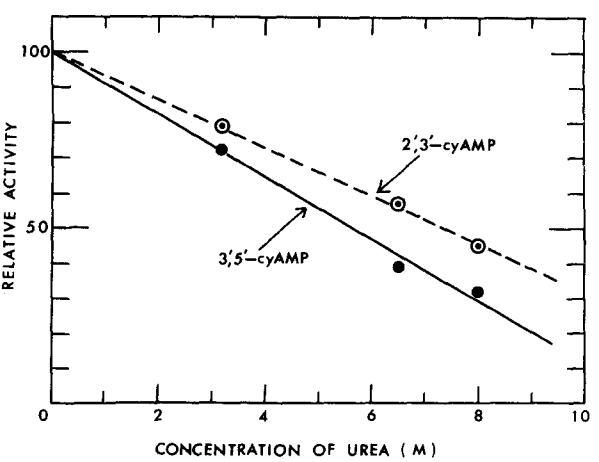


Fig. 10. Effect of the concentration of urea on the activity of pea cyclic nucleotide phosphodiesterase. The standard reaction mixtures containing 20 μg protein were incubated with various concentrations of urea at 37 °C for 1 h. After incubation, excess 3'-nucleotidase was added and the inorganic phosphate released was determined as described under *Methods*. Substrate used: ○---○, 2',3'-cyclic AMP; ●—●, 3',5'-cyclic AMP.

Optimum temperature and heat stability of enzyme activity

The enzyme activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP had an optimum temperature at 40 °C under the standard assay condition. Heat inactivation became appreciable at 50 °C. Activity toward 2',3'-cyclic AMP was apparently more sensitive to high temperature denaturation than that toward 3',5'-cyclic AMP. An Arrhenius plot of data taken from the effect of temperature on enzyme activities showed a change in slope at 40 °C (Fig. 11). The activation energy was calculated to be 8.6 kcal/mole for hydrolysis of 2',3'-cyclic AMP and 7.2 kcal/mole for hydrolysis of 3',5'-cyclic AMP in the temperature range from 20–40 °C. The value for the hydrolysis of 3',5'-cyclic AMP is close to the value of 7.5 kcal/mole reported by Cheung[25] for rat brain enzyme, but quite different from the 19 kcal/mole reported by Nair[26] for dog heart enzyme. The two activities showed a similar heat inactivation. After heating at 60 °C for 5 min, about 50% of the activity was lost.

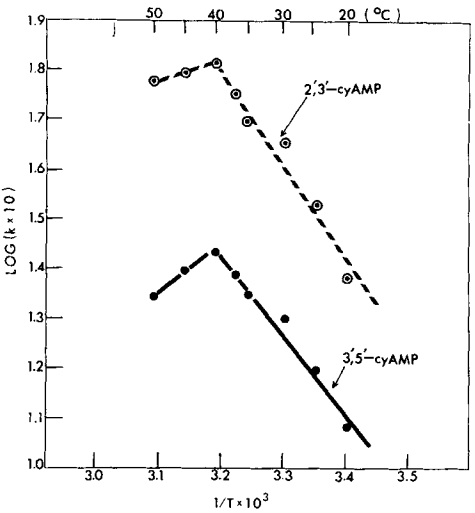


Fig. 11. Arrhenius plots for the activities toward 2',3'-cyclic AMP and 3',5'-cyclic AMP. The standard reaction mixture contained 20 μg protein. Excess 3'-nucleotidase was added after 1 h incubation at various temperatures. Further details were as described in *Methods*. Substrate used: ○---○, 2',3'-cyclic AMP; ●—●, 3',5'-cyclic AMP.

Effect of various compounds on enzyme activity

Unlike their effects of inhibition on animal 3',5'-cyclic AMP phosphodiesterase[2,32,33], caffeine and theophylline at a concentration of 0.1 mM or 4.0 mM had no appreciable effect on enzyme activity as shown in Table III. Imidazole at a concentration of 4 mM has been shown to activate phosphodiesterase in various animal tissues[26,27], but it has no effect on the pea enzyme. Various nucleotides, P_i and PP_i were inhibitory.

K_m values and the relative rates of hydrolysis of cyclic nucleoside monophosphates

With 4 mM substrate, pea enzyme showed the following relative rates (%) of hydrolysis of substrates: 2',3'-cyclic UMP (100%) > 2',3'-cyclic AMP (83%) > 3',5'-cyclic UMP (68%) > 2',3'-cyclic GMP (51%) > 3',5'-cyclic IMP (45%) > 3',5'-cyclic AMP (41%) > 2',3'-cyclic CMP, 3',5'-cyclic GMP, 3',5'-cyclic TMP (26%) > 3',5'-

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON THE ENZYME ACTIVITY USING 3',5'-CYCLIC [^{3}H]AMP AS SUBSTRATE

A 0.25-ml reaction mixture containing 1 μmole of 3',5'-cyclic [^{3}H]AMP ($7 \cdot 10^5$ cpm/μmole), 10 μmoles potassium-acetate buffer (pH 5.4), 4 units of enzyme and the compound to be tested at the concentration indicated was incubated at 37 °C for 1 h and the radioactive end products from a 0.02-ml aliquot were determined as described under *Methods*.

Compound added	*Activity (% of control)*	
	Concentration of compound added:	
	$4 \cdot 10^{-3}$ *M*	$1 \cdot 10^{-4}$ *M*
None	100	100
3',5'-cyclic GMP	41	78
3',5'-cyclic CMP	28	81
2',3'-cyclic AMP	27	95
2',3'-cyclic UMP	61	100
ATP	27	74
2'-AMP	43	106
3'-AMP	33	90
5'-AMP	30	100
Adenosine	103	101
P_i	35	87
PP_i	29	72
Imidazole	116	100
Caffeine	95	100
Theophylline	100	100

cyclic CMP (23%). It is interesting that 2,6-dibutyryl 3',5'-cyclic AMP, an analog of 3',5'-cyclic AMP which is not hydrolyzed by the animal enzyme[2], was hydrolyzed at a rate similar to that of 3',5'-cyclic AMP by the pea enzyme. In general, the enzymes so far isolated and partially purified from animal tissues have been shown to be specific for hydrolysis of 3',5'-cyclic AMP and 3',5'-cyclic GMP[2,28]. Although the diesterase from rabbit brain seems to have activity toward 2',3'-cyclic AMP, it may be due to the contamination of a separate enzyme[29]. The K_m values of the pea enzyme were as follows: 2',3'-cyclic CMP (5.0 mM), 2',3'-cyclic GMP (4.34 mM), 3',5'-cyclic CMP (1.81 mM), 3',5'-cyclic GMP (1.61 mM), 3',5'-cyclic AMP (0.90 mM), 2',3'-cyclic AMP (0.83 mM), 2',3'-cyclic UMP (0.62 mM), 3',5'-cyclic UMP (0.58 mM).

Activity toward other organic phosphates

Although the pea cyclic nucleotide phosphodiesterase from the Sephadex G-200 contained activities toward RNA, DNA, and other organic phosphates as indicated in Fig. 12, such activities were probably not due to the cyclic nucleotide phosphodiesterase itself. For instance, evidence for the contamination of ribonuclease and 3'-nucleotidase in cyclic nucleotide phosphodiesterase preparation was demonstrated by gel electrophoresis (Fig. 3). Furthermore, the sucrose density gradient centrifugation provided evidence that pea cyclic nucleotide phosphodiesterase had no significant activity toward either RNA or 3'-nucleotides (Fig. 13).

DISCUSSION

An enzyme from Alaska pea seedlings hydrolyzes both 2',3'-cyclic nucleoside monophosphate and 3',5'-cyclic nucleoside monophosphate. This cyclic nucleotide

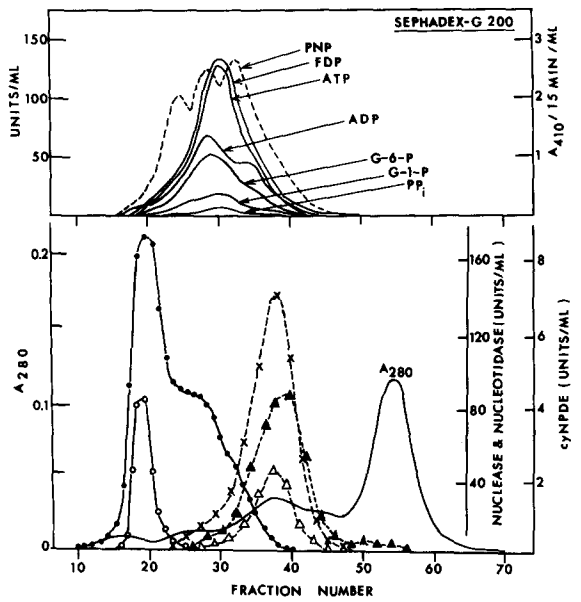


Fig. 12. The elution profiles of enzyme activities from Sephadex G-200 column chromatography. All experimental conditions are the same as described in Fig. 1. Enzyme activities for various substrates were assayed according to *Methods*. In the upper graph, each solid curve represents the enzyme activity for one specific substrate as indicated by the arrow (G = glucose; FDP = fructose diphosphate). The dashed line shows the activity toward *p*-nitrophenol phosphate (PNP) as measured by the absorbance at 410 nm. Each fraction corresponds to the number shown in the lower part of the figure. In the lower graph, the solid curve represents protein concentration as measured by the absorbance at 280 nm. Enzyme activities (in units/ml) on 2′,3′-cyclic AMP (●—●), 3′,5′-cyclic AMP (○—○), 3′-AMP (pH 8.0, ×---×), RNA (▲---▲) and DNA (△---△) were determined as described under *Methods*. cyNPDE = cyclic nucleotide phosphodiesterase.

phosphodiesterase was purified approx. 218-fold with a recovery of about 8% of the total activity toward 2′,3′-cyclic AMP.

Although the enzyme preparation still had detectable activities toward 3′-nucleotides, RNA, DNA, and some organic phosphates, such activities are probably due to the contamination of nucleotidases and other phosphatases rather than to cyclic nucleotide phosphodiesterase itself. Evidence for this is as follows: (1) The enzyme activity toward cyclic nucleoside monophosphate can be clearly distinguished from the activities toward other nucleotides, RNA, DNA, and various organic phosphates by means of gel filtration, sucrose density gradient centrifugation, and gel electrophoresis. (2) Compared to the properties of pea ribonuclease (P. P-C. Lin and J. E. Varner, in preparation), pea cyclic nucleotide phosphodiesterase is quite different with respect to pH optimum, effect of reducing reagents, acid stability, rate of sedimentation in sucrose density gradient, electrophoretic mobility and molecular weight.

Since most of the ribonucleases so far characterized from higher plants are cyclizing enzymes that yield 2′,3′-cyclic nucleoside monophosphates with negligible activity toward 2′,3′-cyclic nucleoside monophosphate[4,5], the pea cyclic nucleotide phosphodiesterase described here may play a crucial role in the degradation of RNA. We suggest that RNA degradation in higher plants may not follow the scheme that is generally accepted in which ribonuclease (cyclizing enzyme) hydrolyzes both RNA

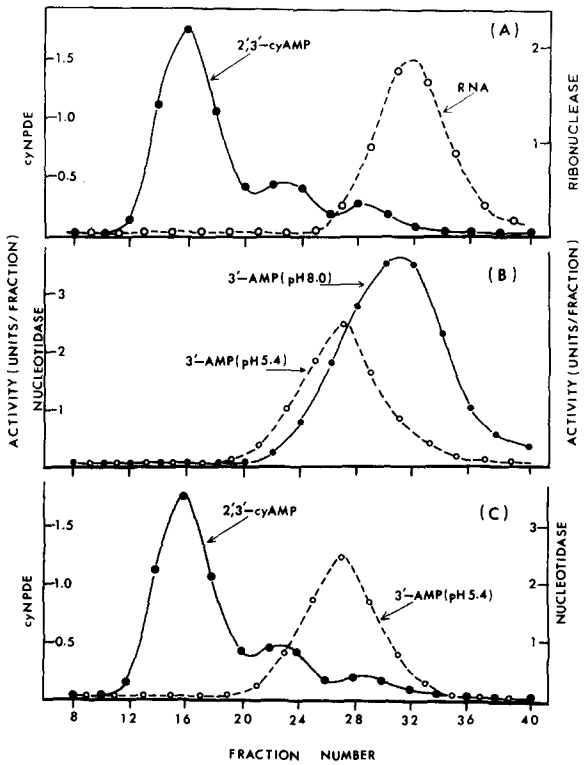
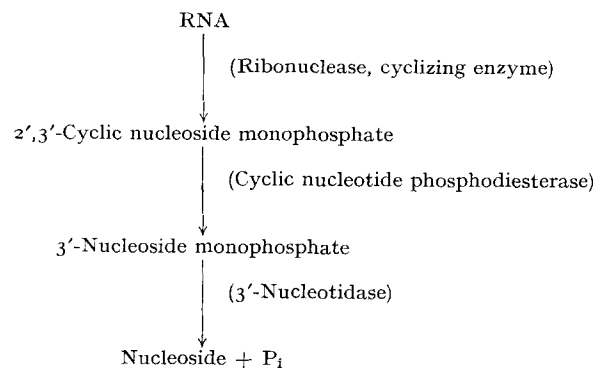


Fig. 13. The elution profiles of cyclic nucleotide phosphodiesterase (cyNPDE), ribonuclease, and 3'-nucleotidases from sucrose density gradient centrifugation. For detailed experimental conditions, refer to the description in Fig. 2A. (A) The elution profile of enzyme activity toward 2',3'-cyclic AMP (●—●) and ribonuclease (○---○). (B) The elution profile of 3'-nucleotidases activities. Substrates and buffers used: 3'-AMP, potassium acetate 0.1 M, pH 5.4 (○---○); 3'-AMP, Tris–acetate 0.1 M, pH 8.0 (●—●). (C) The elution profile of enzyme activity toward 2',3'-cyclic AMP (●—●) and 3'-nucleotidase activity toward 3'-AMP (○---○). 0.1 M potassium acetate buffer (pH 5.4) was used for assays.

and 2',3'-cyclic nucleoside monophosphate[4,5]. We propose that the degradation of RNA in higher plants is as follows:



With respect to the hydrolysis of 3',5'-cyclic AMP, the pea enzyme was purified approx. 470-fold with a recovery of about 19% of the total activity present in the crude extract.

Unlike the enzyme from animal tissues, the pea enzyme exhibited an acidic pH optimum, and insensitivity to caffeine, theophylline, and imidazole. Furthermore, the pea enzyme activity was not dependent upon the presence of Mg^{2+}. Other metal ions had no effect on enzyme activity. The reason for the inhibition by NaF is not clear.

The pea enzyme catalyzes the formation of both 3'-AMP and 5'-AMP with a ratio of 3'-AMP/5'-AMP of about 7:1 while 5'-AMP is the exclusive product of the enzyme from the animal system[2], slime molds[30], and microorganisms[8,23,24,39].

The data suggest that the formation of two products from one substrate is due to a single enzyme. This conclusion is based on the following evidence:

(1) The formation of the two nucleotides was parallel with a constant ratio throughout the time course of the hydrolysis of 3',5'-cyclic AMP. (2) The ratio of the two nucleotides was constant throughout the fractions of the sucrose density gradient. (3) The enzyme activity toward 3',5'-cyclic AMP was inhibited to a similar degree by 3'-AMP and 5'-AMP.

The activities of the enzyme toward 2',3'-cyclic AMP and 3',5'-cyclic AMP were maintained in a rather constant ratio throughout the purification procedures and were quite similar with respect to pH optima, metal ion effects, effect of sulfhydryl reagents, heat stability, temperature optima, and the sensitivity to treatment with urea. Furthermore, both activities had the same electrophoretic mobility, the same rate of sedimentation in sucrose density gradient, the same isoelectric point and the same behavior on gel filtration. Therefore, it is suggested that the hydrolysis of these two cyclic nucleotides was due to the same enzyme molecule.

The possible biological significance of the presence of an enzyme with activity toward 3',5'-cyclic nucleoside monophosphate in higher plants is not known.

ACKNOWLEDGEMENTS

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REFERENCES

1 E. W. Sutherland and T. W. Rall, *J. Biol. Chem.*, 232 (1958) 1077.
2 G. A. Robison, R. W. Butcher and E. W. Sutherland, *Annu. Rev. Biochem.*, 37 (1968) 149.
3 I. Pastan and R. Perlman, *Science*, 169 (1970) 339.
4 E. A. Barnard, *Annu. Rev. Biochem.*, 38 (1969) 677.
5 W. J. Tang and A. Maretzki, *Biochim. Biophys. Acta*, 212 (1970) 300.
6 M. S. Center and F. J. Behal, *J. Biol. Chem.*, 243 (1968) 138.
7 T. Unemoto and M. Hayashi, *Biochim. Biophys. Acta*, 171 (1969) 89.
8 D. Monard, J. Janecek and H. V. Rickenberg, *Biochem. Biophys. Res. Commun.*, 35 (1969) 584.
9 G. I. Drummond, N. T. Iyer and J. Keith, *J. Biol. Chem.*, 237 (1962) 3535.
10 A. M. Crestfield, K. C. Smith and F. W. Allen, *J. Biol. Chem.*, 216 (1955) 185.
11 G. Krishna, B. Weiss and B. B. Brodie, *J. Pharmacol. Exp. Therap.*, 163 (1968) 379.
12 C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 66 (1925) 375.
13 M. R. McDonald, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1957, p. 427.

14 F. Ibuki, A. Aoki and S. Matsushita, *Agric. Biol. Chem.*, 28 (1964) 144.
15 G. A. Bray, *Anal. Biochem.*, 1 (1960) 279.
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
17 L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
18 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
19 A. Chrambach, R. A. Reisfeld, M. Wyckoff and J. Saccari, *Anal. Biochem.*, 20 (1967) 150.
20 R. G. Martin and B. N. Ames, *J. Biol. Chem.*, 236 (1961) 1372.
21 E. F. Brunngraber and E. Chargaff, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 107.
22 E. F. Brunngraber and E. Chargaff, *J. Biol. Chem.*, 245 (1970) 4825.
23 T. Okabayashi and M. Ide, *Biochim. Biophys. Acta*, 220 (1970) 116.
24 A. W. Murray, M. Spiszman and D. E. Atkinson, *Science*, 171 (1971) 496.
25 W. Y. Cheung, *Biochemistry*, 6 (1967) 1079.
26 K. G. Nair, *Biochemistry*, 5 (1966) 150.
27 J. Nakano, R. Oliver and T. Ishii, *Pharmacology*, 3 (1970) 273.
28 J. A. Beavo, J. G. Hardman and E. W. Sutherland, *J. Biol. Chem.*, 245 (1970) 5649.
29 G. I. Drummond and S. Perrott-Yee, *J. Biol. Chem.*, 236 (1961) 1126.
30 Y. Y. Chang, *Science*, 160 (1968) 57.
31 B. Chance and A. C. Maehly, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 764.
32 R. W. Butcher and E. W. Sutherland, *J. Biol. Chem.*, 237 (1962) 1244.
33 L. A. Menahan, K. D. Hepp and O. Wieland, *Eur. J. Biochem.*, 8 (1969) 435.