ENZYMES OF CYCLIC NUCLEOSIDE MONOPHOSPHATE METABOLISM IN PEA SEEDLINGS

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This is to certify that the

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ABSTRACT

ENZYMES OF CYCLIC NUCLEOSIDE MONOPHOSPHATE METABOLISM IN PEA SEEDLINGS

By

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Two 3'-nucleotidases have been isolated and partially purified from germinating pea seedlings. They provide useful tools for the study of pea cyclic nucleotide phosphodiesterase.

The 3'-nucleotidase I with a molecular weight of 70,000 shows maximal activity at pH 5.4-5.7. It catalyzes the hydrolysis of 3'-phosphoryl linkages of 3'-AMP, 3'-GMP, 3'-UMP and 3'-CMP, with little activity toward 2'-AMP and 5'-AMP. Several lines of evidence suggest that it does not catalyze the hydrolysis of RNA, DNA, or cyclic nucleoside monophosphates.

The 3'-nucleotidase II has an optimal pH at 8.0 and a molecular weight of 30,000. It catalyzes the hydrolysis of 3'-AMP, 3'-GMP, and 3'-UMP, but not 3'-CMP, 2'-AMP, and 5'-AMP. This enzyme represents about 0.2% of the total protein of homogenates of seedlings. It seems to have RNase activity associated with it. Results from a variety

of experiments suggest that 3'-nucleotidase II and RNase activities reside in a single protein molecule. RNase from 3'-nucleotidase II preparation catalyzes the formation of 2',3'-cyAMP from polyadenylic acid.

An enzyme capable of hydrolyzing both 2',3'-cyclic nucleoside monophosphate and 3',5'-cyclic nucleoside monophosphate has been found and partially purified from pea seedlings. It has a molecular weight of 350,000 and an optimal pH at 5.4-6.0. It is insensitive to methylxanthines and imidazole. It catalyzes the formation of 3'-AMP exclusively from 2',3'-cyAMP and the formation of 3'-AMP and 5'-AMP with a ratio of 3'-AMP:5'-AMP of about 7:1 from 3',5'-cyAMP. Because there is no interconversion between 3'-AMP and 5'-AMP, both 3'-AMP and 5'-AMP are direct products from 3',5'-cyAMP. The activities toward 2',3'-cyAMP and 3',5'-cyAMP are quite similarly affected by pH, metal ions, sulfhydryl reagents, temperature, and urea. Furthermore, the two activities have similar physical properties. It is suggested, therefore, that a single enzyme molecule is responsible for both activities. Activation energy for hydrolysis of 2',3'-cyAMP is 8.6 Kcal/mole and of 3',5'-cyAMP is 7.2 Kcal/mole.

Since several lines of evidence indicate that pea cyclic nucleotide phosphodiesterase is not the enzyme which hydrolyzes RNA, a new mode of RNA degradation in higher plants, at least in pea seedlings, is proposed. This is

that RNase (cyclizing enzyme) may function only in catalyzing the formation of 2',3'-cyNMP. Further hydrolysis of 2',3'-cyclic nucleoside monophosphate is due to cyclic nucleotide phosphodiesterase.

ENZYMES OF CYCLIC NUCLEOSIDE MONOPHOSPHATE METABOLISM IN PEA SEEDLINGS

By

Paul Po-chao Lin

A THESIS

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DEDICATION

To my mother, my wife, and my son

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I would like to express my sincere gratitude to Dr. J. E. Varner for his guidance and encouragement and for the freedom to learn during the course of this study. Thanks are extended to Drs. James L. Fairley, Hans Kende, and Clarence H. Suelter for serving on my guidance committee.

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ADP	5'-diphosphate of adenosine
ATP	5'-triphosphate of adenosine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetate
FDP	fructose-1,6-diphosphate
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
2'-NMP	2'-nucleoside monophosphate (e.q.2'-AMP, etc.)
3'-NMP	3'-nucleoside monophosphate (e.q.3'-AMP, etc.)
5'-NMP	5'-nucleoside monophosphate (e.q.5'-AMP, etc.)
2',3'-cyNMP	2',3'-cyclic nucleoside monophosphate (e.q.2',3'-cyAMP, etc.)
3',5'-cyNMP	3',5'-cyclic nucleoside monophosphate (e.q.3',5'-cyAMP, etc.)
cyNPDE	cyclic nucleotide phosphodiesterase
P_i	inorganic orthophosphate
PNP	p-nitrophenol phosphate
Poly A	polyadenylic acid
PP_i	inorganic pyrophosphate
RNA	ribonucleic acid
RNase	ribonuclease

$S_{20,w}$	Svedberg unit (sedimentation coefficient in water at 20°). One Svedberg = 1 x 10^{-13}sec.
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl) aminomethane

PART I

THE PURIFICATION AND CHARACTERIZATION OF 3'-NUCLEOTIDASE FROM PEA SEEDLINGS

Introduction

Although 5'-nucleotidase is widely distributed in mammalian tissues (1-7), enzymes capable of nucleotidase activity studied from various plant sources have been shown to have high specificity toward 3'-ribonucleotides (8-15). Furthermore, most of the 3'-nucleotidases in higher plants have been demonstrated to accompany nuclease activities (12, 14, 19). Whether the two activities were due to the same or to different enzyme molecules was not clear.

The 3'-nucleotidases so far isolated and characterized from higher plants in general work best on purine 3'-nucleotides (12, 15, 16). Most of the RNases studied in higher plants have been shown to be cyclizing enzymes which catalyze the formation of 2',3'-cyNMP, with little activity toward 2',3'-cyAMP and 2',3'-cyGMP, but not pyrimidine 2',3'-cyNMP derivatives (12, 19, 28). However, recent studies of rye grass (29) and sugar cane (30) RNases showed that these RNases could not hydrolyze 2',3'-cyNMP.

Preliminary experiments indicated that germinating pea seedlings contained two 3'-nucleotidases. Since little work has been done on pea nucleotidase and RNase and since it was desirable to find enzymes which could serve as tools in the enzyme coupled assay of pea cyclic nucleotide phosphodiesterase as described in the Part II, it was desirable to purify the pea 3'-nucleotidases and to characterize them.

Part I presents the procedures for isolation, purification, and characterization of the general chemical and physical properties of these 3'-nucleotidases. Attempts to separate the activities of 3'-nucleotidase II and RNase by a variety of chemical and physical means will be described. The substrate specificity of 3'-nucleotidase and a suggested mode of the action of RNase are also presented.

Experimental Procedure

Materials

Early Alaska peas (Pisum satirum, Var.) were used for all enzyme preparations and were purchased from the Vaughan's Seed Co., Chicago, Illinois. The following compounds were commercial samples purchased from various suppliers as indicated: nucleotide and nucleoside derivatives, glutathione, dithiothreitol, cysteine, Coomassie blue, tris(hydroxymethyl) aminomethane, Sigma; calf thymus DNA, Worthington; DEAE-cellulose ion exchanger (0.7 meq/gm), Gallard-Schlesinger chemical; Sephadex products and blue

dextran, Pharmacia; Lypogel, Gelman; enzyme protein molecular weight markers, sucrose and ammonium sulfate (special enzyme grade), Mann Research Laboratory; polyadenylic acid and polyadenylic acid-8-C^{14} (0.154 μCi/mg), Miles Laboratory; cellulose powder MN 300 (Brinkmann), Macherey and Nagel Co.; BioRad Ag-1-X 2, 400 mesh, chloride form ion exchanger, BioRad Laboratory; acrylamide, TEMED(N,N,N'-N'-tetramethylethylenediamine), BIS-acrylamide(N,N'-methylene-bisacrylamide), Eastman Kodak. All standard chemicals were reagent grade, and were used without further purification with the exception of acrylamide and BIS-acrylamide which were recrystallized twice from chloroform and acetone, respectively. High molecular weight ribosomal RNA was prepared from commercial yeast by the method of Crestfield et al. (31).

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-liter Erlenmeyer Flask containing moist, sterile vermiculite. After germination at 23° in the dark for about one week, seed 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 contained 0.1M K-acetate buffer, pH 5.4,

or Tris-acetate buffer, pH 8.0, and 2 mM nucleoside monophosphate with a suitable dilution of the enzyme preparation being assayed in a total volume of 0.5 ml. The reaction mixture was incubated at 37° for 10 to 30 minutes, and the reaction terminated by the addition of 0.05 ml of cold 55% TCA. After standing in an ice bath for 15 min, the precipitate formed was removed by centrifugation at top speed of the International clinical centrifuge (2,000 rpm) for 10 min. The resulting supernatant was analyzed for P_i by the method of Fiske and SubbaRow (32), modified as follows: 0.2 ml of 2.5% ammonium molybdate in 5 N H_2SO_4 was added to 1.0 ml aliquot of the supernatant solution (diluted with distilled water). The color was developed by the further addition of 0.1 ml of reducer (100 ml solution contained 0.2 g of 1-amino-2-naphthol-4-sulfonic acid, 1.2 g of sodium bisulfite and 1.2 g of sodium sulfite) and read at 660 mμ on a Beckman D.U. spectrophotometer with a Gilford digital absorbance meter. A standard curve relating the absorbance to the concentration of P_i (KH_2PO_4 as the standard) was constructed for each assay. This standard curve was not affected by the presence of enzyme solution or TCA. 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 RNase and DNase.--RNase was assayed according to the procedures of McDonald (33) and Ibuki

et al. (34). The reaction mixture contained 0.1 M Tris-acetate buffer, pH 6.5, 0.2 mg of yeast ribosomal RNA and an appropriately diluted enzyme preparation in a total volume of 0.5 ml. Incubation was carried out at 37° for 30 min. At the end of the incubation, 0.5 ml of cold 3 mM uranyl acetate in 0.2 N HCl was added. The precipitate formed after standing at 4° for 15 min was removed by centrifugation and the resulting supernatant was diluted to 3.0 ml with distilled water. The absorbance at 260 mμ was then measured. One unit of RNase activity is defined as that amount of enzyme which causes an increase in the absorbance at 260 mμ of 0.1 unit per 30 min incubation under the assay conditions. Assay of DNase activity was essentially the same as that described for the RNase activity with the exception that denatured calf thymus DNA (heated 10 min at 100°, followed by quick cooling) was used instead of ribosomal RNA as substrate. One unit of DNase is defined as previously described for RNase.

Determination of Protein Content.--Protein concentration was determined according to the method of Lowry et al. (35) with crystalline bovine serum albumin as a standard. Colorimetric readings were made at 660 mμ. Specific activity of the enzyme is defined as units per mg of protein.

Preparation of DEAE-Cellulose Ion Exchange Resin.--DEAE-cellulose with a capacity of 0.7 meq/g was readied for

use (without acid and base treatments) by suspending 30 g in 2 liters of deionized water and pouring off the finer particles five times. The slurry was then washed with two 500 ml of 0.01 M Tris-acetate buffer, pH 7.5 and stored at 4° in the same buffer prior to use.

Polyacrylamide Disc-Gel Electrophoresis.--The apparatus used in this gel electrophoresis was similar to that described by Ornstein (36) and Davis (37) with the following modifications. The glass tubes were 0.5 cm i.d.x 11.5 cm long. The height of the polyacrylamide gel columns were 8.5 cm and spacer gels were 0.5 cm. The concentration of all the running gels were 7% (w/v). The stock solutions were prepared as follows:

a. 48 ml of 1 N HCl, 36.6 g of Tris, 0.23 ml of TEMED, and water to 100 ml.
b. 28.0 g of acrylamide (2x crystallized), 0.735 g of BIS-acrylamide (2x crystallized), and water to 100 ml.
c. 4 mg of riboflavin, and water to 100 ml.
d. 48 ml of 1 N HCl, 5.98 g of Tris, 0.46 ml of TEMED, and water to 100 ml.
e. 10 g of acrylamide (2x crystallized), 2.5 g of BIS-acrylamide (2x crystallized), and water to 100 ml.
f. 40 g of sucrose, and water to 100 ml.

The running gel contained 0.5 part (a), 2 parts (b), 1 part (c), and 4.5 parts water. The spacer gel contained 1 part (d), 2 parts (e), 1 part (c), and 4 parts (f). Buffer for electrodes contained 0.6 g of Tris, 2.9 g of glycine and water to 1 liter, pH 8.5. Sample, 0.3 ml of enzyme preparation, was routinely layered onto the spacer gel by displacement of electrode buffer.

Electrophoresis was performed at 4° for 45 min with a constant current of 2 mA per tube. On completion of the electrophoresis, the gels were carefully removed under water by needling and air pressure. The protein bands were located by a method similar to that described by Chrambach et al. (38). The gel was stained for a minimum of 2 hr in 0.05% Coomassie blue (prepared in 12.5% TCA) and destained by diffusion in either distilled water or 5% TCA. In some cases, the gel was removed from the tube and divided into two parts along the longitudinal axis. One-half of the gel was stained for protein bands. The other half was sliced and assayed for enzyme activities under the assay conditions previously described.

Sucrose Density Gradient Centrifugation.--The linear sucrose density gradient was prepared according to the method of Martin and Ames (39) by a device which consists of two chambers, A and B interconnected with each other when a needle valve was opened. Chamber A, loaded with 2.2 ml of 20% (w/v) sucrose, was that one from which

the gradient solution was delivered. Chamber B was filled with 2.4 ml of 5% sucrose. In general the sucrose solutions also contained a buffer. The gradient was made in 1/2" x 2" of Beckman cellulose nitrate tube and allowed to stand at least 1 hr at 4° to smooth out before the sample (0.2-0.25 ml) was layered on the gradient.

A swinging bucket rotor, SW 65 LTi (Beckman) was used for centrifugation which was routinely performed at 2° in a Beckman L-2 65B ultracentrifuge. Upon completion of the run, 10-drop fractions were collected after needle puncture of the bottom of the tube. Enzyme assays were carried out in alternate fractions with two different substrates for each gradient set.

Electrofocusing Column Chromatography.--An LKB model 8101 electrofocusing column with a total capacity of 110 ml was used. Ampholyte carrier solution (Ampholine, LKB) and sucrose were used in order to establish a pH gradient with a density gradient. Electrofocusing was done according to the methods described in the LKB manual.

The composition of gradient solution and electrode solution for the electrofocusing in the pH range from 3.0 to 6.0 were as follows:

1. Dense gradient solution:

    Ampholyte (40%) --------- 1.9 ml

    Sucrose ---------- 28 g

    Distilled water --------- to 55 ml

2. Less dense gradient solution:

Ampholyte (40%)	0.6 ml
Enzyme solution	varied
Distilled water	to 55 ml

3. Dense electrode solution:

NaOH	0.3 g
Sucrose	18 g
Distilled water	21 ml

4. Less dense electrode solution:

H_2SO_4 (conc.)	0.1 ml
Distilled water	to 10 ml

A potential of 350 volts (kept constant throughout the whole procedure) was applied to the column for a period of 48 hr with the aid of a Buchler model No. 3-1014 A voltage and current regulated D.C. power supply. The working temperature was maintained at 2° by circulating water and methanol solution from a thermostat water cooler, LAUDA model WB-20/R (Brinkman Instruments) through the external and internal jackets.

The electric current of the electrofocusing unit gradually dropped as proteins settled at their isoelectric points along the linear pH gradient. Eventually, the current stabilized at less than 1.0 mA indicating nearly all the proteins in the electric field have been neutralized at their isoelectric points. After completion of the run, 80-drop fractions were collected by draining the gradient

solution from the bottom of the column at a flow rate of 1 ml/min with the aid of a Gilson fraction collector.

Since ampholine was found to form a precipitate with ammonium molybdate (2.5% in 5 N H_2SO_4) which was used for P_i assay as previously described in the method of Fiske and SubbaRow (32), it was removed from the fractions before enzyme assays were carried out. Therefore, soon after the determination of pH, fractions within the pH range from 3.0 to 6.0 were dialyzed against 1 M NaCl solution at 4° over night, then against deionized water for 6 hr. The resulting dialyzed fractions were free of ampholine and were assayed for enzyme activity.

Dowex Ion Exchange Chromatography.--Although 2'-AMP, 3'-AMP, and 2',3'-cyAMP could be separated by thin-layer chromatography, the quantitative detection was not sensitive enough to tell whether 2',3'-cyAMP was the exclusive product from the action of pea RNase on synthetic polyadenylic acid. Volkin and Carter (40) have described the separation of 2'-AMP and 3'-AMP on Dowex-1-Cl^-, 400 mesh column. For that procedure, a column of BioRad Ag-1-X2, chloride form, 400 mesh, 0.5 x 5 cm, was first equilibrated with 2 mM HCl and then calibrated by chromatographing the mix of authentic compounds 2' and 3' isomers of adenylic acid and 2',3'-cyAMP. The same ionic strength of HCl was used as the eluting solvent. Fractions of 60-drops (approximately 3.4 ml) were collected with a flow rate of 12 drops per min and

the absorbance measured at 260 mμ. The column was regenerated by washing with 50 ml of 1 N NaOH, 100 ml of distilled water, 50 ml 1 N HCl and 200 ml of 2 mM HCl, in that order.

Experimental Results

Purification of Enzymes

Preparation of Crude Enzyme Extract.--(All procedures in enzyme fractionation were performed at 4° or in ice bath unless otherwise specified.)

Routinely, 100 g of a week-old pea seedlings, germinated in sterile vermiculite in the dark, was homogenized with 100 ml of deionized water for 1-2 min in a commercial Waring Blender. The homogenate was first squeezed through double-layered cheesecloth to remove the bulk of insoluble material, then centrifuged at 4,000 x g for 30 min. The resulting supernatant fluid was decanted through deionized water washed glass wool. The filtrate was taken as the crude enzyme extract for further purification.

Ammonium Sulfate Fractionations.--The crude extract was brought to 50% saturation by slowly adding solid ammonium sulfate (29.5 g per 100 ml of enzyme extract) (41). The solution was stirred for 30 min, and the precipitate was removed by centrifugation at 10,000 x g for 20 min and discarded. The clear supernatant fluid was decanted and brought to 80% saturation with the addition of 19.7 g of

solid ammonium sulfate per 100 ml of extracted solution. After stirring and standing for at least 1 hr, the precipitate was collected by centrifugation at 10,000 x g for 30 min, and dissolved in 2 mM Tris-acetate buffer, pH 7.5, by vigorous mechanical stirring. The resulting enzyme solution was then taken for dialysis.

Dialysis and Freezing of 50-80% Ammonium Sulfate Fraction.--The resulting enzyme solution was dialyzed in 2.3 cm diameter dialysis tubing (Union Carbide) against 20 volumes of 2 mM Tris-acetate buffer, pH 7.5, with constant agitation for 24 hr, with three changes. After dialysis, the enzyme solution was centrifuged at 10,000 x g for 10 min in order to remove a small amount of precipitate which often formed during the dialysis. Although the precipitate contained some activity, the specific activity was too low to be saved. The supernatant fluid was then fractionated further or was frozen at -20°.

DEAE-cellulose Chromatography.--The dialyzed solution, 25 ml (47 mg of protein/ml) was applied to a DEAE-cellulose column (2.5 x 25 cm) which had previously been equilibrated with 2 liters of 0.01 M Tris-acetate buffer (pH 7.5) at a flow rate of 1 ml per min, regulated with a polystaltic pump (Bucher Instruments). After loading the enzyme solution on the column, the adsorbent was washed with 120 ml of the same buffer with which it was equilibrated.

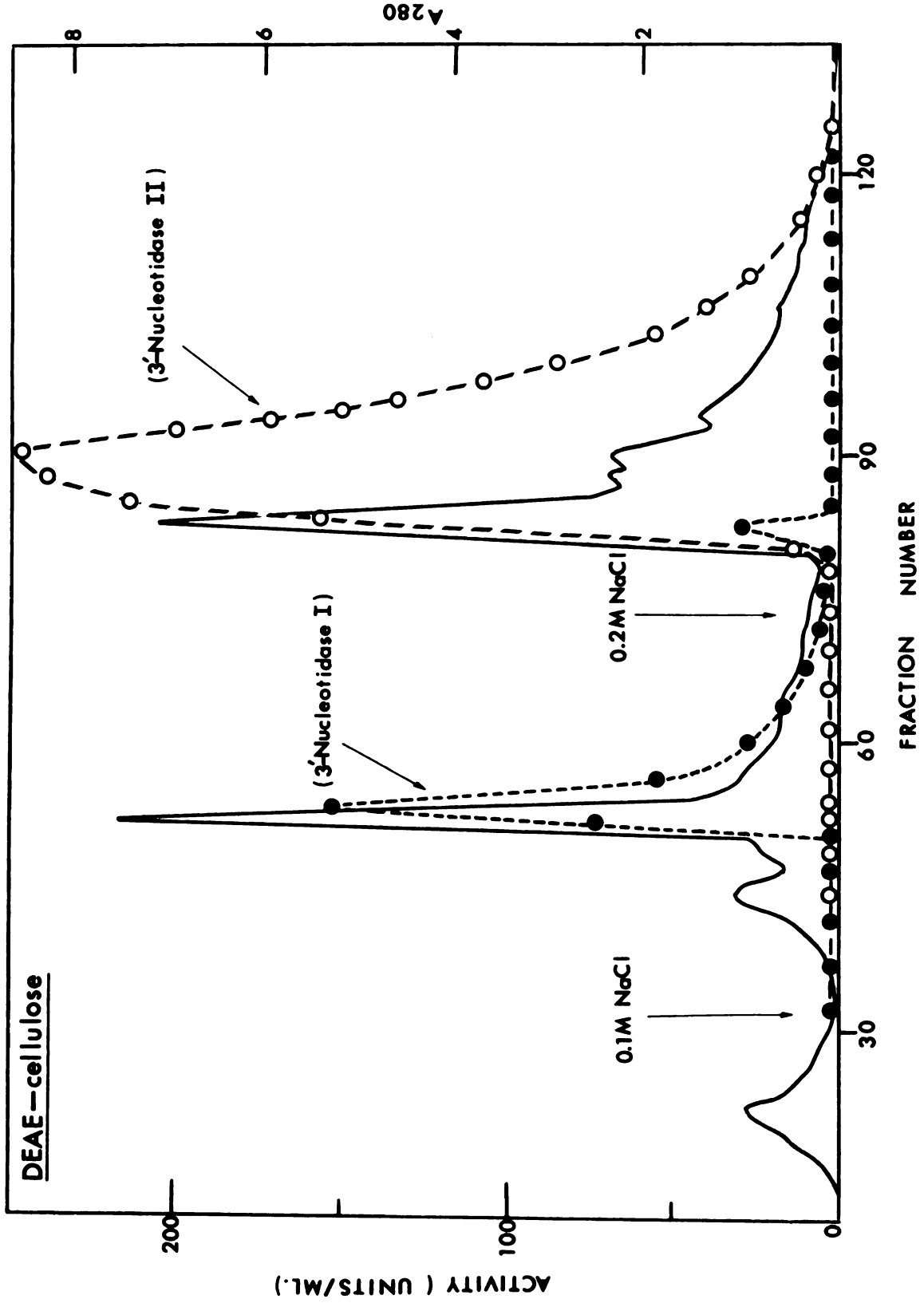
A step-gradient of NaCl solution (prepared in the same buffer as previously mentioned) was applied to the column. Fractions (8 ml) were collected and the absorbance measured at 280 mμ. Enzyme assays were performed as previously described. The residual material in the column was washed out with 1 M NaCl solution, and the column regenerated by the method of Peterson and Sober (42).

The elution profile of protein and enzyme activities is shown in Figure 1. The 3'-nucleotidase activity was located by assaying 0.1 ml of each fraction with either 3'-CMP or 3'-AMP as substrate. Buffer of 0.1 M K-acetate, pH 5.4, was used for the assay of 3'-nucleotidase I activity when 3'-CMP served as substrate. A reaction mixture containing 0.1 M Tris-acetate buffer (pH 8.0), 2 mM $ZnCl_2$, and 2 mM 3'-AMP was used for the assay of 3'-nucleotidase II activity.

Evidently, 3'-nucleotidase I activity was eluted by 0.1 M NaCl solution between fractions 52 and 70, without detectable contamination of 3'-nucleotidase II activity. With 0.2 M NaCl solution, 3'-nucleotidase II activity was eluted between fractions 83 and 104. Although there was a minor amount of 3'-nucleotidase I in the fraction of 3'-nucleotidase II, this impurity could be eliminated by further purification (Sephadex gel filtration). RNase and DNase activities (not shown in the figure) were located mainly between fractions 82 and 120. Fractions containing

Figure 1.--Elution profile of pea 3'-nucleotidase activities from DEAE-cellulose column chromatography.

Dialyzed ammonium sulfate fraction, 25 ml (47.2 mg protein/ml) was applied to a DEAE-cellulose column (2.5 x 25 cm) previously equilibrated with 2 liters of 0.01 M Tris-acetate buffer (pH 7.5) at a flow rate of 1 ml/min. After layering the enzyme preparation on the column and washing with 120 ml of the same buffer. Two step gradients of NaCl concentration (prepared in the same buffer), 0.1 M and 0.2 M, were applied to the column as indicated by vertical arrows. Eight-ml fractions were collected, the absorbance measured at 280 mμ, and the fractions assayed for enzyme activity. The solid line indicates protein concentration as measured by absorbance at 280 mμ. Dashed lines represent enzyme activities (in units/ml) of 3'-nucleotidase I (3'-CMP as substrate, pH 5.4, ●----●) and 3'-nucleotidase II (3'-AMP as the substrate, pH 8.0, 0-----0). Detailed experimental procedures are given in the text and "Methods."


DEAE—cellulose
(3'-Nucleotidase II)
(3'-Nucleotidase I)
0.2M NaCl
0.1M NaCl
A280
ACTIVITY (UNITS/ML.)
FRACTION NUMBER
0
100
200
30
60
90
120
2
4
6
8

25 units or more per ml for 3'-nucleotidase I and 50 units or more per ml for 3'-nucleotidase II were pooled separately. The recoveries of enzyme activities were 20% for 3'-nucleotidase I, 78.5% for 3'-nucleotidase II and 80.1% for RNase (Tables 1 and 2). However, about 15.6% of the applied protein was present in the fraction of 3'-nucleotidase I while 45.5% was present in the fraction of 3'-nucleotidase II and RNase. Therefore, the specific activities for these enzymes were not much increased.

Chromatography of 3'-Nucleotidase I on Sephadex G-100 Column.--Sephadex G-100 gel, 40-120 μ bead size, was allowed to swell in an excess of water on boiling water bath for 5 hr (43). The resulting swollen gel was cooled and packed in a column 1.5 x 90 cm which was equilibrated by washing with 1 liter of 0.01 M Tris-acetate buffer, pH 7.5, at a flow rate of 12 ml per hr.

The 3'-nucleotidase I preparation from DEAE-cellulose column chromatogram was concentrated from 20 ml (1.1 mg of protein per ml) to 5.5 ml with 3.1 g of lyphogel at 4° for 12 hr. The specific activity was maintained about 56 units per mg of protein after concentration. Following the application of the concentrated enzyme solution (5.5 ml), the column was eluted with 0.01 M Tris-acetate buffer, pH 7.5, in 3-ml fractions. The elution profile of protein and enzyme activity is shown in Figure 2.

TABLE 1.--Summary of purification of 3'-nucleotidase I from 100 g of pea seedlings.

Fraction	Total Protein	Total Activity	Specific Activity	Yield
	mg	units	units/mg	%
4,000 x g supernatant	1569	27,787	18	100
50-80% $(NH_4)_2SO_4$	277	12,140	44	43.7
DEAE-cellulose	43.2	2,430	56	8.7
Sephadex G-100 filtration	2.6	262	100[a]	0.9

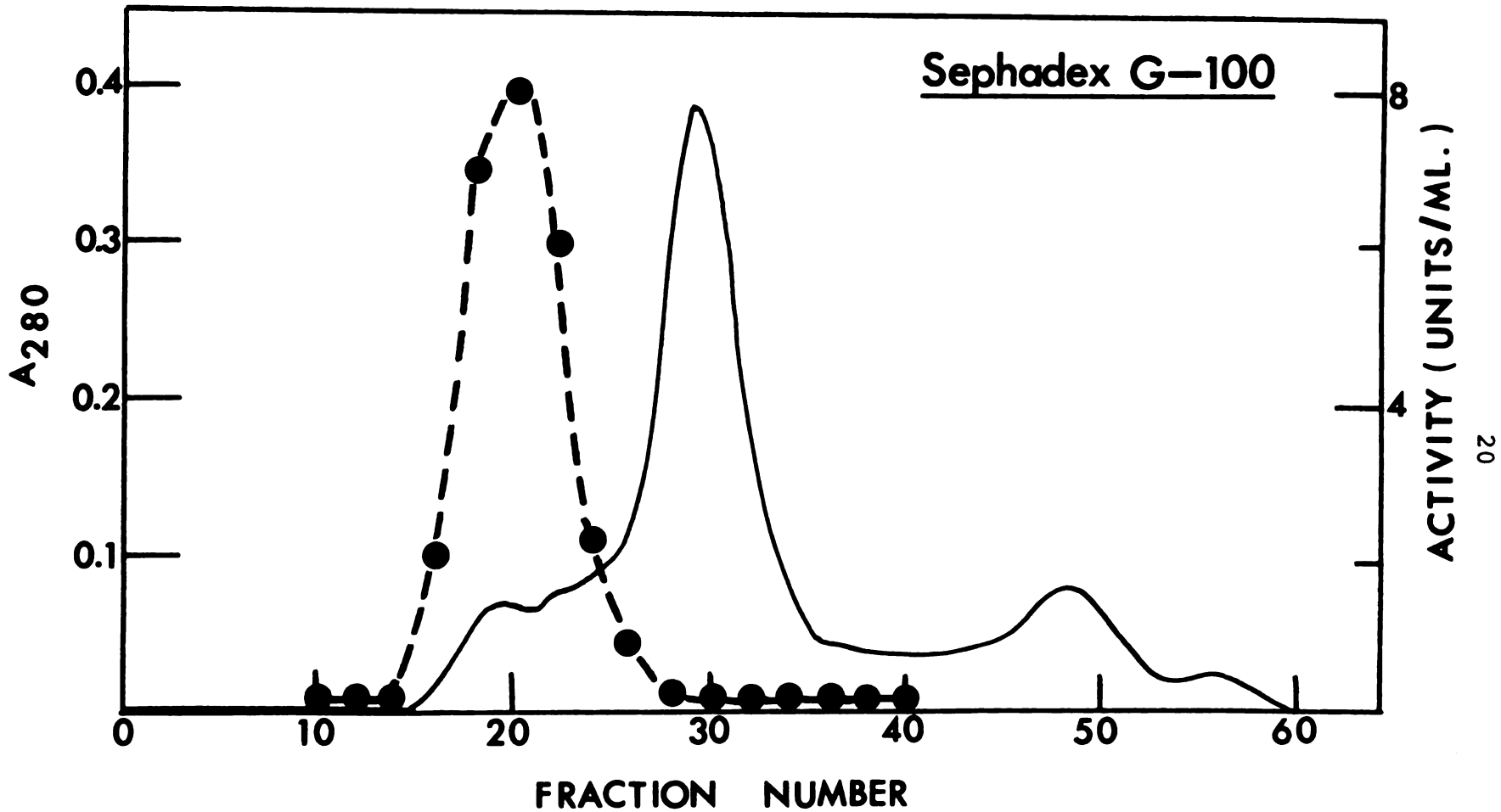
[a]The low specific activity was due to enzyme denaturation or to the presence of protein species which showed similar electrophoretic mobilities as described in Figure 7.

TABLE 2.--Summary of purification of 3'-nucleotidase II and RNase from 100 g of pea seedlings.

Fraction	Total Protein	3'-Nucleotidase II			RNase			Ratio of B'-Nucleotidase II / RNase in Sp. Act.
		Total Activity	Specific Activity	Yield	Total Activity	Specific Activity	Yield	
	mg	units	units/mg	%	units	units/mg	%	
4,000 x g Supernatant	1569	64,125	41	100	44,000	28	100	1.5
50-80% $(NH_4)_2SO_4$	277	24,400	88	38	15,470	56	35	1.6
DEAE-cellulose	126	19,200	152	30	12,426	99	28	1.5
Sephadex G-75	24	14,594	605	22	10,025	416	22	1.5

Figure 2.--Chromatography of 3'-nucleotidase I on Sephadex G-100 column.

An amount of 5.5 ml of lyphogel concentrated DEAE-cellulose enzyme fraction containing 20 mg of protein was applied to a column (1.5 x 90 cm) which had been equilibrated with 0.01 M Tris-acetate buffer (pH 7.5) and eluted with the same buffer in 3 ml fractions. The flow rate was maintained at 12 ml/hr. Enzyme assays were performed as described under "Methods." Protein concentration was measured as the absorbance at 280 mμ, ———. 3'-Nucleotidase I activity (3'-CMP as substrate, pH 5.4), ●------●.


Sephadex G—100
0.4
0.3
0.2
0.1
A_{280}
8
4
ACTIVITY (UNITS/ML.)
0
10
20
30
40
50
60
FRACTION NUMBER

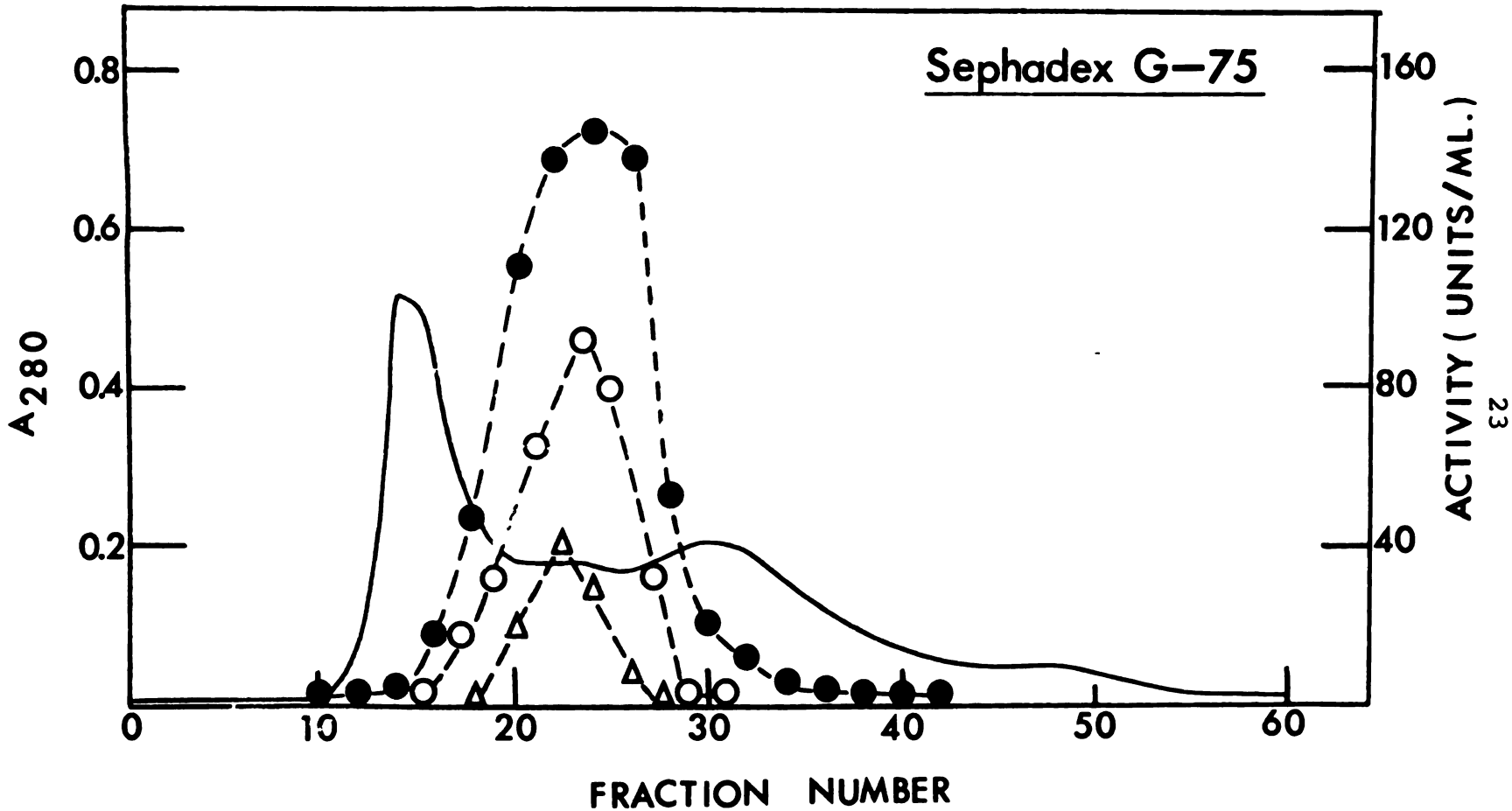
Although about 90% of the applied protein was removed from the major enzyme fraction, the recovery of enzyme activity was only 10%. Therefore, it was only about 2-fold increased in specific activity. The reason for such low recovery is given in the "Discussion." The enzyme preparation was stored in 3 ml quantities at -20°. The summary of the purification of 3'-nucleotidase I from 100 g of pea seedlings is given in Table 1, to which all values were calculated based on 3'-CMP as substrate.

Chromatography of 3'-Nucleotidase II on Sephadex G-75 Column.--The procedure for the preparation of a Sephadex G-75 column (1.5 x 60 cm) was essentially the same as described for Sephadex G-100 column with the exception that the flow rate was 14 ml per hr. The 3'-nucleotidase II preparation from the DEAE-cellulose column chromatogram, 5 ml, containing about 40 mg of protein, was applied to a column which had been equilibrated with 0.01 M Tris-acetate buffer (pH 7.5) and eluted in 4-ml fractions. Figure 3 shows the elution profile of protein and enzyme activities. This purification step routinely gave 80% recovery of 3'-nucleotidase II and RNase activities applied to the column, and an increase in specific activity of about 4-fold.

The enzume activities toward 3'-AMP, RNA and denatured DNA were eluted between fractions 15 and 30. The peaks of the three activities coincided at the same fraction. Further attempts to separate these three activities

Figure 3.--Chromatography of 3'-nucleotidase II on Sephadex G-75 column.

Five ml of lyphogel concentrated DEAE-cellulose enzyme preparation containing 40 mg protein was applied to a column (1.5 x 60 cm) which was equilibrated with 0.01 M Tris-acetate buffer (pH 7.5) and eluted with the same buffer in 4 ml fractions. The flow rate was 14 ml/hr. Enzyme assays were performed as described under "Methods." Protein concentration was measured as the absorbance at 280 mμ, ———. The 3'-nucleotidase II activity (3'-AMP as substrate, pH 8.0), •----•; RNase, ∘----∘; DNase, Δ----Δ.


Sephadex G—75
0.8
0.6
0.4
0.2
A280
160
120
80
40
ACTIVITY (UNITS/ML.)
0
10
20
30
40
50
60
FRACTION NUMBER

on Sephadex G-200 was unsuccessful. The enzyme preparation at this step was stored at -20°, over a period of 5 months without any significant loss of activity. The summary of the purification of 3'-nucleotidase II and RNase from 100 g of pea seedlings is given in Table 2.

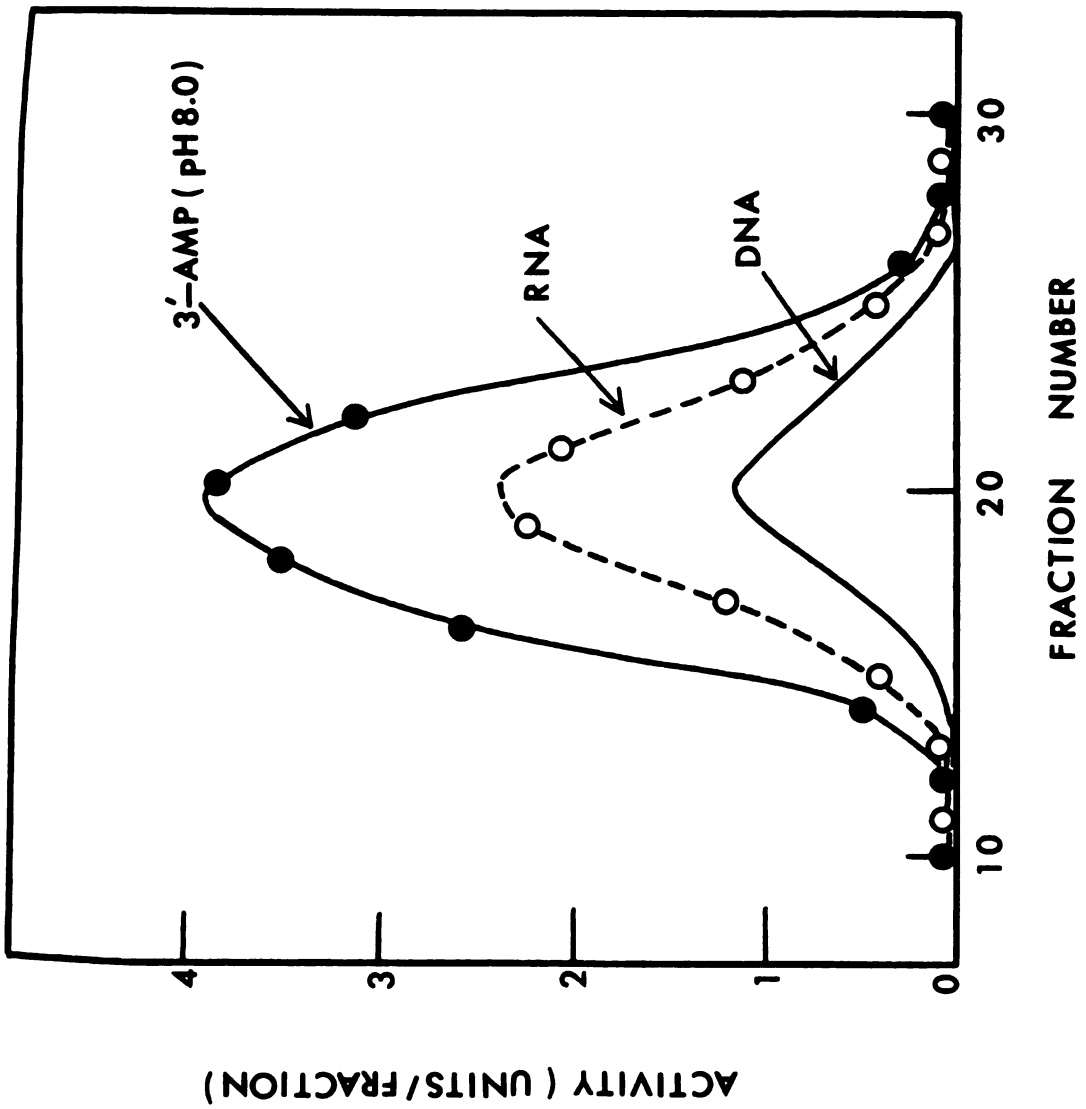
Sucrose Density Gradient Centrifugation.--Sucrose density gradients were prepared as described in "Methods." An amount of 0.2 ml of lyphogel concentrated enzyme solution of 3'-nucleotidase II which had been partially purified from Sephadex G-75 gel filtration (210 enzyme units toward 3'-AMP per ml) was layered on a precooled and equilibrated sucrose density gradient. The tube was centrifuged for 18 hr at 60,000 r.p.m. in a Beckman L2-65B ultracentrifuge with the temperature maintained at 2°. After centrifugation, 10-drop fractions were collected and assayed for enzyme activities as previously described.

The enzyme activities toward 3'-AMP, RNA and denatured DNA were eluted in an identical pattern (Figure 4). Recovery in each case was about 57%.

Electrofocusing Column Chromatography of 3'-Nucleotidase II.--Although the activities toward 3'-AMP, RNA, and denatured DNA appeared to purify together as in the previous results, it was desirable to have additional evidence bearing on the question of their identity. I therefore attempted further purification by the technique of electrofocusing.

Figure 4.--Elution pattern of 3'-nucleotidase II, RNase, and DNase from sucrose density gradient centrifugation.

The lyphogel concentrated enzyme solution, 0.2 ml (210 units toward 3'-AMP at pH 8.0/ml) of 3'-nucleotidase II from Sephadex G-75 preparation, was layered over a 5 to 20% sucrose density gradient (4.6 ml). The sucrose density gradient was prepared in 0.1 M Tris-acetate buffer, pH 7.5. Centrifugation was performed at 60,000 rpm with SW 65 L Ti swinging bucket rotor in a Beckman L2-65B ultracentrifuge for 18 hr. Temperature was maintained at 2°. After centrifugation, 10-drop fractions, total of 40 fractions, were collected and assayed for enzyme activity with different substrates in the alternative fractions. Details are given in the text. Enzyme activity of 3'-nucleotidase (3'-AMP as substrate), ●——●; RNase, 0----0; DNase, ———.


3'-AMP (pH 8.0)
RNA
DNA
ACTIVITY (UNITS/FRACTION)
FRACTION NUMBER
0
1
2
3
4
10
20
30

Ten ml of enzyme of 3'-nucleotidase II preparation (partially purified from Sephadex G-75) was dialyzed against 20 volumes of deionized water overnight in order to reduce the salt content to less than 0.5 μmoles. The small amount of precipitate formed during dialysis was removed by centrifugation and discarded. The resulting clean supernatant enzyme solution was subjected to electrofocusing as described under "Methods." After applying a potential of 350 volts to the column for a period of 48 hr, 80-drop fractions were collected. As mentioned in "Methods," the fractions in the pH range of 3.6-6.0 were dialyzed against 1 M NaCl and deionized water in order to remove the ampholine. After dialysis, enzyme assays were performed with the standard methods. As shown in Figure 5-B, it is evident that 3'-nucleotidase II (with 3'-AMP as substrate) and RNase were eluted in an identical pattern with an isoelectric point at pH 4.8. However, the ratio of enzyme activities toward 3'-AMP and RNA was not the same as those previously observed in the earlier steps of the enzyme purification scheme. The recovery of activity was 30% for 3'-nucleotidase II and 11% for RNase. This discrepancy may be due to enzyme instability at low pH (Table 6).

Electrofocusing Column Chromatography of 3'-Nucleotidase I.--Ten ml of enzyme solution of 3'-nucleotidase I (dialyzed ammonium sulfate fraction with low salt content, containing 200 units of activity toward 3'-CMP per ml) was

added to the column. After completion of the electrofocusing, the eluted fractions (dialyzed against 1 M NaCl overnight and against deionized water for 6 hr) were assayed for enzyme activity. With 3'-CMP as substrate, bands of activity were found at pH 4.7 and 5.3 (Figure 5-A). The low recovery (7%) of enzyme activity was probably due to the absence of reducing reagents (sulfhydryl compounds) during the electrofocusing and dialysis.

Polyacrylamide Disc-Gel Electrophoresis of 3'-Nucleotidase II.--With a 7% polyacrylamide gel at pH 8.5 as described under "Methods," 0.3 ml of 3'-nucleotidase II enzyme preparation from Sephadex G-75 fraction containing 54 µg of protein (with 36 units toward 3'-AMP, or 24 units toward RNA) was subjected to electrophoresis. The electrophoresis was performed with the addition of one drop of 0.005% of bromophenol blue (Fisher Scientific Co.) as a tracking dye. When the dye band reached the end of the gel, the current was turned off, and the gels removed and cut in half horizontally and either stained with Coomassie blue or assayed for enzyme activities. For assay of enzyme activities, one of the half gels was cut into 3 mm segments and assayed for 3'-nucleotidase II and RNase activities in alternate segments at 37° for 10 hr.

Figure 6 shows the staining pattern of protein and the distribution of enzyme activities within the gel after electrophoresis. It is evident that 3'-nucleotidase II and

Figure 5.--Elution profiles of pea 3'-nucleotidase from an electrofocusing column chromatography.

(A) Elution profile of 3'-nucleotidase I.

Ten ml of enzyme preparation (200 units toward 3'-CMP at pH 5.4/ml) as described in the text was applied to the column. Enzyme activity was assayed with 3'-CMP as substrate. Detailed experimental procedures are described under "Methods." Solid line represents the pH gradient. Enzyme activity (in units/fraction) is shown by 0----0. Ampholyte, pH 3.0 to 6.0, was used in this study.

(B) Elution profile of 3'-nucleotidase II.

Ten ml of 3'-nucleotidase II preparation (120 units/ml toward 3'-AMP at pH 3.0, partially purified from sephadex G-75 and dialyzed against 20 volumes of deionized water over night) was applied to the column. Ampholyte, pH 3.0 to 6.0, was used in this study. pH gradient, ———; 3'-nucleotidase (assayed with 3'-AMP at pH 8.0) ●———●; RNase, 0----0.

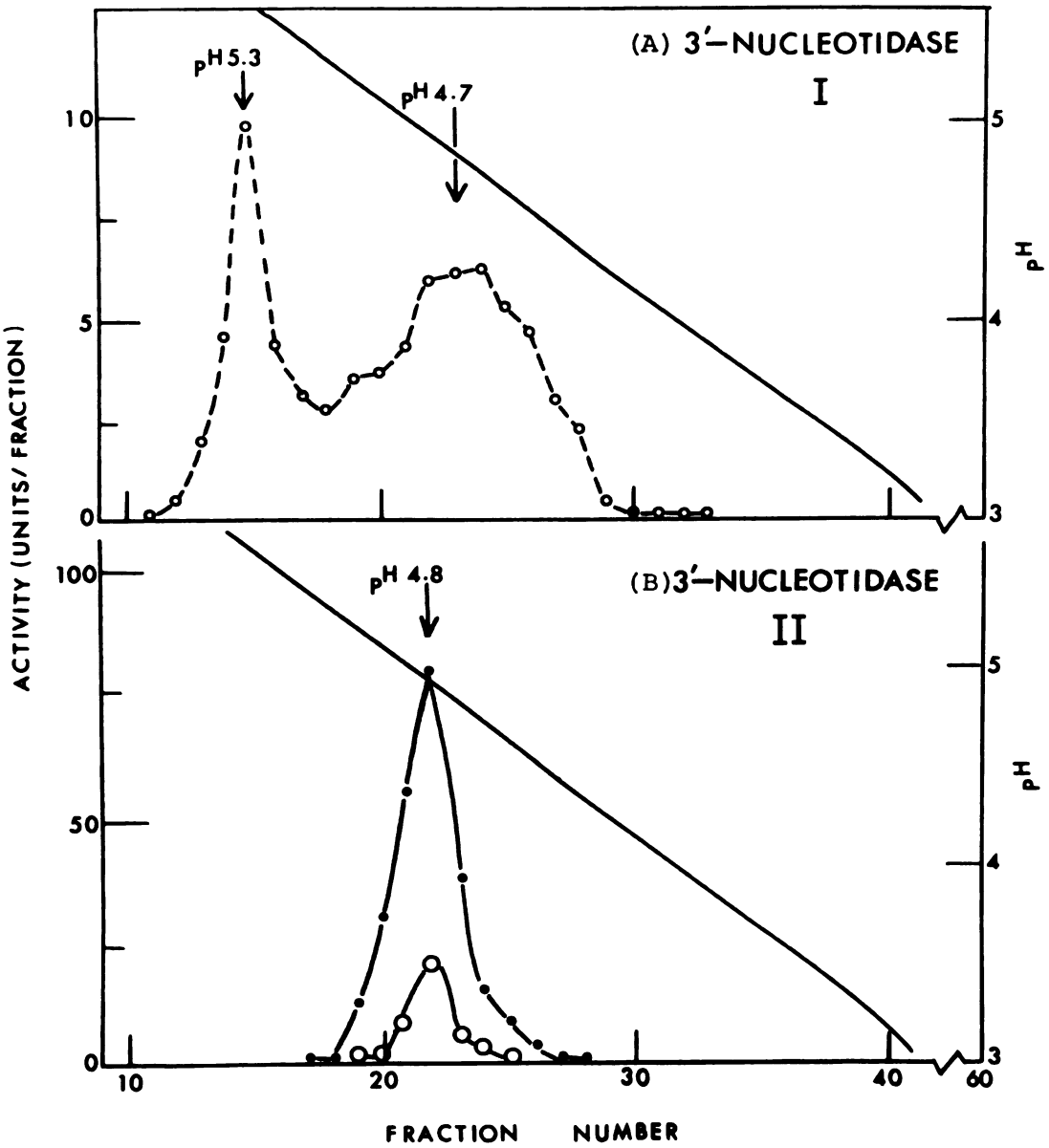
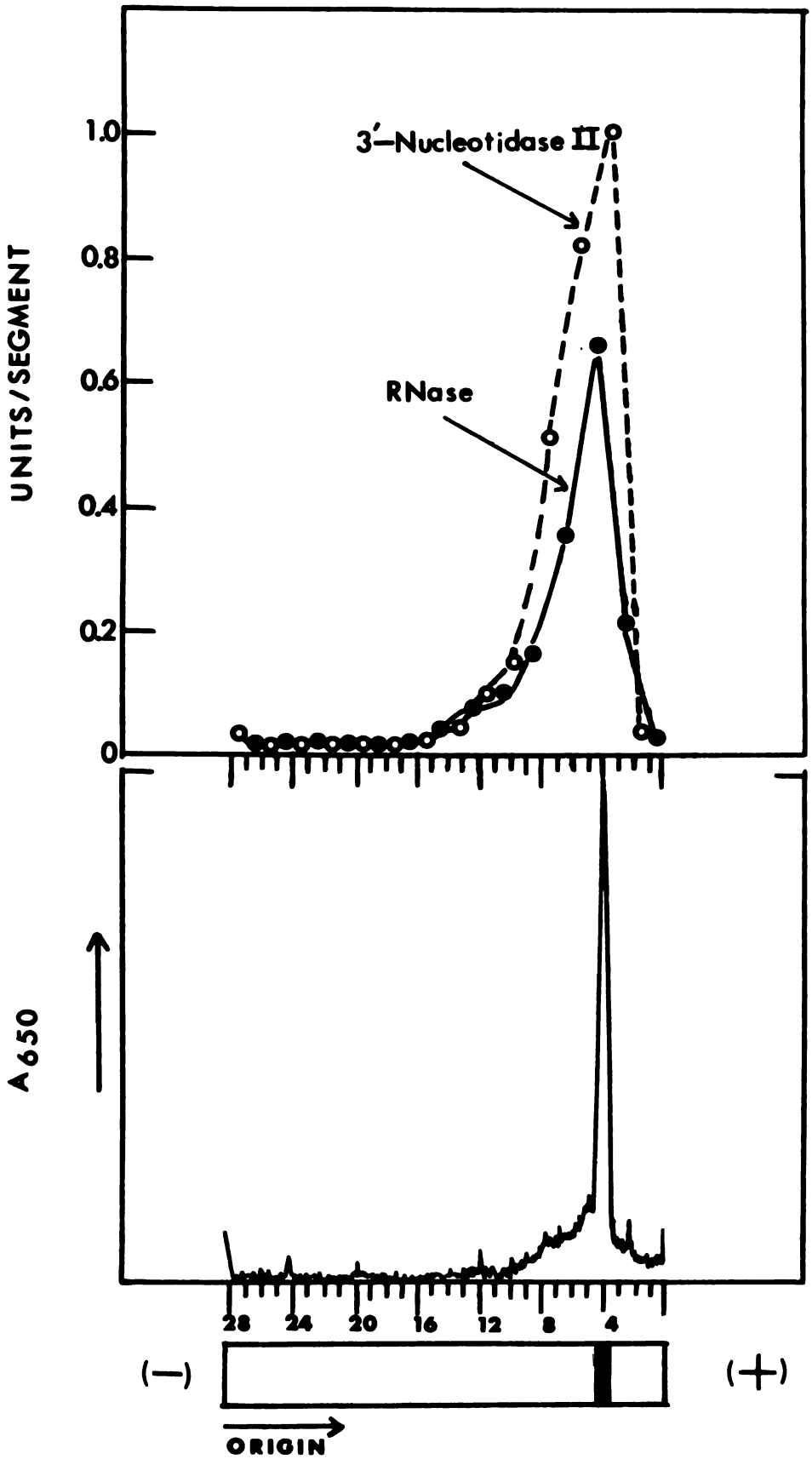

(A) 3′–NUCLEOTIDASE
I
pH 5.3
pH 4.7
(B) 3′–NUCLEOTIDASE
II
pH 4.8
ACTIVITY (UNITS/ FRACTION)
pH
FRACTION NUMBER
0
5
10
50
100
3
4
5
10
20
30
40
60

Figure 6.--The staining pattern of protein and the distribution of RNase and 3'-nucleotidase II on a polyacrylamide gel after electrophoresis.

A 0.3 ml quantity of enzyme preparation (3'-nucleotidase II) containing 54 µg of protein was subjected to electrophoresis in 7% gel as described under "Experimental Procedures." Electrophoresis at pH 8.5 was performed at 4° for 45 min with an applied current of 2 mA per tube. After the run, the gel was cut in half; one half was stained for protein with Coomassie blue dye, and the other half was cut into 3 mm segments. The 3'-nucleotidase and RNase activities were assayed in alternate segments with 3'-AMP and ribosomal RNA as substrate, respectively. Detailed procedures for enzyme assays are described under "Methods." The absorbance of the protein stained at 650 mµ was measured as described in the text. Enzyme activity: 3'-nucleotidase, 0----0; RNase ●——●.

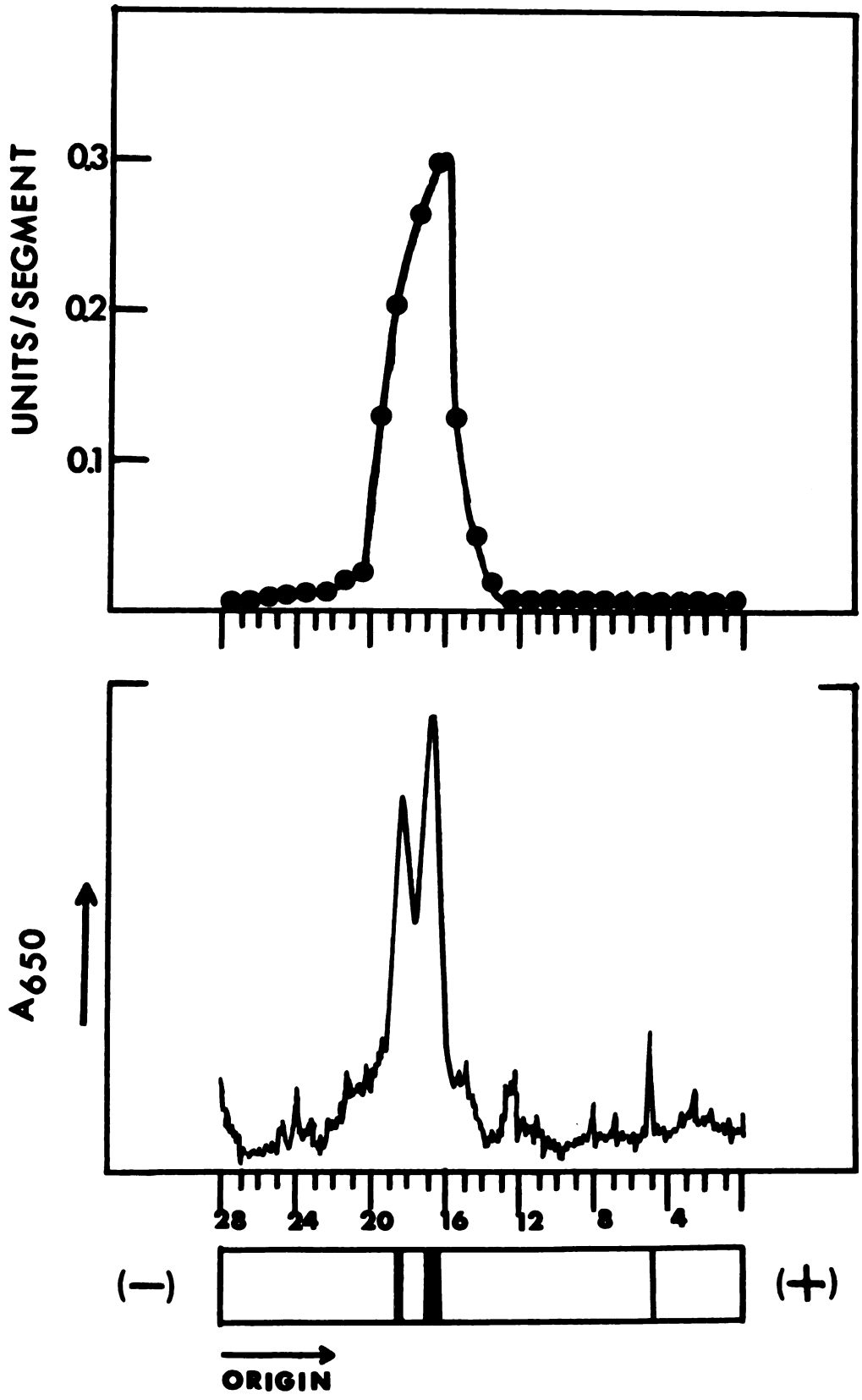

UNITS/SEGMENT
1.0
0.8
0.6
0.4
0.2
0
3'-Nucleotidase II
RNase
A650
28
24
20
16
12
8
4
(−)
(+)
ORIGIN

RNase activities were found to be associated with the only major detectable protein band and their recoveries were similar, 22% and 24%, respectively. The resulting protein profile of the gel as measured at 650 mμ indicated about 90% of total protein in the preparation was located in the band containing 3'-nucleotidase II and RNase activities. Because there was 1.6% recovery of protein as shown in Table 2, the 3'-nucleotidase from Sephadex G-75 fraction seems to represent 1.4% of total protein in the 4,000 x g supernatant (or 0.2% of protein of seedlings homogenate). Separation with a 10% gel gave essentially the same pattern in protein staining and enzyme distribution as that observed in the 7% gel.

Polyacrylamide Disc-Gel Electrophoresis of 3'-Nucleotidase I.--With the same procedures as previously described for 3'-nucleotidase II, 0.3 ml of lyphogel concentrated enzyme preparation of 3'-nucleotidase I from Sephadex G-100 fraction containing 20 μg of protein and 5.2 units toward 3'-CMP was subjected to electrophoresis in 7% acrylamide gel. The staining pattern of protein and the distribution of enzyme activity is shown in Figure 7. Apparently, the enzyme preparation still contained a number of protein species. One major contaminating protein bands was found. About 30% of the applied enzyme activity was recovered.

Figure 7.--The staining pattern of protein and distribution of 3'-nucleotidase I on a polyacrylamide gel after electrophoresis.

A 0.3 ml of lyphogel concentrated enzyme of 3'-nucleotidase I preparation containing 20 µg of protein was applied to a 7% polyacrylamide gel. Details were the same as described in Figure 6. Enzyme activity was assayed with 3'-CMP as substrate.


UNITS/SEGMENT
0.3
0.2
0.1
A650
28
24
20
16
12
8
4
(—)
(+)
ORIGIN

Properties of the Enzyme Preparations

Rate of Hydrolysis as a Function of pH and Zn^{++}.--Figure 8 shows the enzyme activity as a function of pH using K-acetate and Tris-acetate as buffers. The pH optimum of 3'-nucleotidase I (with 3'-AMP as substrate) as shown in Figure 8-A was in the range of pH 5.4-5.7 with no significant activity at pH 7.5 or higher pH values. In contrast to 3'-nucleotidase I, 3'-nucleotidase II was shown to have an optimum pH at 8.0, with less than 50% activity at pH 6.5 (Figure 8-B). However, with RNA as substrate, 3'-nucleotidase II showed a pH optimum around 6-7.

Furthermore, as shown in Table 3, the pH optimum of 3'-nucleotidase I varied from 5.0 to 5.7 depending on the substrate used. The addition of $ZnCl_2$ at a concentration of 2 mM shifted the optimal pH to a lower pH value, about 4.7, with 50% inhibition on enzyme activity. However, the pH optima for 3'-nucleotidase II were the same, 8.0, on all 3'-nucleotides except 3'-CMP which the enzyme could not attack. The addition of 2 mM $ZnCl_2$ apparently had no effect on the pH optimum of 3'-nucleotidase II, but slightly increased (20%) the enzyme activity.

Effect of Metal Ions and Inorganic Ions on Enzyme Activity.--The effect of various metal ions and inorganic ions on 3'-nucleotidase activity was tested. All metal ions and inorganic ions were added at zero time to the

Figure 8.--Effect of pH on the activity of pea 3'-nucleotidase.

The reaction mixture and experimental procedures were as described for the standard assay. 3'-nucleotidase I, (A); 3'-nucleotidase II, (B). Buffer used: 0.1 M K-acetate, 0——0; 0.1 M Tris-acetate, ●——●.

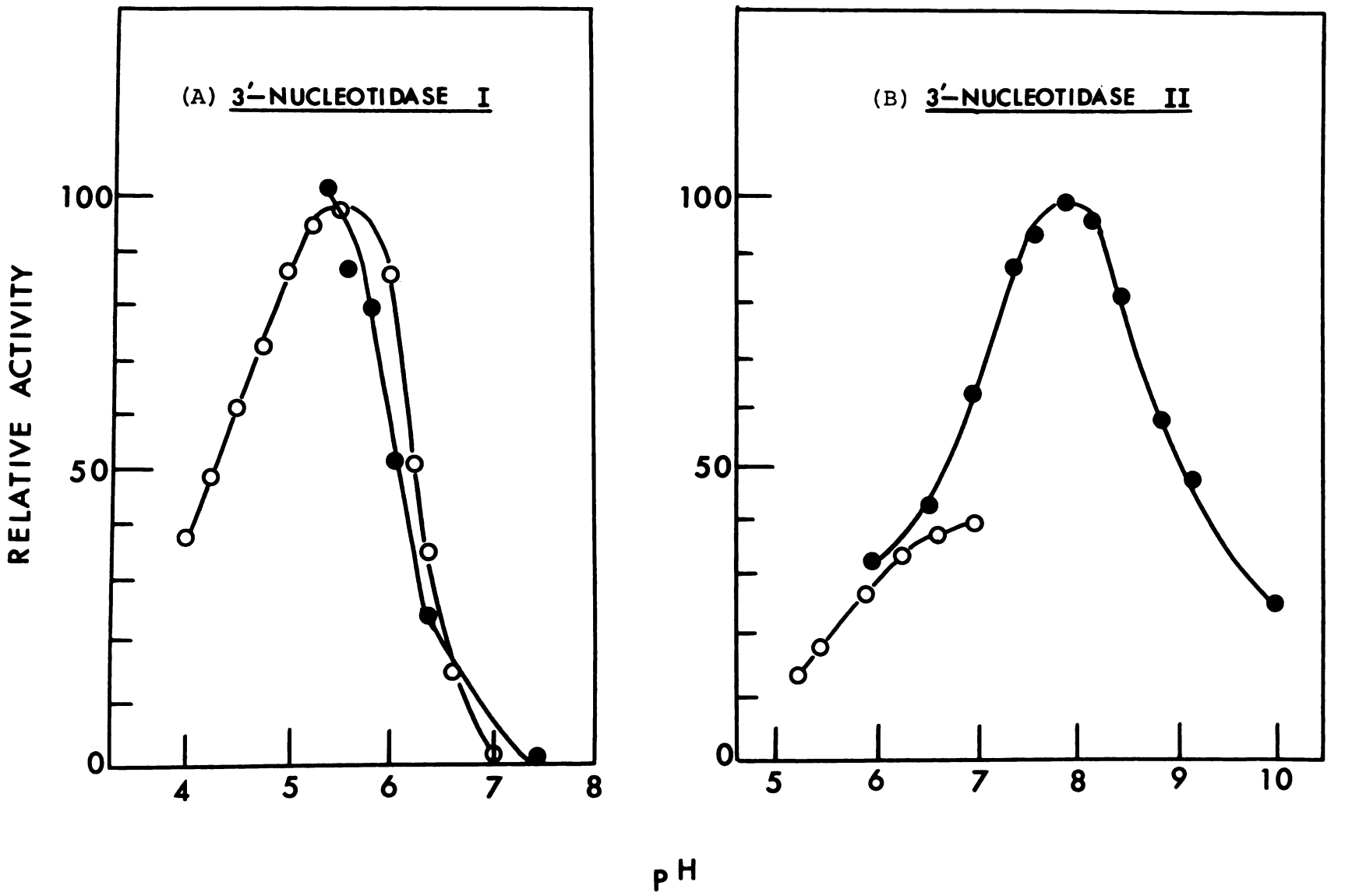

(A) 3′-NUCLEOTIDASE I
(B) 3′-NUCLEOTIDASE II
RELATIVE ACTIVITY
100
50
0
4
5
6
7
8
5
6
7
8
9
10
pH

TABLE 3.--Effect of Zn^{++} on the optimum pH for the activities of 3'-nucleotidase I and 3'-nucleotidase II.[a]

Substrate Used	Optimum pH of 3'-Nucleotidase I		Optimum pH of 3'-Nucleotidase II	
	$-Zn^{++}$	$+Zn^{++}$	$-Zn^{++}$	$+Zn^{++}$
3'-AMP	5.7	4.8	8.0	8.0
3'-GMP	5.4	4.7	8.0	8.0
3'-UMP	5.0	4.6	8.1	8.0
3'-CMP	5.4	4.7	---	---
2'-AMP	5.6	4.7	---	---
5'-AMP	5.6	4.7	---	---

[a]Experimental conditions were as described for the standard assay system except with or without addition of 2 mM $ZnCl_2$ in the reaction mixture. Buffers used as given in Figure 8: 0.1 M K-acetate, pH 4.0 to 7.0; 0.1 M Tris-acetate, pH 5.5 to 10.0. Inorganic phosphate released was determined as described under "Methods." --- denotes no detectable inorganic phosphate released.

standard reaction mixture as described in the "Methods" except that the buffer used was Tris-acetate for the assay of both 3'-nucleotidase I and 3'-nucleotidase II. The final concentration of the additives were 1 mM and the reactions were carried out at 37° for 30 min with 5 units of enzyme preparation. The variation in enzyme activity due to the presence of metal ion or inorganic ion is summarized in Table 4. It is evident that divalent cations, Mg^{++}, Mn^{++}, Co^{++}, and Zn^{++} showed 4%, 22%, 28%, and 50% inhibition respectively on 3'-nucleotidase I activity while EDTA and monovalent cations (K^{+}, NH_4^{+}, Na^{+}) showed no effect. Imidazole caused a 33% inhibition.

In contrast to 3'-nucleotidase II activity none of the ions lead to major increase or decrease in 3'-nucleotidase I activity except EDTA which gave an inhibition of 60%.

Effect of Sulfhydryl Compounds on Enzyme Activity.--Effect of various sulfhydryl compound such as glutathione, cysteine, and dithiothreitol on 3'-nucleotidase activity was determined with a standard reaction mixture at 37° for 30 min. Table 5 shows that there was little effect of sulfhydryl compounds on 3'-nucleotidase I activity except that at 4 mM there was 10-50% activation. However, sulfhydryl compounds at concentrations as low as 0.4 mM gave almost complete inhibition of 3'-nucleotidase II activity.

TABLE 4.--Effect of inorganic ions, caffeine and theophylline on 3'-nucleotidase activity.

Addition[a]	3'-Nucleotidase I	3'-Nucleotidase II
	Activity Remaining (%)[b]	
None	100	100
$MgCl_2$	96	102
$MnCl_2$	78	---
$CoCl_2$	72	118
$ZnCl_2$	50	109
$(NH_4)_2SO_4$	92	100
KCl	101	100
NaCl	100	100
Imidazole	67	---
EDTA	100	42
NaF	42	83
Caffeine	104	100
Theophylline	101	101

[a]The final concentration of the added reagent was 1 mM.

[b]All reaction mixtures contained 0.05 M Tris-acetate buffer, pH 5.6 for assay 3'-nucleotidase I or 0.05 M Tris-acetate buffer, pH 8.0 for assay 3'-nucleotidase II.

TABLE 5.--Effect of various sulfhydryl compounds on 3'-nucleotidases activity.[a]

Concentration of Sulfhydryl Compound Added	3'-Nucleotidase I	3'-Nucleotidase II
	Relative Activity (%)	
None	100	100
Glutathione		
4×10^{-4}M	100	2.5
1×10^{-3}M	102	1.8
2×10^{-3}M	103	1.2
4×10^{-3}M	111	0.9
Cysteine		
2×10^{-4}M	101	5.4
4×10^{-4}M	100	2.0
1×10^{-3}M	126	1.5
2×10^{-3}M	131	0.9
4×10^{-3}M	135	0.2
Dithiothreitol		
4×10^{-4}M	102	2.2
1×10^{-3}M	114	1.0
2×10^{-3}M	126	0.8
4×10^{-3}M	151	0.6

[a]The standard reaction mixture (0.5 ml) contained 10 units of enzyme and assayed for 3'-nucleotidase activity as described under "Methods."

Effect of Glycine and Zn^{++} on the Inactivation of 3'-Nucleotidase II at pH 5.0.--From the previous studies, 3'-nucleotidase II was shown to be unstable at pH values below 6.0. Since glycine and Zn^{++} have been suggested to be factors that can prevent such an inactivation of 3'-nucleotidase in mung bean sprouts (12), it was desirable to test whether such protection occurred in the pea enzyme system.

The test proceeded as follows. Reaction mixture, 0.5 ml, containing 10 units of enzyme, 0.1 M K-acetate buffer and additions as shown in Table 6 was allowed to stand at 23° for 14 hr. After standing, 0.5 ml of 0.5 M Tris-acetate buffer, pH 8.0, was added to the reaction mixture and enzyme assay was carried out with the addition of 2 mM of 3'-AMP as previously described. The result as summarized in Table 6 indicated that Zn^{++} protected both 3'-nucleotidase II activity and RNase activity. But, glycine showed no effect on enzyme activity.

Substrate Specificity, Relative Activities and Km Study.--Since nucleotidases prepared from other plant sources have been shown to exhibit specificity toward purine 3'-nucleotides, it was desirable to know whether or not the pea nucleotidases also demonstrated such a specificity. Standard assay conditions were employed for each substrate which was present at a final concentration of 2 mM. An amount of enzyme (about 5 units) which was within

TABLE 6.--Effect of various concentrations of Zn^{++} and glycine on acidic inactivation of 3'-nucleotidase II and RNase.[a]

Additions	Activity Remaining (%)	
	3'-Nucleotidase II	RNase
Control (pH 7.5)	100	100
pH 7.0 treatment	98.7	94.3
pH 6.5 treatment	94.3	92.5
pH 6.0 treatment	36.2	45.2
pH 5.0 treatment (no addition)	10.8	7.5
Zn^{++}, 2×10^{-2}M	13.6	19.2
Zn^{++}, 2×10^{-3}M	67.8	48.5
Zn^{++}, 2×10^{-4}M	62.8	40.7
Zn^{++}, 2×10^{-5}M	17.2	25.6
Zn^{++}, 2×10^{-6}M	16.5	14.2
Zn^{++}, 2×10^{-7}M	11.6	12.7
Glycine, 2×10^{-2}M	11.2	---
Glycine, 2×10^{-3}M	13.2	---
Glycine, 2×10^{-3}M + Zn^{++}, 2×10^{-5}M	18.5	---

[a]The solution, 0.5 ml, containing 10 units of enzyme purified from Sephadex G-75, 0.1 M Tris-acetate buffer and the reagents shown in the table were allowed to stand at room temperature for 14 hr. The solution was assayed as described under "Methods."

the linear range of experiment was added to each reaction mixture. The relative rates of hydrolysis, expressed as percent of maximum activity, for all nucleotides tested is given in Table 7. It shows clearly that 3'-nucleotidase I has a high specificity toward 3'-nucleotides and not toward 2'- or 5'-nucleotides. However, it did not show specificity toward purine or pyrimidine 3'-nucleotides. The 3'-nucleotidase II showed specificity for purine 3'-nucleotides. That appreciable cleavage of 3'-UMP also occurred. There was no detectable hydrolysis of 3'-CMP, 2'-AMP, and 5'-AMP.

The effect of substrate concentration on the rate of hydrolysis was studied with the standard assay condition. The Km values were calculated from the Lineweaver and Burk plot of 1/S vs. 1/V and found to be as follows for 3'-nucleotides with 3'-nucleotidase I as enzyme source: 0.6 mM for 3'-AMP or 3'-CMP, 0.75 mM for 3'-GMP, and 0.8 mM for 3'-UMP. With 3'-nucleotidase II, the Km value was 0.35 mM for 3'-AMP, 0.45 mM for 3'-GMP, and 0.62 mM for 3'-UMP. It is apparent that the affinity constants of the respective substrate decrease in following order: 3'-AMP, 3'-CMP > 3'-GMP > 3'-UMP for 3'-nucleotidase I and 3'-AMP > 3'-GMP > 3'-UMP for 3'-nucleotidase II.

Activity Toward Cyclic Nucleoside Monophosphates.--RNases purified from various plant sources have been shown to have a rather weak activity toward 2',3'-cyNMP, the

TABLE 7.--Relative 3'-nucleotidase activities toward ribonucleoside monophosphates.

Mononucleotide Assayed[a]	Relative Activity (%)		
	Pea		Rye Grass
	3'-Nucleotidase I	3'-Nucleotidase II	3'-Nucleotidase[b]
3'-AMP	83	100	60
3'-GMP	95	85	100
3'-UMP	100	48	5
3'-CMP	80	0	0
2'-AMP	15	0	0
5'-AMP	12	0	0

[a]Assays were carried out as described under "Methods" with 4 mM substrate.

[b]Rye grass 3'-nucleotidase (purchase from Sigma Co.) was assayed at pH 7.5.

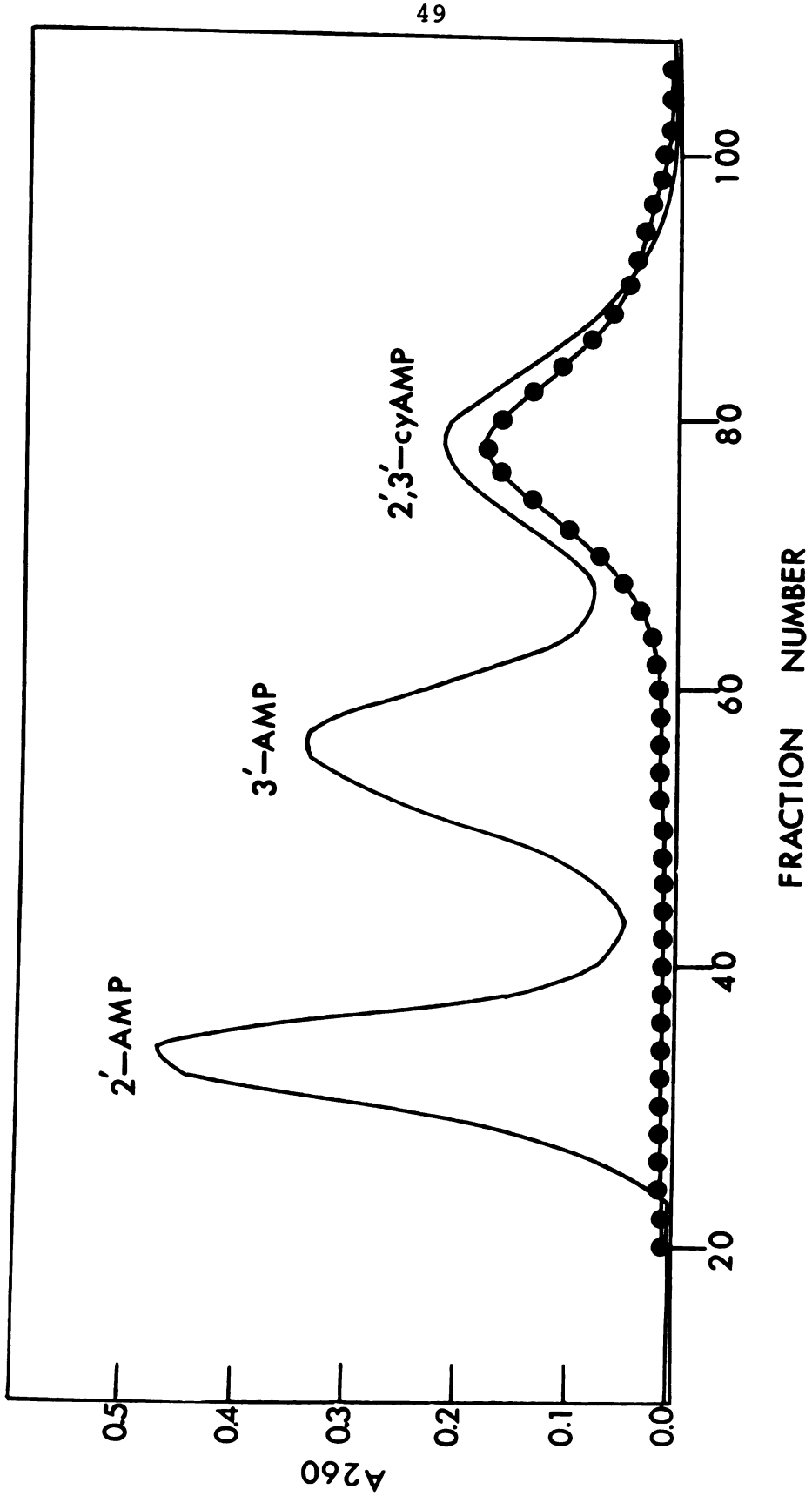
product from RNA degradation (12, 19, 30, 44). However, I found that 3'-nucleotidase II of pea had no activity toward either 2',3'-cyNMP or 3',5'-cyNMP (described in the second part of this thesis).

The Mode of the Action of 3'-Nucleotidase II on Poly A.--Evidence suggests that the 3'-nucleotidase II from pea characterized in this study may be associated with the RNase activity as observed in the previous results. Most of the RNases so far purified and characterized from higher plants, including the enzyme from pea leaves (21, 22), are cyclizing enzymes (phosphotransferases) which catalyze the formation of 2',3'-cyNMP from RNA. The purine 2',3'-cyNMPs are hydrolyzed slowly to give nucleoside 3'-phosphates, while the pyrimidine derivatives are apparently inert to further action of the enzyme. The small amounts of hydrolysis of purine derivatives of 2',3'-cyNMP are of doubtful significance because it is probable that small amounts of cyclic nucleotide phosphodiesterase (not RNase in this case) contamination was present and accounted for the cleavage found.

With the synthetic polymer of adenylic acid as substrate, it seemed possible to analyze the end products formed from the action of RNase in a more precise way. In order to avoid contaminating cyclic nucleotide phosphodiesterase, 0.2 ml of 3'-nucleotidase II preparation from Sephadex G-75 fraction was subjected to a 5-20% sucrose

Figure 9.--Ion exchange chromatography of the hydrolysis product of poly A.

The column of BioRad Ag-1-X2(Cl^-), 400 mesh, 0.5 x 5.0 cm, was first calibrated with a various combination of authentic compounds (0.4 mg of 2'-AMP, 0.5 mg of 3'-AMP, and 0.4 gm of 2',3'-cyAMP). With 2 mM HCl as the equilibration and elution solvent, 60-drop (app. 3.4 ml) fractions were collected with a flow rate of 12 drops/min. and the absorbance was measured at 260 mμ. The calibration curve is shown by ———. The column was then regenerated as described under "Methods." The hydrolysis products obtained from the action of pea RNase (3'-nucleotidase II) on the synthetic poly A as described in the text was applied to the regenerated column. The elution pattern of this hydrolysate is represented by ●——●. RNase or poly A alone shows no detectable formation of 2'-AMP, 3'-AMP, and 2',3'-cyAMP.


2′-AMP
3′-AMP
2′,3′-cyAMP
A260
0.0
0.1
0.2
0.3
0.4
0.5
20
40
60
80
100
FRACTION NUMBER

density gradient as described under "Methods." Centrifugation was carried out at 35,000 r.p.m. for 20 hr with a Beckman SW 39 rotor in Beckman L-65 ultracentrifuge. The temperature was maintained at 2° throughout the whole procedure. After collecting 10-drop fractions, fractions of 32-34 were added to a reaction mixture containing 0.1 ml of 0.2 M Tris-acetate buffer, pH 6.5, and 0.2 ml of poly A (3 mg/ml). Incubation was carried out at 37° for 5 hr and terminated by the addition of 0.1 ml of 3 mM uranyl acetate in 0.2 N HCl. The resulting supernatant from the centrifugation at 10,000 x g for 10 min was subjected to a BioRad Ag-1-X2 (chloride form 400 mesh) column as described in "Methods." Pea RNase catalyzed the formation of 2',3'-cyAMP from poly A, with no formation of 2'-AMP and 3'-AMP. Poly A alone gave no detectable amounts of 2',3'-cyAMP, 2'-AMP, and 3'-AMP. In a subsequent experiment using thin-layer chromatography, 0.05 ml of the resulting supernatant as mentioned above was applied to a cellulose precoated thin-layer plate and developed in the solvent system as described in Figure 10. The result indicated that 2',3'-cyAMP was formed from poly A. A further experiment with ^{14}C-poly A as substrate gave essentially the same result as observed in Figures 9 and 10. Thus, it is suggested that pea 3'-nucleotidase associated with RNase activity does not have catalytic activity toward 2',3'-cyNMP. The summary of well-characterized RNase from higher plants is given in Table 8.

Figure 10.--Thin-layer chromatography of the hydrolysis products obtained from the action of RNase (3'-nucleotidase II) on poly A.

Details of the reaction mixtures and procedures are described in the text. The chromatogram was developed in solvent system of isopropanol-0.03 M NH_4HCO_3, pH 8.6 (3:1 v/v) for 4 hr at room temperature, 25°. The locations of poly A and hydrolysate were detected as two black bands shown in the figure under u.v. light. One centimeter intervals of the cellulose were received and eluted with 1.5 ml of distilled water in boiling water bath for 30 min. After centrifugation, the absorbance of the supernatants were measured at 257 mμ. Shadowed area represents poly A alone as the control. Open area shows the result obtained from poly A incubated with RNase. O, origin of chromatogram; SF, solvent front. The positions that would be occupied by adenine and adenosine are indicated by arrows.

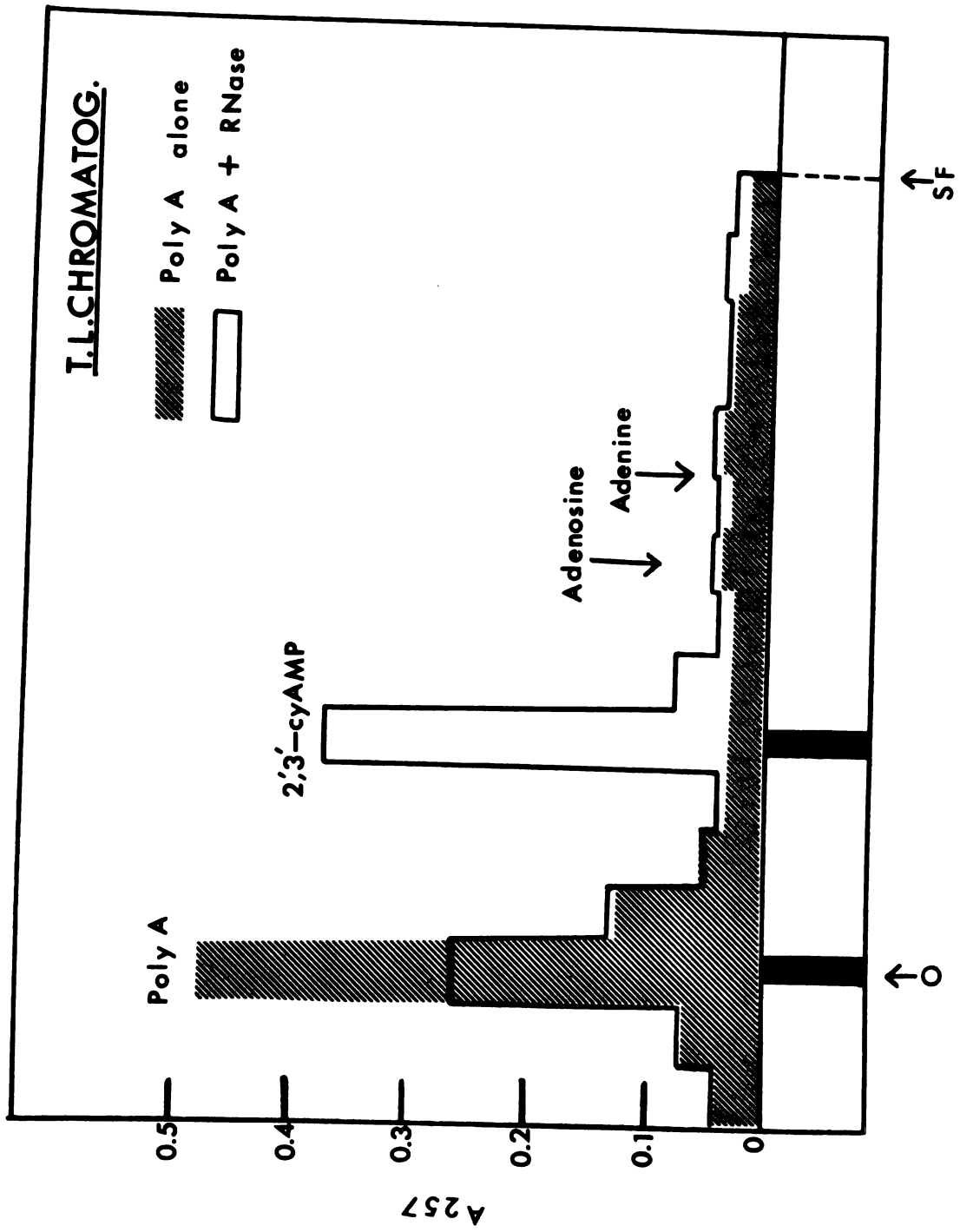

T.L.CHROMATOG.
Poly A alone
Poly A + RNase
Poly A
2',3'-cyAMP
Adenosine
Adenine
O
SF
A257
0.5
0.4
0.3
0.2
0.1
0

TABLE 8.--Summary of well-characterized RNase from higher plants.

Source	Products Formed[a]	Hydrolyzing 2',3'-cyNMP[b]		Specificity[c]	pH	Reference
		Purine	Pyrimidine			
Tobacco leaves	2',3'-cyNMP	Very slow,3'	None	G>A,U>C	5.1	Schuster et al. (1959)
Pea leaves	2',3'-cyNMP	Very slow,3'	None	G>A,U>C	5.1	Markham et al. (1956) Hoden et al. (1955)
Garlic (Alium)	2',3'-cyNMP	Very slow,3'	None	---	6-7	Carlsson et al. (1964)
Spinach leaves	2',2'-cyNMP	Very slow,3'	None	G>A,U,C	5-6	Tuve et al. (1960)
Mung bean sprouts (M_1)	2',3'-cyNMP	Very slow,3'	None	---	5.5	Walter et al. (1966)
Soy bean	2',2'-cyNMP	Very slow,3'	None	G>A,U,C	5.2	Merola et al. (1962)
Cucumber seedling	2',3'-cyNMP	Very slow,3'	None	U,C,G	5.6	Kado (1968)
Corn (root, seed)	2',3'-cyNMP	Very slow,3'	None	G,A>U,C	5-6	Wilson (1967)
Avena leaves	2',3'-cyNMP	Very slow,3'	None	---	5.5	Udvardy et al. (1969)
Rye grass	2',3'-cyNMP	None	None	---	5.2	Freeman (1964)
Sugar cane	2',3'-cyNMP	None	None	---	5.0	Tang et al. (1970)
Pea seedling[d]	2',3'-cyNMP	None	None	---	6-7	This study

[a]At first 2',3'-cyclic nucleoside monophosphates, then very slowly purine cyclic phosphates further hydrolyzed.

[b]None indicates no detectable hydrolysates found after 24 hr incubation. The 3' denotes the final phosphate product formed.

[c]Nucleotide bonds adjacent to the bases shown are split, in the relative order shown. A large difference is marked by >.

[d]RNase is the 3'-nucleotidase II purified from Alaska pea seedlings.

Estimation of Molecular Weight, Diffusion Constant and Stokes' Radius for 3'-Nucleotidases.--Kulkarni and Mehrotra (45) modified the equation described by Determann et al. (46) for the estimation of the molecular weight, diffusion constant, and Stokes' radius of a protein in relation to its reduced elution volume (Ve/Vo) using Sephadex G-150 column. I derived similar equations for a Sephadex G-200 column which was used for study of pea 3'-nucleotidases.

A Sephadex G-200 column (1.5 x 120 cm) with 40-120μ bead size was used (47, 48) and a bed height of 120 cm was kept throughout the study. An 0.01 M of Tris-acetate buffer, pH 7.5, was used for elution of the proteins. To assure reproducible results and to maintain the flow rate at 9 ml/hr, it was essential to keep the hydrostatic pressure at 40 cm. Fractions were collected at 20 min intervals at 4°. The column was calibrated with various combinations of standard proteins (2 mg for each protein) as shown in Table 9. The void volume (Vo) used in the calculation is the elution volume of blue dextran (Ve = 48.0 ml). With the least-squares method, the data of standard proteins shown in Table 9 were analyzed and gave the following three regression equations:

a. $\log \text{Molecular Weight} = (6.2392 \pm 0.7428 \pm 0.0252)(Ve/Vo)$.

TABLE 9.--Physical properties and elution data (from Sephadex G-200) of standard proteins and pea 3'-nucleotidases.

Protein	Mol. Wt.	Diffusion Constant ($D^{s}_{20,w} \times 10^{7}$ cm^2/sec)	Stokes' Radius ($r_s \times 10^{8}$)	Elution Volume (ml)	Ve/Vo
Cytochrome c	12,400 (49)	---	---	138.2	2.88
Myoglobin	17,800 (56)	11.3 (57)	18.8 (57)	129.1	2.69
Trypsin	23,800 (50)	11.0 (57)	19.4 (57)	120.5	2.51
Chymotrypsinogen A	25,000 (51)	9.5 (57)	22.4 (57)	119.5	2.49
Ovalbumin	45,000 (52)	7.8 (57)	27.3 (57)	102.7	2.14
Albumin (bovine serium)	67,000 (53)	6.0 (49)	36.1 (54)	90.2	1.88
γ-Globulin	160,000 (53)	3.8 (57)	55.5 (57)	68.6	1.43
Apo-ferritin	480,000 (55)	3.0 (55)	---	50.9	1.06
Blue dextran	2,000,000	---	---	48.0	1.00
3'-nucleotidase I (pea)[a]	70,000	5.9	36.2	90.0	1.88
3'-nucleotidase II (pea)[a]	30,000	9.1	23.2	114	2.38

[a]The values of molecular weight, diffusion constant, and Stokes' radius are calculated from the following equations:

log Mol. Wt. = 6.2392 - 0.7428 (Ve/Vo).

log Diffusion constant = 0.0645 + 0.3769 (Ve/Vo).

log Stokes' radius = 2.2664 - 0.3778 (Ve/Vo).

b. log Diffusion Constant ($D°_{20W} \times 10^7$ cm^2/sec.)

$= (0.0645 \pm 0.0618) + (0.3769 \pm 0.0277)$ (Ve/Vo).

c. log Stokes' Radius ($r_s \times 10^8$) = $(2.2664 \pm 0.0587) - (0.3778 \pm 0.0263)$(Ve/Vo).

Thus, using only the experimental value of Ve for any protein (assumed to have similar shape) under study and Vo of the column of Sephadex G-200 without having a calibration curve, one can determine the molecular parameters mentioned above for a protein by the above equations. For example, the value of such molecular parameters for pea 3'-nucleotidases are shown in Table 9. Apparently, 3'-nucleotidase I has a molecular weight about 70,000 and 3'-nucleotidase II has 30,000. In addition, a typical plot of the correlation between log molecular weight and reduced elution volume (Ve/Vo) is shown in Figure 11.

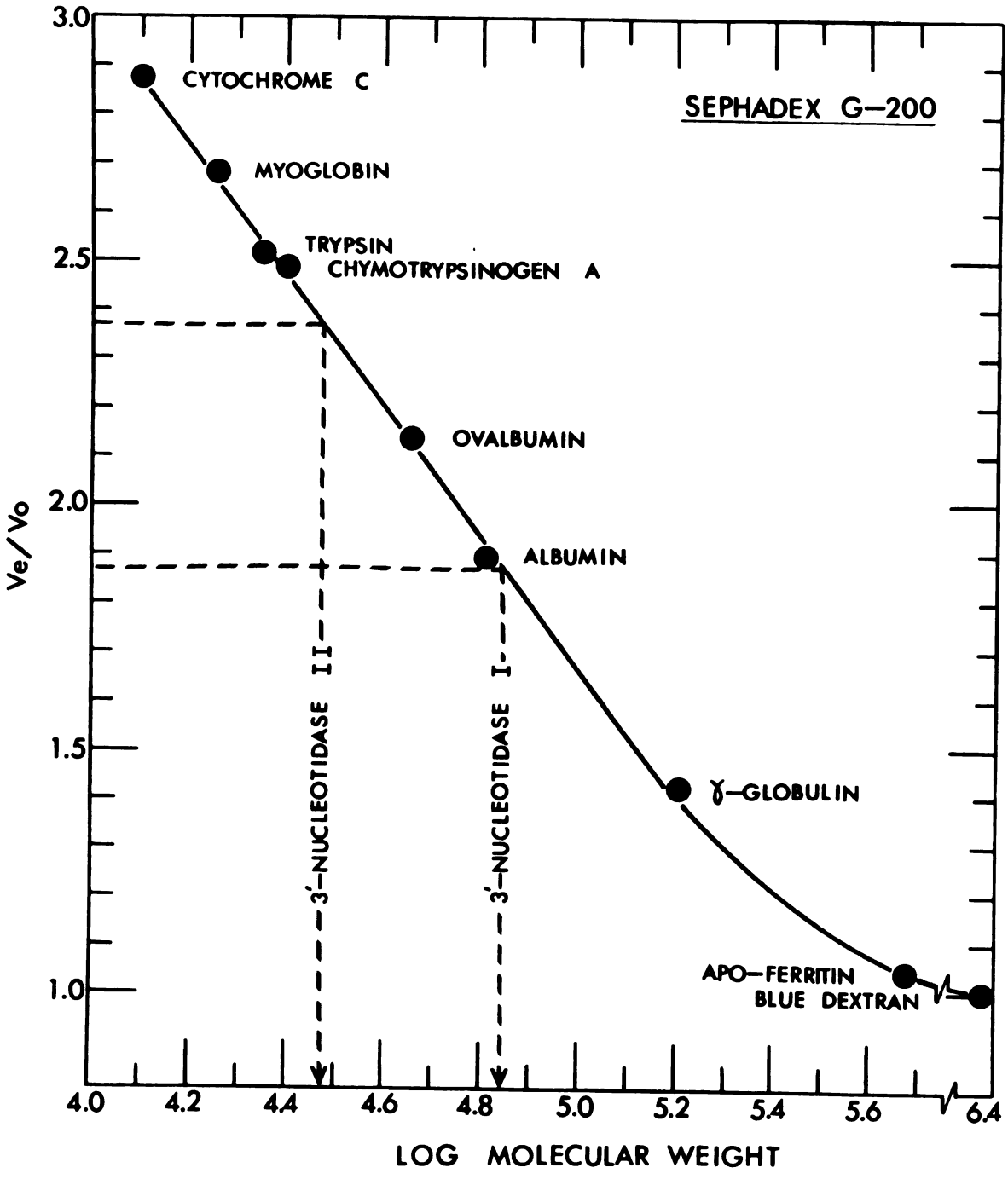
Discussion

Two 3'-nucleotidase activities were found in pea seedlings and were readily separable by DEAE-cellulose column chromatography. These two enzymes were further purified by Sephadex gel filtration.

The 3'-nucleotidase I was purified approximately 6-fold with a recovery of about 1% of that enzyme activity present in the 4,000 x g supernatant. Although the result

Figure 11.--Calibration curve for molecular weight determination on Sephadex G-200 column chromatography.

A Sephadex G-200 column (1.5 x 120 cm) was equilibrated with 0.01 M Tris-acetate buffer, pH 7.5, at a flow rate of 9 ml/hr at 4°. The column was calibrated with various combinations of standard proteins as shown in Table 9. The elution volume (Ve) of each protein was determined by extrapolating to the center of the peak. The void volume (Vo) used in the calibration is the Ve of blue dextran. A typical plot of the correlation between log molecular weight and reduced elution volume (Ve/Vo) is shown in this figure. The values of log molecular weight for 3'-nucleotidase I and II are indicated by -----→. Apparently, 3'-nucleotidase I has a molecular weight about 70,000 while 3'-nucleotidase II has 30,000. For further details, refer to the description in the text.


SEPHADEX G—200
CYTOCHROME C
MYOGLOBIN
TRYPSIN
CHYMOTRYPSINOGEN A
OVALBUMIN
ALBUMIN
γ—GLOBULIN
APO—FERRITIN
BLUE DEXTRAN
3'-NUCLEOTIDASE II
3'-NUCLEOTIDASE I
Ve/Vo
3.0
2.5
2.0
1.5
1.0
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
6.4
LOG MOLECULAR WEIGHT

shows that 3'-nucleotidase I does not need the presence of reducing reagent such as cysteine, glutathione, and dithiothreitol for maximal activity, it may need them for stabilization during its purification procedures as is the case for 3'-nucleotidase in wheat seedlings (14). Thus, the lack of reducing reagents during purification procedures may be the main reason for the low recovery. Furthermore, Zn^{++} may also stabilize the enzyme even though it inhibits enzyme activity during the assay. Unlike the enzyme purified from wheat seedlings (14) and muskmelon seeds (15), the pea 3'-nucleotidase I preparation was nearly free of contaminating proteins.

The addition of Zn^{++} shifted the optimum pH and decreased the specific activity.

The pea 3'-nucleotidase I is quite different from other plant nucleotidases (12, 16) which have been well-characterized and shows no significant activity toward pyrimidine 3'-nucleoside monophosphates. However like other plant 3'-nucleotidases (12, 15, 16), pea 3'-nucleotidase I shows little activity toward 2'- or 2'-AMP and no activity toward cyclic nucleoside monophosphates. Based on these characterizations, 3'-nucleotidase I could serve as a tool for an enzyme coupled assay of cyclic nucleotide phosphodiesterase in higher plants. The molecular weight 70,000 of pea 3'-nucleotidase I is higher than that of most plant nucleotidases which in general have

molecular weights of 15-30,000 (15). Further studies will be required to establish whether the two isoelectric points observed for 3'-nucleotidase I are due to enzyme dissociation or the presence of two enzymes.

A rather unusual combination of nuclease and 3'-nucleotidase activities purify as one species from a variety of plant sources. These include the enzyme from rye grass (16, 29), muskmelon seed (15), rice bean (17), mung bean sprouts (12), wheat seedlings (14), and possibly soy bean (18), and germinating garlic (19). The enzyme of 3'-nucleotidase II preparation from pea seedlings in the present study was suggested to have RNase and 3'-nucleotidase combination.

Although the enzyme has been purified approximately 15-fold with a recovery of 23% of the total activity present in the 4,000 x g supernatant, about 90% of the protein in the preparation is 3'-nucleotidase II. Based on this high yield and purity, further steps of purification with either DEAE-cellulose or Sephadex G-200 may provide a good source for the study of the physical and chemical properties of this unusual enzyme.

The ability of Zn^{++} to prevent both 3'-nucleotidase II and RNase inactivation at pH 5.0, and the elimination of enzyme activity by EDTA, imidazole, cysteine and other reducing reagents provide strong evidence that the enzyme concerned may be a metalloprotein, probably a zinc-containing enzyme. However, added Zn^{++} has no significant

effect on enzyme activity; added Zn^{++} may only function to stabilize the proper tertiary or quaternary structure of the protein. A similar role of Zn^{++} has been observed in several enzymes such as Escherichia coli alkaline phosphatase (58), horse liver alcohol dehydrogenase (59), Bacillus subtilis α-amylase (60), wheat seedling 3'-nucleotidase (14), and mung bean 3'-nucleotidase (12).

Pea 3'-nucleotidase II, like other plant nucleotidases, has high specificity for purine 3'-nucleoside monophosphates and also catalyzes appreciable cleavage of 3'-UMP. The enzyme shows no activity toward 3'-CMP, 2'- and 5'-AMP.

Like most of the RNases so far characterized from higher plants, as described in Table 8, pea RNase (3'-nucleotidase II) catalyzes the formation of 2',3'-cyAMP from poly A, with no further formation of 2'-AMP and 3'-AMP under assay condition. Since the finding of a cyclic nucleotide phosphodiesterase in the same tissue (as described in Part II), the pea RNase (3'-nucleotidase II preparation) failure to hydrolyze 2',3'-cyclic nucleoside monophosphates is probably due to the lack of the enzyme mentioned above rather than because of the contamination of nucleotides bound to the RNase. Therefore, the mode of RNA degradation in higher plants may not follow the way which is generally accepted (20, 30, 44, 61).

As mentioned earlier, the results of the present study suggest that both 3'-nucleotidase II and RNase

activities reside in a single protein molecule. Evidence for this suggestion is based on the following criteria.

1. The two activities maintain a constant ratio throughout the purification procedures.
2. Attempts to separate the two activities by means of gel filtration, sucrose density gradient centrifugation, polyacrylamide gel electrophoresis, and electrofocusing have been unsuccessful.
3. Both activities are lost during treatment at pH 5.0. Zn^{++} stabilizes both activities.

Both 3'-nucleotidase I and 3'-nucleotidase II should be useful to study the end products of cyclic NMP diesterases.

Summary

Two 3'-nucleotidases have been isolated and partially purified from germinating pea seedlings.

The 3'-nucleotidase I shows maximal activity at pH 5.4-5.7 with no addition of Zn^{++}. However, the optimal pH is lowered to 4.7 and enzyme activity is decreased about 50% with the addition of 2 mM Zn^{++}. The relative rates of hydrolysis of the respective nucleotides are 3'-UMP (100%)> 3'-GMP(95%)>3'-AMP(83%) 3'-CMP(80%)>2'-AMP(15%)>5'-AMP(12%). The values of Km for the 3'-nucleotides are 0.6-0.8 mM. The enzyme does not require the presence of metal ions or sulfhydryl compounds for maximal activity. The molecular

weight of the enzyme is 70,000. Two isoelectric points, pH 4.7 and 5.3, were found for the enzyme which was able to hydrolyze 3'-CMP at pH 5.4.

In contrast to 3'-nucleotidase I, pea 3'-nucleotidase II has maximal activity at pH 8.0. Zn^{++} protects against inactivation of enzyme at pH 5.0. However, metal ions are not required for full activity. Sulfhydryl compounds at 0.4 mM give about 98% inhibition. The relative rates of hydrolysis of respective nucleotides is 3'-AMP (100%)>3'-GMP(85%)>3'-UMP(48%)>3'-CMP(0%), 2'-AMP(0%), 5'-AMP(0%). The Km for 3'-AMP, 3'-GMP, and 3'-UMP were 0.35 mM, 0.45 mM, and 0.62 mM, respectively. The 3'-nucleotidase II preparations showed RNase activity. Attempts to separate RNase activity from nucleotidase activity by a variety of chemical and physical means have been unsuccessful. It is, therefore, suggested that 3'-nucleotidase and RNase activities reside in a single protein molecule. The enzyme has a molecular weight of 30,000 and isoelectric point of pH 4.8. It catalyzes the formation of 2',3'-cyAMP from poly A.

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PART II

THE PURIFICATION AND CHARACTERIZATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM PEA SEEDLINGS

Introduction

Since the discovery of 3',5'-cyAMP 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 (3-6), knowledge about this cyclic nucleotide in higher plants is meager.

Preliminary attempts to detect adenyl cyclase (7) and to incorporate radioactive adenine and adenosine into 3',5'-cyNMP in pea and barley tissues were unsuccessful. However, an enzymatic system for the degradation of 3',5'-cyAMP in both pea and barley tissues has been found. It seemed necessary to study such a 3',5'-cyAMP phosphodiesterase in more detail in order to have a better idea how to assay for adenyl cyclase or endogeneous cyclic nucleoside

monophosphates in higher plants. The partially purified phosphodiesterase hydrolyzed not only 3',5'-cyNMP but also 2',3'-cyNMP.

The general accepted mode of RNA degradation in higher plants is that RNase is the enzyme which hydrolyzes both RNA and 2',3'-cyNMP (8, 9). Although a specific enzyme for the hydrolysis of 2',3'-cyNMP, but not RNA, has been found in both animal and bacterial systems (10-15), it has not yet been found in higher plants.

This paper presents the detailed procedures for isolation and purification of cyNPDE, together with a description of the general chemical and physical properties of the enzyme with respect to its action on the substrates 2',3'-cyAMP and 3',5'-cyAMP. The biological significance of cyNPDE and the degradation of RNA in higher plants are discussed.

Experimental Procedures

Materials

Most of the materials used were the same as described in Part I with the following additions. Silica gel G (acc. to Stahl) was purchased from Brinkmann Instruments. The products of Packard are PPO and POPOP. The ^{3}H-3',5'-cyAMP 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}H-3',5'-cyAMP which was purified according to the following procedures. With thin-layer chromatography, it was shown that most of the 2% impurity appeared to be located in the area of authentic adenosine, adenine, and 3'-AMP or 3'-AMP.

The method used for purification was similar to that described for the assay of adenyl cyclase in animal system by Krishna et al. (16). A commercial sample was first applied to a Dowex-50-H^{+}, 200-400 mesh, column (1.5 x 8 cm) and eluted with deionized water in 2 ml fractions. In general, the third and fourth fractions contained 95% of 3',5'-cyAMP were combined and freeze-dried with the aid of VIRTIS lyaphylizer (Model No. 10-145 MR-BA). Deionized water was used for dissolving the purified material. With thin-layer chromatography in a two-dimensional separation system as described under "Methods," purified ^{3}H-3',5'-cyAMP was shown to be free of bases, nucleosides, and other nucleotides. About 80-85% recovery was achieved in these procedures.

Methods

Some of the methods used in this part of the study were the same as described in the Part I. These included the growth of pea seedlings, assay of 3'-nucleotidase, assays of RNase and DNase, determination of protein content, preparation of ion exchange resin, polyacrylamide gel

electrophoresis, sucrose density gradient centrifugation, and electrofocusing.

Thin-Layer Chromatography.--Although thin-layer chromatography on ECTEOLA cellulose (17), silica gel (18, 19), cellulose powder and anion exchange cellulose (20) have been shown useful for the separation of bases, nucleosides and nucleotides, these methods did not completely separate either 2',3'-cyAMP or 3',5'-cyAMP from other ultraviolet light-absorbing substances, especially xanthine, xanthosine, hypoxanthine, and inosine. Mixed cellulose-silica gel thin-layer (21-23) has these properties; in aqueous solvent the silica gel is deactivated and remains inert resulting in chromatograms typical of cellulose systems, in organic solvent systems the cellulose is inert and chromatograms typical of silica gel system are observed.

The plates were prepared by mixing 7.5 g of silica gel G and 7.5 g of cellulose powder in 90 ml of deionized water in a Waring Blender at top speed for 1 min. The resulting slurry was spread over five glass plates (20 x 20 cm) at a thickness of 250 μ using a spreader in the conventional manner and dried overnight at room temperature.

On the thin-layer plate, substances located with ultraviolet light (Mineralight UVS. 11) were scraped out and eluted with 0.5 deionized distilled water in boiling water bath for 10 min. After centrifugation to remove insoluble material, the resulting supernatant, if radioactive

substrate was used, was directly poured into a scintillation vial to which 15 ml of scintillation fluid was added. The radioactivity was counted in a Beckman liquid scintillation spectrometer. The scintillation fluid, Bray's solution (24), consisted of 60 g of naphathalene, 4 g of PPO (2,5'diphenyloxazole in scintillation grade). An 0.2 g of POPOP (1,4-bis-(2-(4-methyl-5-phenyloxazolyl)))-benzene, 100 ml of absolute methanol, 20 ml of ethylene glycol and p-dioxane to make 1 liter.

For other purposes, the following thin-layer, and solvent systems were used (Table 1).

1. For one dimensional separation of 3',5'-cyAMP and 2',3'-cyAMP from other compounds:
   Thin-layer; cellulose powder MN300.
   Solvent system 1; isopropanol:0.03M NH_4HCO_3 (pH 8.6) (3:1 v/v)
   Solvent system 2 (14); isopropanol:NH_4OH: 0.1 M boric acid (7:1:2 v/v)
   Running time and temperature; 4-5 hr at 23°.
2. For two-dimensional separation of 3',5'-cyAMP and 2',3'-cyAMP for their relative compounds:
   Thin-layer: cellulose (MN300):silica gel G (1:1 w/w)
   Solvent systems and running time:
   First dimension: H_2O, 40 min.
   Second dimension: isopropanol:0.03 M NH_4HCO_3 (pH 8.6) (3:1 v/v) 4-5 hr.

TABLE 1.--R_f values of bases, nucleosides, and nucleotides in thin-layer chromatography.

Compound	R_f Values { A: Cellulose Thin-layer B: Cellulose-silica Gel G Thin-layer)				
	Solvent System 1[a]		Solvent System 2[b]	Solvent System 3[c]	Solvent System 4[d]
			A	B	A
Adenine	0.63	0.79	0.74	0.28	--
Adenosine	0.57	0.75	0.75	0.53	--
5'-AMP	0.07	0.07	0.06	0.93	0.42
3'-AMP	0.08	0.10	0.20	0.93	0.23
2'-AMP	0.08	0.10	0.19	0.93	0.35
2',3'-cyAMP	0.43	0.57	0.47	0.93	0.10
3',5'-cyAMP	0.38	0.52	0.44	0.93	--
ADP	0.04	0.01	0.02	0.93	--
ATP	0.02	0.01	0.02	0.93	--
Poly A	0.00	0.00	0.00	--	--
Xanthine	--	0.42	--	0.45	--
Hypo-xanthine	--	0.68	--	0.58	--
Xanthosine	0.30	0.45	--	0.93	--
Inosine	--	0.62	--	0.93	--
6-methyl purine	0.80	0.78	0.76	--	--
Caffeine	0.84	0.90	0.85	0.77	--
Theo-phylline	0.78	0.80	0.76	0.65	--

[a]Solvent system 1: Isopropanol--0.03 M NH_4HCO_3, pH 8.6 (3:1 v/v).

[b]Solvent system 2: Isopropanol--NH_4OH - 0.1 M Boric acid (7:1:2 v/v).

[c]Solvent system 3: H_2O.

[d]Solvent system 4: Isopropanol-$(NH_4)_2SO_4$(saturated) -0.1 M NH_4Ac (1:40:10 v/v).

3. For the separation of 3'-AMP, 5'-AMP, and 3',5'-AMP:

   Thin-layer: cellulose MN 300

   Solvent system: isopropanol:NH_4OH:0.1 M boric acid (7:1:2 v/v)

   Running time and temperature: 6 h4, 23°

4. For the separation of 2'-AMP, 3'-AMP, and 2',3'-cyAMP:

   Thin-layer: cellulose MN 300

   Solvent system: isopropanol:saturated $(NH_4)_2SO_4$:0.1 M NH_4Ac (1:40:10 v/v).

   Running time and temperature: 5 hr, 23°.

Enzymatic and Chemical Assays of cyNPDE.--Two assays were performed in the measurement of cyNPDE activity depending on the assay condition required. "Assay method-1," an enzymatic assay, was based on the colorimetric measurement of P_i released from the following enzyme coupled system:

$$\text{Cyclic Nucleoside Monophosphate} \xrightarrow{\text{pea cyNPDE}}$$

$$\text{3'-NMP} \xrightarrow[\text{3'-nucleotidase}]{\text{pea}} \text{Nucleoside} + P_i$$

Unless otherwise stated, 3'-nucleotidase purified from pea seedlings as previously described was used in this coupled assay system. The standard assay was carried out in a total volume of 0.5 ml reaction mixture containing 0.1 M

K-acetate buffer, pH 5.4, 2 mM substrate and a suitable amount of enzyme preparation. In general, the reaction was conducted at 37° for a total incubation time of 1 hr. An 0.1 ml of 3'-nucleotidase preparation with excess amount of activity was added at 45 min and incubated for the remaining 15 min. In certain cases, the reaction was terminated first by heating in a boiling water bath for 3 min. After cooling, the reaction mixture was then incubated with 3'-nucleotidase for 15 min. Since 3'-nucleotidase I has been shown to have a maximal activity at the same pH as that used for the assay of cyNPDE, the pH of the reaction mixture was kept constant at 5.4 throughout the whole procedure. However, when 3'-nucleotidase II was used, the pH of the reaction mixture had to be changed to pH 8.0 with the addition of 0.1 ml of 1 M Tris-acetate buffer, pH 8.0, after 1 hr incubation at pH 5.4. The whole reaction was then terminated by the addition of 0.05 ml of cold 70% TCA solution according to the method shown in the preceding part. After centrifugation, P_i in the resulting supernatant was determined by the method of Fiske and SubbaRow (25) with slight modification as described in the previous part. Heat denatured enzyme preparation was used for the control assay. In general, 3'-nucleotidase I was used in the coupled enzyme assay system for enzymatic hydrolysis of 3',5'-cyNMP. One unit of enzyme activity is defined as 0.1 μmole of P_i released per hr of incubation. Specific activity of enzyme is defined as units per mg of protein.

"Assay method-2" measured the rate of formation of ^{3}H-3'-AMP and ^{3}H-5'-AMP from ^{3}H-3',5'-cyAMP. If the enzyme preparation was contaminated with 3'-nucleotidase, the rate of formation of ^{3}H-3'-AMP, ^{3}H-5'-AMP, and ^{3}H-adenosine from ^{3}H-3',5'-cyAMP was measured. The standard assay was conducted in 0.5 ml of reaction mixture containing 0.1 M K-acetate buffer, pH 5.4, 2 mM 3',5'-cyAMP, suitable amount of tritium labeled 3',5'-cyAMP and enzyme preparation. After incubation at 37° for a period of time, carrier nucleotides and nucleosides (3'-AMP, 5'-AMP, and adenosine) were added and a suitable aliquot was applied to a cellulose thin-layer plate. The chromatogram was developed in the solvent system of isopropanol:NH_4OH:0.1 M boric acid (7:1:2 v/v) or in isopropanol:0.03 M NH_4HCO_3, pH 8.6 (3:1 v/v). The remaining procedures were the same as described in the method of "Thin-layer chromatography."

Experimental Results

Purification of Enzyme

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

Preparation of Crude Extract of cyNPDE.--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

cheesecloth to remove the bulk of insoluble material. The resulting filtrate was taken as the crude extract of cyNPDE. The crude extract was centrifuged at 10,000 x g for 10 min.

Ammonium Sulfate Fractionation.--The supernatant was brought to 50% saturation by slowly adding solid ammonium sulfate (29.5 g per 100 ml of fluid) (26). The solution was stirred for 30 min and the precipitate was removed and discarded by centrifugation at 10,000 x g for 20 min. The resulting supernatant was decanted and then brought to 80% saturation with the addition of 19.7 g of solid ammonium sulfate per 100 ml of the extracted solution. After stirring for 2 hr, the precipitate was collected by centrifugation at 10,000 x g for 30 min and dissolved in 2 mM Tris-acetate buffer, pH 7.5. The resulting solution was then dialyzed in 2.3 cm diameter dialysis tubing (Union Carbide) against 20 volumes of 2 mM Tris-acetate buffer, pH 7.5, with constant agitation for 24 hr, with 3 changes. After dialysis, the solution was centrifuged at 10,000 x g for 10 min in order to remove a small amount of precipitate which formed during the dialysis. The resulting supernatant was then fractionated further or was frozen at -20°. Enzyme activity was retained at the original level after 3 months at -20°. The enzyme up to this step still contained appreciable amount of 3'-nucleotidase.

Treatment at pH 5.0.--The pH of the dialyzed enzyme preparation 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 x g for 10 min. The supernatant 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.

Chromatography of cyNPDE on Sephadex G-200 Column.--After pH 5.0 treatment, enzyme preparation was first concentrated with Centriflo membrane cone (Amicon) in an International portable refrigerated centrifuge (Model PR-2) and then applied to a Sephadex G-200 column (1.5 x 120 cm) which had been equilibrated with 0.01 M Tris-acetate buffer, pH 7.5. With a 40 cm hydrostatic pressure, enzyme was eluted by the same buffer and collected in 3 ml fractions at a flow rate of 9 ml per hr. Enzyme activities were determined by the standard methods.

The elution profile of protein and enzyme activities is shown in Figure 1. Although only 28.2% of the total activity toward 2',3'-cyAMP or 41.5% toward 3',5'-cyAMP was recovered, about 95% of the protein originally applied to the column was removed from the major enzyme fractions. On the other hand, there was about 5-fold increase in the specific activity toward 2',3'-cyAMP and 8-fold increase for 3',5'-cyAMP as can be seen in Table 2. The summary of the purification of cyNPDE from 300 g of pea seedlings is

Figure 1.--Elution profile of pea cyNPDE from Sephadex G-200 column chromatography.

Lyphogel concentrated and pH 5.0 treated enzyme preparation, 5 ml, containing 6 mg protein, 120 units toward 3',5'-cyAMP and 380 units toward 2',3'-cyAMP was applied to a column (1.5 x 120 cm) which had been equilibrated with 0.1 M Tris-acetate buffer, pH 7.5. Elution was carried out with the same buffer at the flow rate of 9 ml/hr at 4°. Three ml fractions were collected. Enzyme activities (in units/ml) on 2',3'-cyAMP (●——●) and on 3',5'-cyAMP (0——0) were determined as described under "Methods." Protein concentration was measured as the absorbance at 280 mμ (·····).

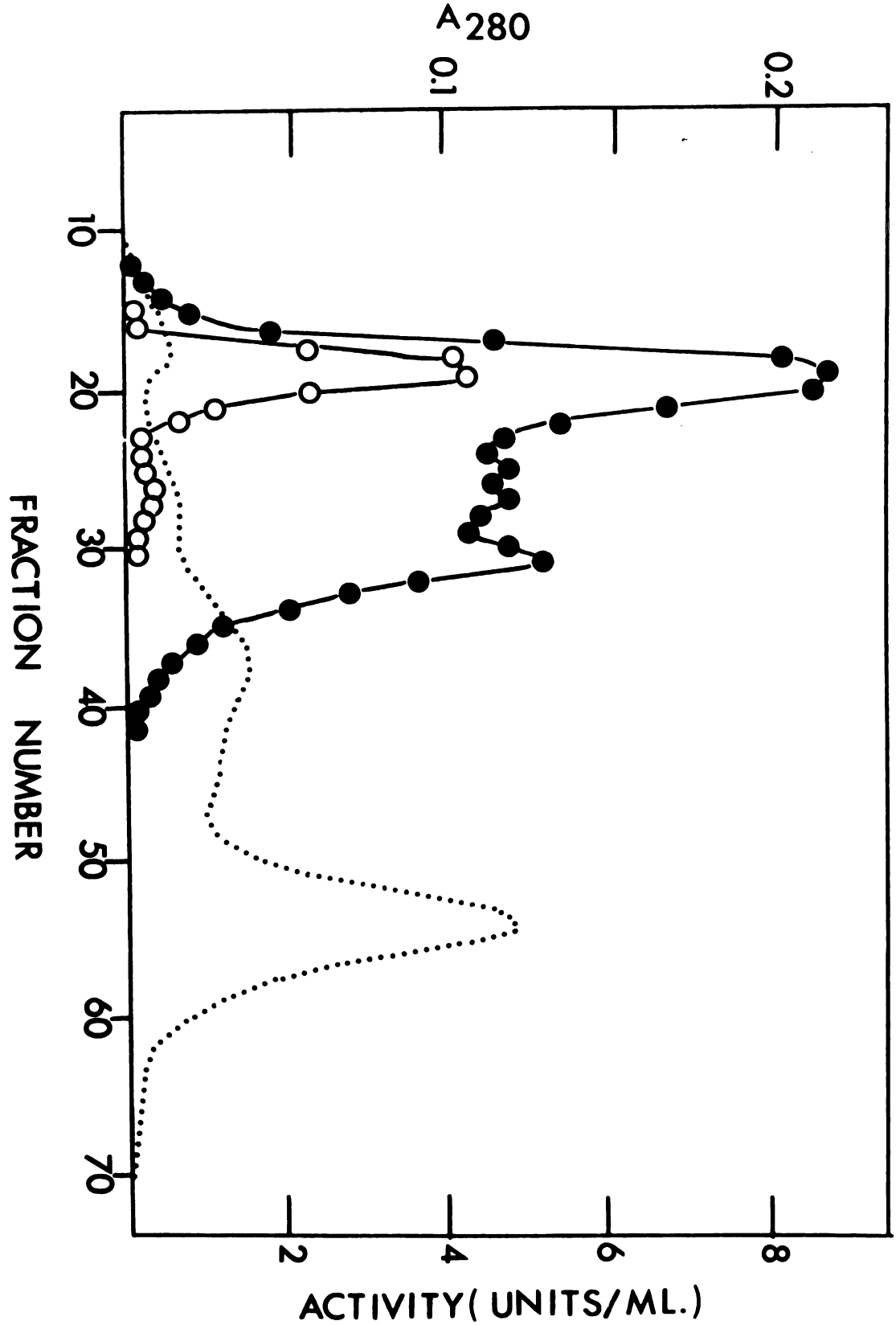

A280
0.1
0.2
10
20
30
40
50
60
70
FRACTION NUMBER
2
4
6
8
ACTIVITY(UNITS/ML.)

TABLE 2.--Summary of purification of cyclic nucleotide phosphodiesterase from 300 g of pea seedlings.

Fraction	Total Protein	2',3'-cyAMP as Substrate				3',5'-cyAMP as Substrate				Ratio of Sp. Act. (2',3'-cyA/3',5'-cyA)
		Total Activity	Specific Activity	Fold Purification	Yield	Total Activity	Specific Activity	Fold Purification	Yield	
	mg	units	units/mg		%	units	units/mg		%	
Crude extract	5510	8816	1.6	1.0	100	1874	0.3	1.0	100	5.3
10,000 x 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 (#17-26)	2.2	762	346	217	8.7	349	159	539	18.6	2.2

also shown in Table 2. Three peaks of enzyme activity toward 2',3'-cyAMP and one having activity toward 3',5'-cyAMP were consistently observed.

Enzyme preparation from this purification step was further concentrated with lyphogel and stored at -20° for further use. At that temperature, the enzyme preparation was stable for at least 4 months. Although the profile of enzyme activities in Sephadex G-200 chromatogram suggested that the enzymatic hydrolysis of 2',3'-cyAMP and 3',5'-cyAMP might be due to the same protein molecule, further attempts were made to separate the two activities.

Further Attempts to Separate the Two Activities

Sucrose Density Gradient Centrifugation.--A 5-20% linear sucrose density gradient was prepared according to the method of Martin and Ames (27). A swinging bucket rotor, SW 39 (Beckman), was used for the centrifugation which was performed at 34,000 r.p.m. in a Beckman L-2 65B ultracentrifuge for 12 hr. Ten-drop fractions were collected after centrifugation and assayed for enzyme activities according to "Assay method-1" as previously described.

As shown in Figure 2-A, three peaks of enzyme activity toward 2',3'-cyAMP 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'-cyAMP. The major peak represented 50% and 80%

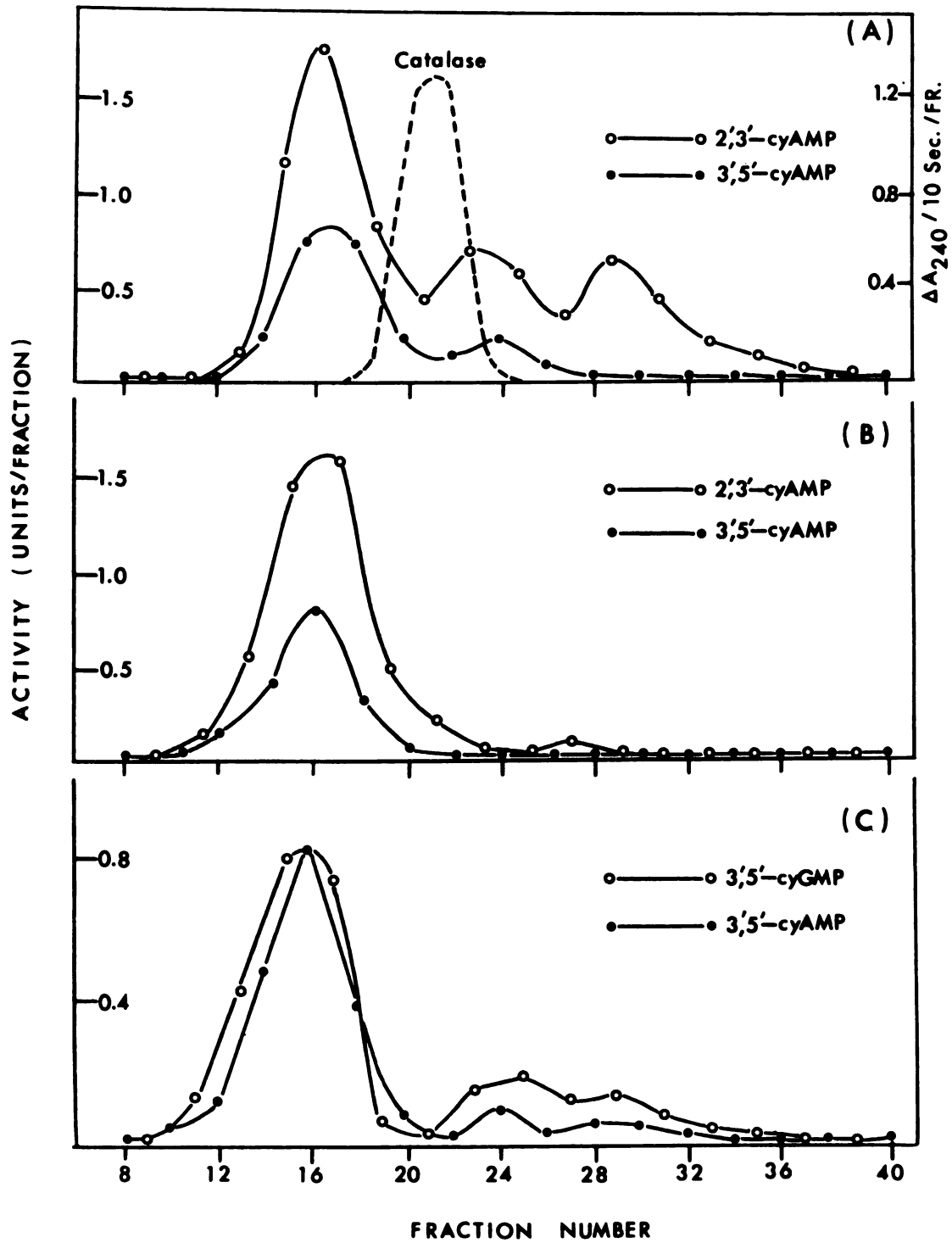
Figure 2.--The elution profiles of cyNPDE activities from sucrose density gradient centrifugation.

Partially purified enzyme preparation (pH 5.0 treated fraction), 0.3 ml, containing 0.39 mg protein (24 units toward 2',3'-cyAMP) was layered over a 5 to 20% sucrose density gradient. Centrifugation was performed as described in the text. Ten-drop fractions, total of 40 fractions, were collected. Enzyme assays were carried out in the alternative fractions with two different substrates for each gradient set. 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 cyNPDE.

(A) Elution profile of cyNPDE activities toward 2',3'-cyAMP (O——O) and 3',5'-cyAMP (●——●) under 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. (51).

(B) Same condition as described in (A) except the sucrose density gradient was prepared in 0.1 M K-acetate buffer, pH 5.4.

(C) Elution profile of cyNPDE activities toward 3',5'-cyAMP (●——●) and 3',5'-cyGMP (O——O). Tris-acetate buffer, 0.1 M, pH 7.5, was used for the preparation of sucrose density gradient.


(A)
Catalase
2',3'-cyAMP
3',5'-cyAMP
1.5
1.0
0.5
1.2
0.8
0.4
ΔA240 / 10 Sec. /FR.
(B)
2',3'-cyAMP
3',5'-cyAMP
1.5
1.0
0.5
(C)
3',5'-cyGMP
3',5'-cyAMP
0.8
0.4
ACTIVITY (UNITS/FRACTION)
8
12
16
20
24
28
32
36
40
FRACTION NUMBER

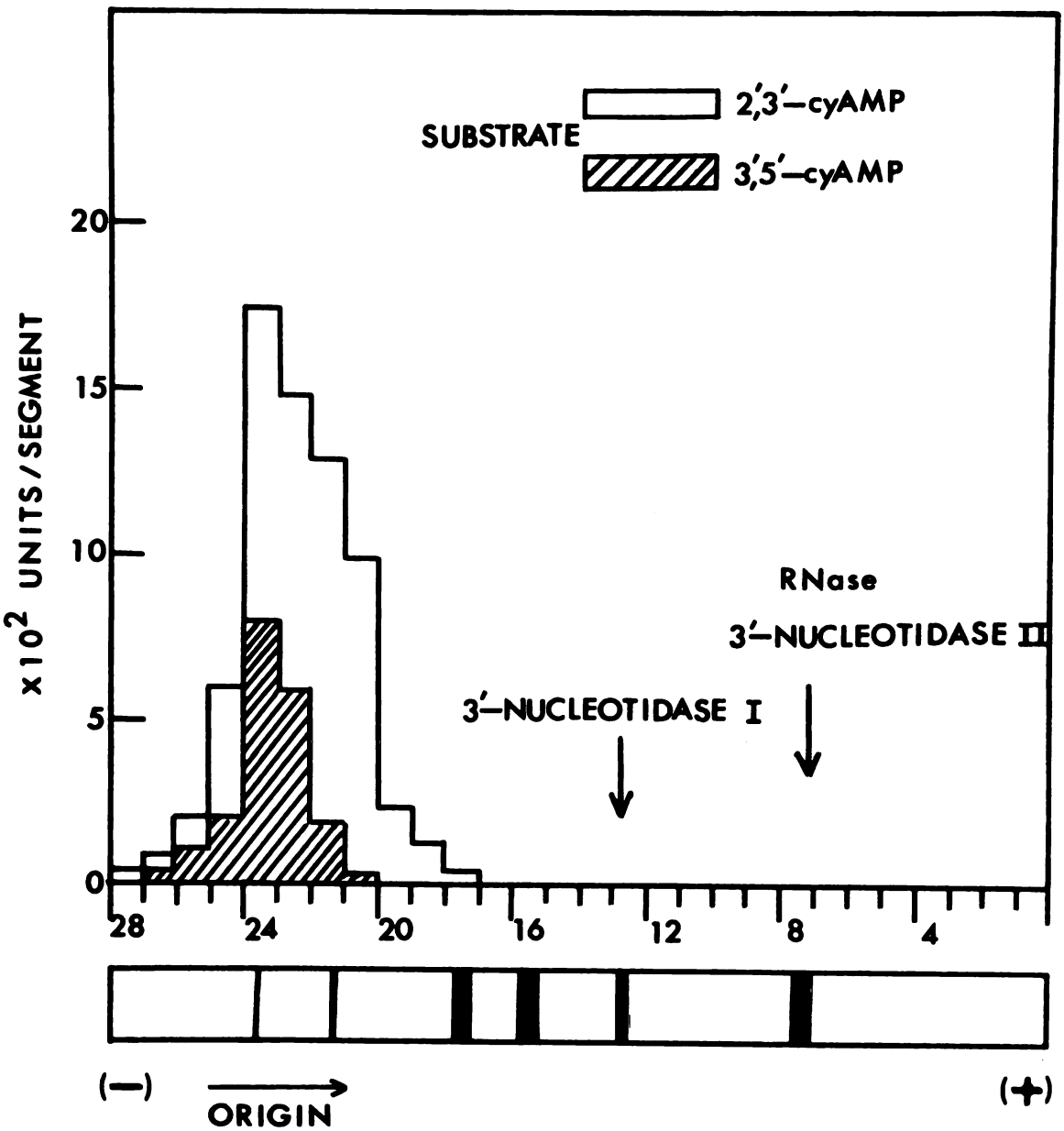
of the total activity toward 2',3'-cyAMP and 3',5'-cyAMP, respectively. About 60-70% of enzyme activity was recovered after centrifugation. Compared to bovine liver catalase (sedimentation constant = 11.3S, molecular weight = 247,500), the major cyNPDE had a sedimentation constant of 14.27S and molecular weight of about 350,000. If acidic sucrose density gradient (prepared in 0.1 M K-acetate buffer, pH 5.4) was used, only one peak of enzyme activity was obtained for either 2',3'-cyAMP or 3',5'-cyAMP (Figure 2-B). Once again, the two activities sedimented in an identical pattern with maximal activity in the same fraction, 16. Because it also represented a 60-70% of recovery in enzyme activity, it is suggested that 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 enzyme association. This result also suggested that the dissociation may not occur in alkaline pH as the result shown in Figure 2-A. Three molecular forms of cyNPDE activity against 3',5'-cyAMP are also observed in animal systems (28-32). The elution profiles of enzyme activity toward 3',5'-cyAMP and 3',5'-cyGMP were essentially the same (Figure 2-C). An experiment using 2',3'-cyUMP as substrate gave the same distribution pattern of enzyme activity as for 2',3'-cyAMP in Figures 2-A and 2-B.

Polyacrylamide Disc-Gel Electrophoresis.--With 7% polyacrylamide gel as described under "Methods," 0.3 ml quantity of lyphogel concentrated cyNPDE preparation from the Sephadex G-200 chromatogram, containing about 10 μg of protein and 3.0 units toward 2',3'-cyAMP and 1.2 unit toward 3',5'-cyAMP, was subjected to electrophoresis at pH 8.5. The staining pattern of protein and the distribution pattern of enzyme activities are shown in Figure 3. It is evident that the enzyme preparation still contained a number of protein species. The enzyme activity of cyNPDE was separated from the contaminated enzymes such as 3'-nucleotidases, RNase and most of the acidic phosphatase. However, enzyme activities toward both 2',3'-cyAMP and 3',5'-cyAMP were still found to be associated. About 23% and 15% enzyme activities were recovered for hydrolysis of 2',3'-cyAMP and 3',5'-cyAMP, respectively.

Electrofocusing Column Chromatography of cyNPDE.--Using the same procedures described in the methods of the preceding paper, 10 ml of pH 5.0 treated and dialyzed enzyme preparation, containing 85 units toward 2',3'-cyAMP and 26 units toward 3',5'-cyAMP per ml, was applied for an electrofocusing experiment. Ampholyte with pH values from 3.0 to 6.0 was used as protein carrier. After operation in 350 volts for 36 hr, 80-drop fractions were collected and dialyzed. Following dialysis, enzyme activities were assayed according to the standard methods. The elution

Figure 3.--Staining pattern of protein and distribution of enzyme activities within a polyacrylamide gel after electrophoresis.

An 0.3 ml of cyNPDE 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° 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'-cyAMP and 3',5'-cyAMP were performed in the alternative segments as described in "Experimental Procedures."


SUBSTRATE
2',3'–cyAMP
3',5'–cyAMP
x10² UNITS/SEGMENT
20
15
10
5
0
RNase
3'–NUCLEOTIDASE II
3'–NUCLEOTIDASE I
28
24
20
16
12
8
4
(–)
ORIGIN
(+)

profile of pH gradient and enzyme activities is shown in Figure 4. Activity toward 2',3'-cyAMP was found at three points, pH 4.8, 4.6, and 4.3. With 3',5'-cyAMP as substrate, enzyme showed isoelectric point at pH 4.8. Recoveries were 10% of the activity toward 2',3'-cyAMP and 8% of the activity toward 3',5'-cyAMP.

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'-cyAMP and 2,3'-cyUMP respectively was provided by the coupled assays with 3'-nucleotidase (Table 3). As described in the preceding part, pea 3'-nucleotidase I and II were rather specific for 3'-nucleoside monophosphates with activities toward both 3'-AMP and 3'-CMP. 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 was due to the presence of small amount of 3'-nucleotidase in cyNPDE preparation from Sephadex G-200 chromatogram.

Further evidence that the reaction product was exclusively 3'-AMP was obtained from ion exchange chromatogram. The enzyme preparation from the sucrose density gradient (fraction 16 in Figure 2) was incubated with 0.1 ml of 0.01 M of 2',3'-cyAMP and 0.05 ml of 0.2 M K-acetate

Figure 4.--Elution profile of pea cyNPDE from an electrofocusing column chromatography.

The pH 5.0 treated enzyme preparation containing 850 units toward 2',3'-cyAMP and 260 units toward 3',5'-cyAMP was subjected to electrofocusing. Detailed procedures are given in the text and the "Methods." Solid curve without circle represents the pH profile. Substrate used for the determination cyNPDE activity: 2',3'-cyAMP, 0——0; 3',5'-cyAMP, ●——●.

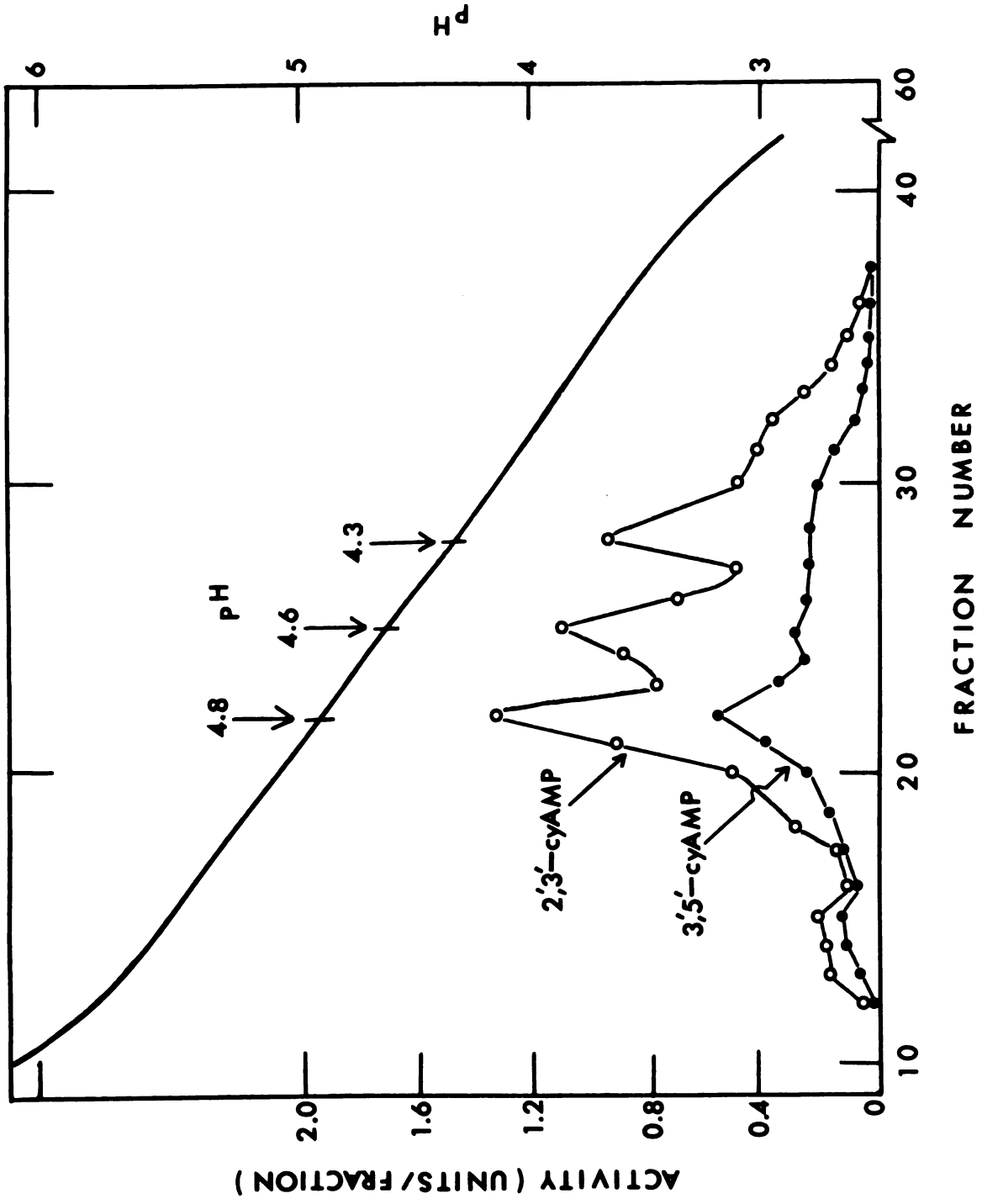

pH
6
5
4
3
60
40
30
20
10
0
4.8
4.6
4.3
pH
2',3'-cyAMP
3',5'-cyAMP
2.0
1.6
1.2
0.8
0.4
ACTIVITY (UNITS/FRACTION)
FRACTION NUMBER

TABLE 3.--Enzymatic analysis of the hydrolysis product formed from 2',3'-cyNMP.[a]

Addition	Substrate Assayed	
	2',3'-cyAMP	2',3'-cyUMP
	x 10 μmoles P_i released	
None	1.105	1.500
3'-nucleotidase I (pea)	3.815	4.291
3'-nucleotidase II (pea)	3.647	4.145
3'-nucleotidase (rye grass)	3.600	1.638

[a]Activity was determined in a 0.5 ml reaction mixture containing 2 mM substrate, 0.1 ml enzyme solution (concentrated fraction 18 from Sephadex G-200 column chromatography, 0.01 mg protein/ml) and 0.1 M K-acetate buffer, pH 5.4. Incubation was performed at 37° for 1 hr. After incubation, the reaction mixture was then heated in boiling water for 3 min to terminate the reaction. Then 3'-nucleotidase in excess amount was added and incubated under its optimal pH for 15 min. Inorganic phosphate released was determined according to the "Methods."

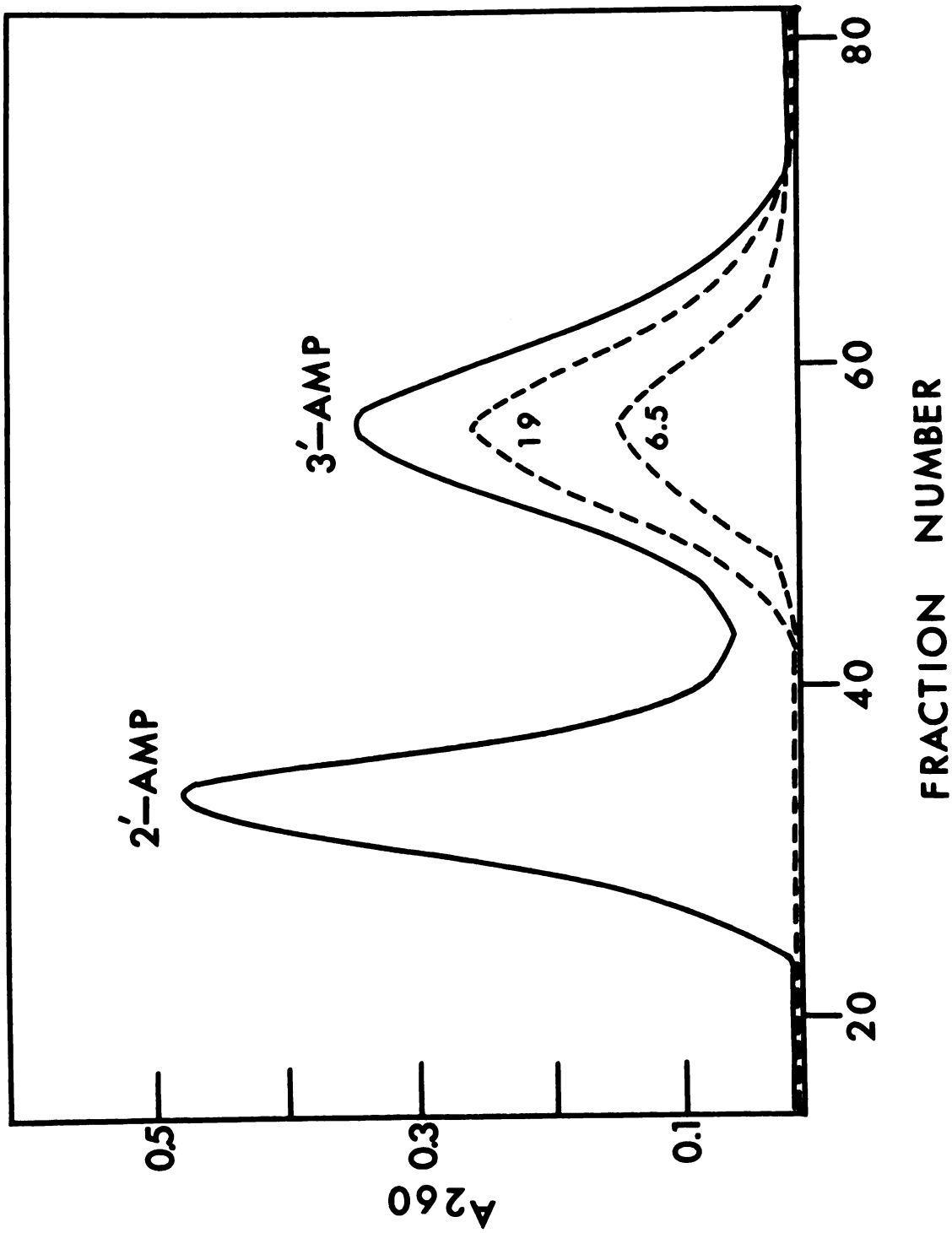
buffer, pH 5.4, in a final volume of 0.3 ml. The incubation temperature was at 37° (Figure 5). After termination by heating the reaction solution in boiling water bath for 3 min, the whole reaction mixture was applied to an ion exchange column as described in the "Methods." The amount of 3'-AMP formed from 2',3'-cyAMP increased with time while there was no apparent formation of 2'-AMP. The values shown in the figure have been corrected by the values obtained in the zero time incubation. Another experiment using ^{14}C-2',3'-cyAMP (prepared from ^{14}C-polyadenylic acid by the action of pea RNase as described in the preceding part) gave the same result shown above without any detectable formation of ^{14}C-2-AMP.

Action of 3',5'-cyclic Nucleoside Monophosphates.--Enzymes specific for the hydrolysis of 3',5'-cyclic nucleoside monophosphates so far isolated and characterized from animal tissues (33-36, 48), slime molds (37), and microorganisms (13, 38, 39) have been demonstrated to catalyze the formation of 5'-AMP exclusively from 3',5'-cyAMP. It was desirable to know whether the pea cyNPDE was similarly specific.

In order to have a more sensitive assay, purified ^{3}H-3',5'-cyAMP was used for this study. The cyNPDE preparation must be free of 3'-nucleotidase. This was accomplished by sedimenting 0.3 ml of concentrated enzyme preparation from Sephadex G-200 chromatogram, containing 11

Figure 5.--Ion exchange chromatography of the hydrolysis product of 2',3'-cyAMP.

Enzyme preparation from sucrose density gradient centrifugation (Fraction 16 in Figure 2) was incubated with 0.1 ml of 10 mM 2',3'-cyAMP and 0.05 ml of 0.2 M K-acetate buffer, pH 5.4 at 37° for 6.5 hr or 19 hr. Fraction 10 from the same figure was used as the control enzyme preparation. After incubation, the heated reaction mixture was directly applied to a BioRad Ag-1-X2 (Cl^-) column (0.5 x 5.0 cm) which was first calibrated with authentic compounds (0.4 mg of 2'-AMP and 0.5 mg of 3'-AMP). Detailed procedures are described in the text and the "Methods." The elution pattern of the hydrolysis product of 2',3'-cyAMP is represented by the dashed line (------). The time (hr) for incubation is indicated within the figure.


2′-AMP
3′-AMP
19
6.5
0.5
0.3
0.1
A260
20
40
60
80
FRACTION NUMBER

units toward 3',5'-cyAMP, in a sucrose density gradient. This separated the major cyNPDE fractions from pea 3'-nucleotidases (Figure 21). The experiment was conducted as follows. The enzyme of fraction 14 from Figure 2 was incubated with a reaction mixture containing 0.05 ml of 10 mM ^{3}H-3',5'-cyAMP (5 x 10^{7} cpm/ml) and 0.28 ml of deionized water at 37° for 5 hr. After incubation, 0.02 ml of aliquot was streaked on a cellulose thin-layer plate (5 x 20 cm). The chromatogram was developed as described under "Methods." The resulting chromatogram was then taken for scanning with the aid of Packard Strip scanner. Enzyme of fraction 10 from the Figure 2 was used as control enzyme preparation, since it contained only a negligible amount of cyNPDE. The cyNPDE from fraction 14 catalyzed the formation not only of ^{3}H-3'-AMP but also of ^{3}H-5'-AMP with a ratio of 3'-AMP: 5'-AMP of 6.8:1 (Figure 6-a). The enzyme preparation in this fraction was essentially free of 3'-nucleotidases, since there was no detectable ^{3}H-Adenosine. That the formation of 5'-AMP was due to enzymatic hydrolysis rather than nonenzymatic degradation of ^{3}H-3',5'-cyAMP can be concluded from the result shown in Figure 6-b, the result for fraction 10, in which no detectable ^{3}H-5'-AMP and ^{3}H-adenosine were formed. The minor amount of ^{3}H-3'-AMP formed in the control fraction 10 was because of the presence of a small amount of cyNPDE.

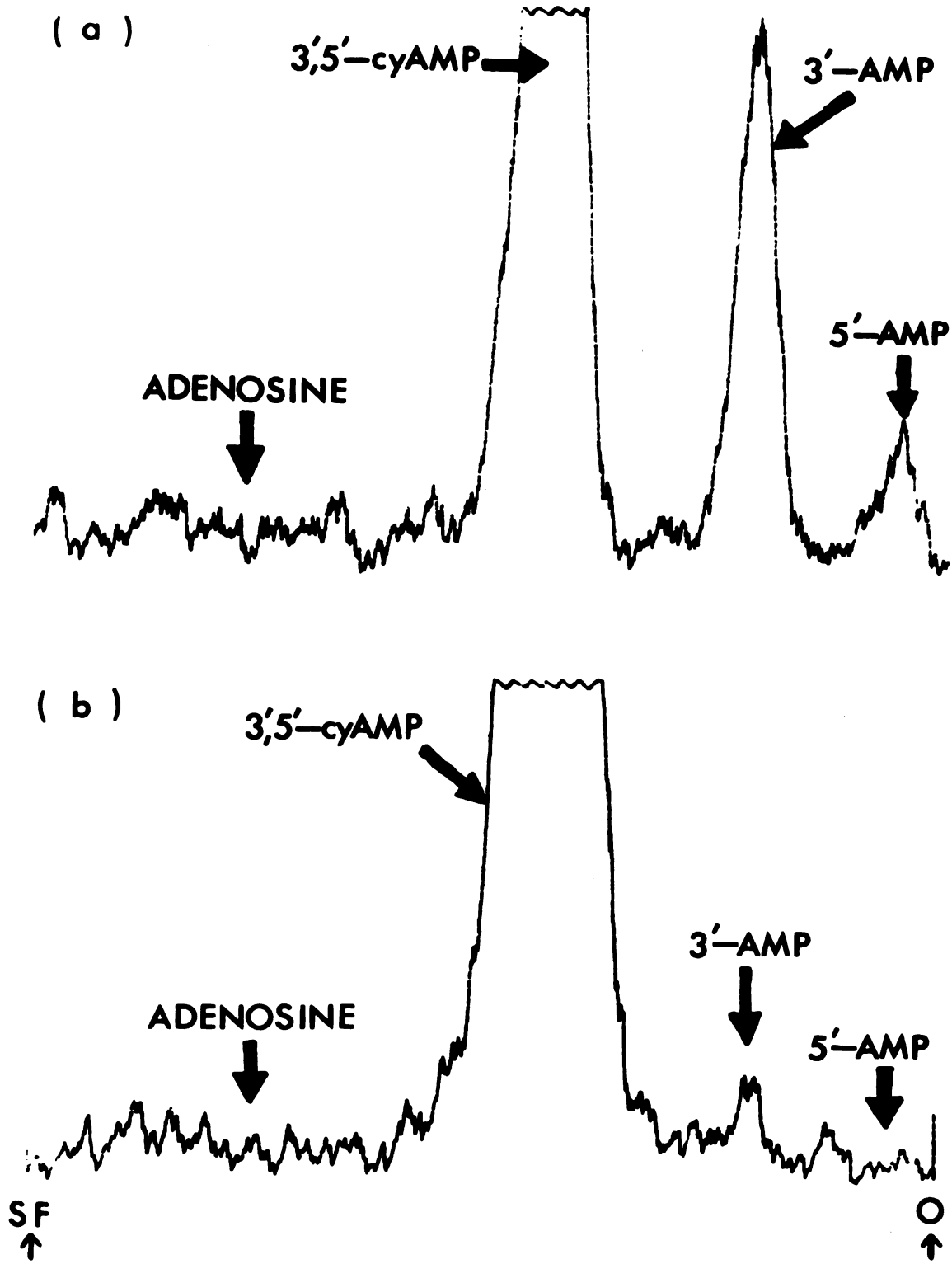
In order to check the result shown above in a different enzyme preparation and to make sure of the location

Figure 6.--Thin-layer chromatography of the hydrolysis products obtained from the action of pea cyNPDE on ^{3}H-3',5'-cyAMP.

Enzyme preparation from sucrose density gradient centrifugation (Fraction 14 in Figure 2) was incubated with 0.05 ml of 1 M K-acetate buffer, pH 5.4, 0.05 ml of 10 mM ^{3}H-3,5'-cyAMP (5 x 10^{7} cpm/ml) and 0.28 ml of distilled water at 37° for 5 hr. After incubation, 0.02 ml of aliquot was streaked on a thin-layer plate (5 x 25 cm). The chromatogram was developed for 5 hr in solvent system of isopropanol-NH_4OH-0.1 M boric acid (7:1:2 v/v). Fraction 10 in Figure 2 was run with the same procedures as previously described, for the control experiment. The resulting thin-layer chromatograms were scanned and traced as described under "Methods." 0; origin of chromatogram, S.F., solvent front. The full scale was 300 c.p.m.

(a) Radiochromatogram of the hydrolysis products obtained from Fraction 14. The locations of authentic 3',5'-cyAMP, 3'-AMP, 5'-AMP, and Adenosine are indicated by the arrows.

(b) Radiochromatogram of the hydrolysis products obtained from Fraction 10 (control).


(a)
3',5'—cyAMP
3'—AMP
5'—AMP
ADENOSINE
(b)
3',5'—cyAMP
3'—AMP
5'—AMP
ADENOSINE
SF
O

of the end products in thin-layer chromatogram, the following experiment was performed. Pea cyNPDE preparation from gel electrophoresis (segment 24 in Figure 3) with a little contamination of 3'-nucleotidase was used in a reaction mixture which contained 0.3 ml of 10 mM ^{3}H-3',5'-cyAMP (1.4×10^{7} cpm/ml), 0.6 ml of 0.2 M K-acetate buffer, pH 5.4, cyNPDE preparation and deionized water to a final volume of 1.6 ml. After 24 hr incubation at 37°, the reaction mixture was separated into three equal parts, 0.5 ml in each part, and incubated separately with excess amount of 5'-nucleotodase (snake venom, Sigma) or pea 3'-nucleotidase I or with equal amount of buffer (0.2 M K-acetate, pH 5.4) for further 1 hr incubation. At the end of the incubation, 0.01 ml of aliquots from each part were separately subjected to thin-layer chromatography with the addition of authentic compounds. As shown in Figure 7-a, ^{3}H-3'-AMP, ^{3}H-5'-AMP, and ^{3}H-Adenosine were formed from ^{3}H-3',5'-cyAMP. With the presence of excess amounts of 5'-nucleotidase, 5'-AMP but not 3'-AMP was converted to adenosine as seen in Figure 7-b. However, 3'-AMP with a little amount of 5'-AMP was converted to adenosine under the presence of excess amount of pea 3'-nucleotidase I as shown in Figure 7-c. The ratio of the formation of 3'-AMP: 5'-AMP was 6.8-7.0:1. These results clearly demonstrated that both 3'-AMP and 5'-AMP were products from enzymatic hydrolysis of 3',5'-cyAMP. A subsequent experiment utilizing the enzyme preparation from fraction 14-15 of Sephadex

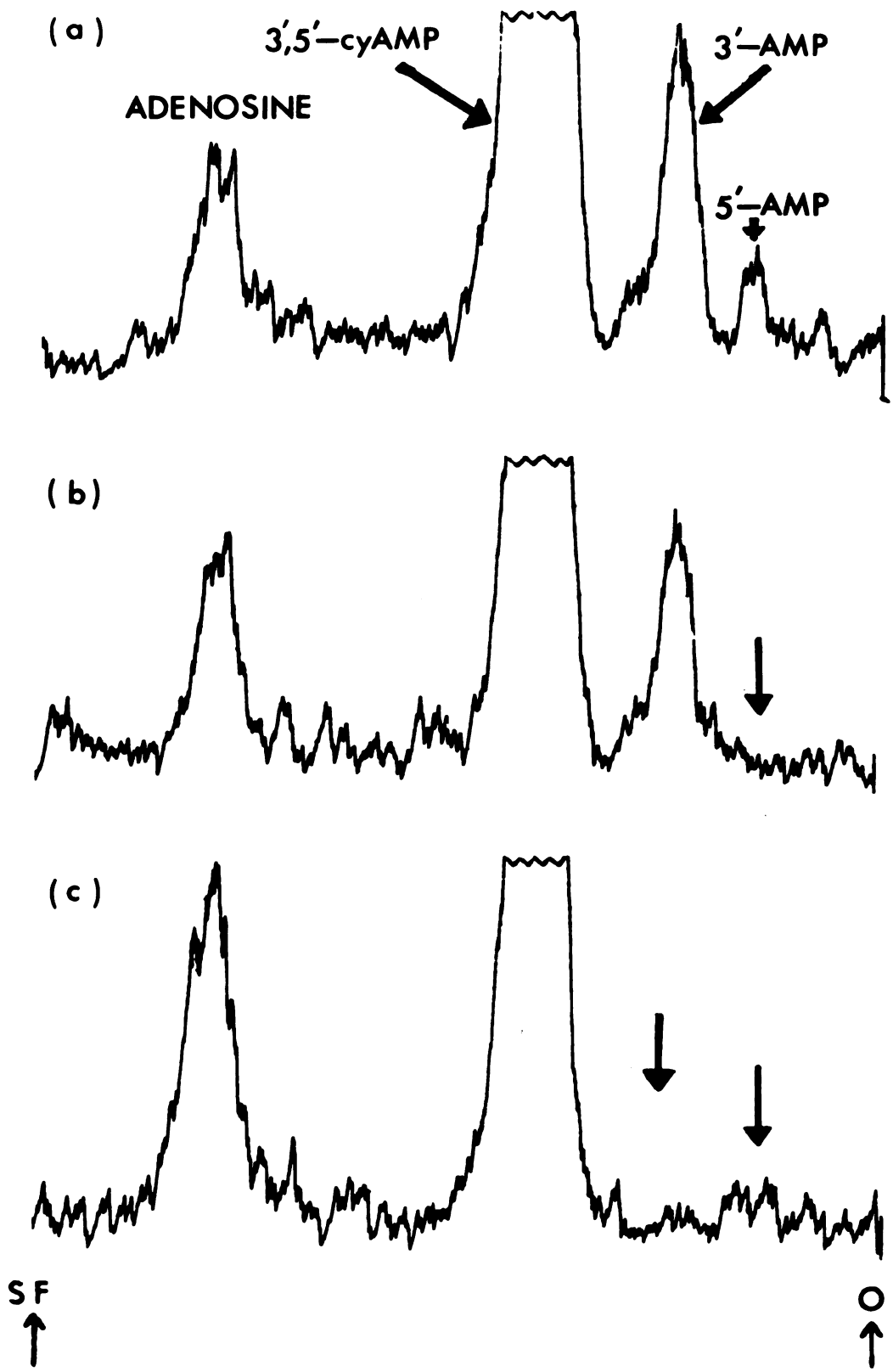
Figure 7.--Thin-layer chromatography and enzymatic analysis of the hydrolysis products obtained from the action of pea cyNPDE on ^{3}H-3',5'-cyAMP.

Enzyme preparation from acrylamide gel electrophoresis (Segment 12 in Figure 3) which has little activity toward 3'-nucleotides was incubated with 0.6 ml of 0.2 M K-acetate buffer, pH 5.4, 0.3 ml of 10 mM ^{3}H-3',5'-cyAMP (1.4×10^{7} cpm/ml) and distilled water to a final volume of 1.6 ml at 37° for 24 hr. After 24 hr incubation, the reaction mixture was separated into 3 parts (0.5 ml for each part) and further incubated with or without excess 3'-nucleotidase or 5'nucleotidase at 37° for 1 hr. After the incubation, 0.01 ml of aliquot from each treatment was separately subjected to thin-layer chromatography. The resulting chromatograms were scanned and traced as described under "Methods." O, origin of chromatogram; S.F., solvent front. The full scale was 300 c.p.m.

(a) Radiochromatogram of the hydrolysis products from ^{3}H-3',5'-cyAMP 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.


(a)
3',5'—cyAMP
3'—AMP
ADENOSINE
5'—AMP
(b)
(c)
SF
O

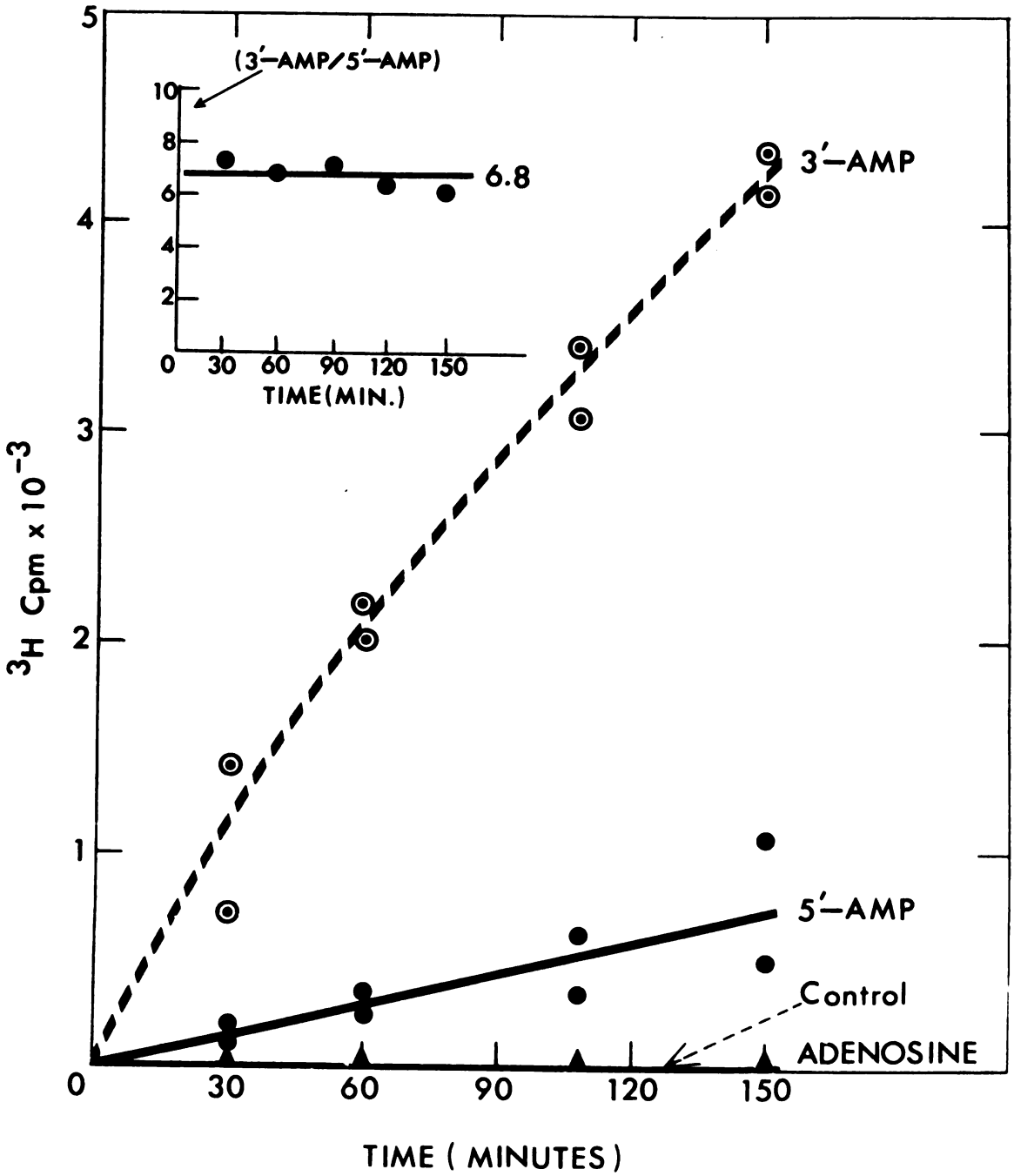
G-200 chromatogram as shown in Figure 1 gave essentially the same result as that observed in Figure 6.

Sucrose Density Gradient Study of the cyNPDE and Time Course of the Formation of 3'-AMP and 5'-AMP from 3',5'-cyAMP.--Although both 3'-AMP and 5'-AMP appeared to be products of the hydrolysis of 3',5'-cyAMP, it was desirable to know whether this was due to the presence of two different enzymes. Therefore, sucrose density gradient centrifugation was used to provide the enzyme preparation for study end products analysis throughout the gradient fractions and for study of the time course of the end products formation.

In the study of the time course of the formation of hydrolysis products from 3',5'-cyAMP, enzyme preparation of fraction 16 as shown in Figure 2 was incubated at 37° with the standard reaction mixture containing 1.8×10^7 cpm of ^{3}H-3',5'-cyAMP. Fraction 8 from the same enzyme preparation was used as control enzyme source. During the time courses, 0.01 ml of aliquot was taken for thin-layer chromatography and developed, eluted, and counted as described in the previous methods. As shown in Figure 8, both 3'-AMP and 5'-AMP increased gradually with time for the reaction mixture containing enzyme preparation of fraction 16 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 as observed

Figure 8.--Analysis of end product formation as a function of time from enzymatic hydrolysis of ^{3}H-3',5'-cyAMP.

Enzyme preparation from sucrose density gradient centrifugation (Fraction 16 in Figure 2) was incubated in the standard reaction mixture containing 1.5 x 10^{7} cpm of purified ^{3}H-3',5'-cyAMP at 37°. Following the time courses, 0.01 ml aliquot was taken for thin-layer chromatography and the areas corresponding to the authentic compounds (3'-AMP, 5'-AMP, and Adenosine) were recovered, eluted, and counted for radioactivity as described under "Methods." Duplicate samples were performed. Fraction 10 from the same figure was used as the control enzyme preparation. The value of ^{3}H-3'-AMP/^{3}H-5'-AMP is shown on the left corner of the figure. ^{3}H-3'-AMP, ⊙----⊙; ^{3}H-5'-AMP, ●——●; ^{3}H-Adenosine, ▲——▲. The control fraction 10 shows no detectable amounts of tritiated products formed.


(3'–AMP/5'–AMP)
6.8
TIME(MIN.)
3'–AMP
5'–AMP
Control
ADENOSINE
^{3}H Cpm x 10^{-3}
TIME (MINUTES)

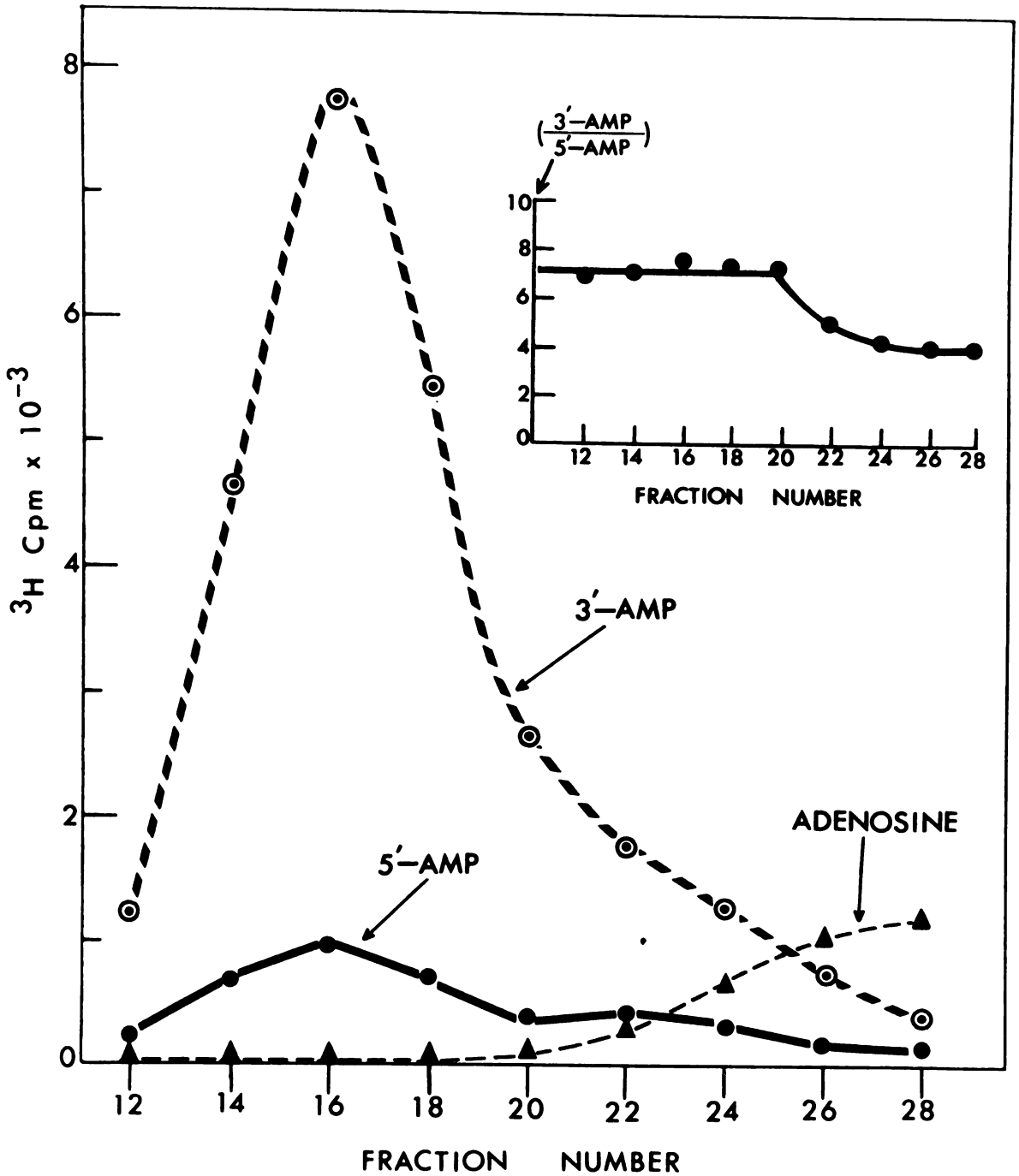
in the previous experiments. This indicates that both 3'-AMP and 5'-AMP are enzymatic products from 3',5'-cyAMP. However, this result does not rule out the possibility of the presence of two enzymes. In an attempt to separate the two activities, enzyme preparation of fractions 12-28 from sucrose density gradient centrifugation as shown in Figure 2-A were assayed for cyNPDE with the standard reaction mixture containing 3 x 10^6 cpm of ^{3}H-3',5'-cyAMP as described in the previous experiments. An 0.01 ml of aliquots were taken for thin-layer chromatography analysis after incubating the reaction mixture at 37° for 5 hr.

The radioactivity profile of reaction products is shown in Figure 9. It is evident that 3'-AMP and 5'-AMP are produced in identical ratios throughout all the fractions. The ratio of 3'-AMP:5'-AMP was 6.8-7.0:1 from fraction 12 to 20. 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 form adenosine.

Although there was no contaminating 3'-nucleotidase from the fraction 12 to 20, it was still questioned that 5'-AMP (or 3'-AMP) might be formed from 3'-AMP (or 5'-AMP) by transferase enzyme, which has been reported in E. coli (40, 41) and carrot leaves (42). With purified ^{3}H-3'-AMP from enzymatic hydrolysis of ^{3}H-3',5'-cyAMP as described under "Materials," the reaction mixture containing enzyme preparation of fraction 16 (in Figure 2), 0.1 M K-acetate

Figure 9.--End products formed from enzymatic hydrolysis of ^{3}H-3',5'-cyAMP.

The enzyme of fractions 12 to 28 from sucrose density gradient centrifugation (Figure 2-A) were assayed for cyNPDE activity with the standard reaction mixture containing 3 x 10^{6} cpm of purified ^{3}H-3',5'-cyAMP. After incubation at 37° for 5 hr, 0.01 ml of aliquot was then taken for thin-layer chromatography as described under "Methods." The areas corresponding to the areas of the authentic compounds (3'-AMP, 5'-AMP, and Adenosine) were eluted and counted. The ration of ^{3}H-3'-AMP/^{3}H-5'-AMP is shown on the right corner of the figure. Detailed procedures are given in the text. ^{3}H-3'-AMP, ⊙----⊙; ^{3}H-5'-AMP, ●——●; ^{3}H-Adenosine, ▲----▲.


8
6
4
2
0
^{3}H Cpm x 10^{-3}
12
14
16
18
20
22
24
26
28
FRACTION NUMBER
3′–AMP
5′–AMP
ADENOSINE
(3′–AMP / 5′–AMP)
10
8
6
4
2
0
12
14
16
18
20
22
24
26
28
FRACTION NUMBER

buffer, pH 5.4, 1 mM 3',5'-cyAMP, and 2.2 x 10^4 cpm of ^{3}H-3'-AMP in a total volume of 0.5 ml was incubated at 37°. During the time courses, 0.05 ml of aliquot was streaked on a thin-layer plate and developed with authentic compounds as described under "Methods." The result showed that there was no formation of ^{3}H-5'-AMP even at 2.5 hr. With ^{3}H-5'-AMP, there was also no formation of ^{3}H-3'-AMP. Therefore, it is suggested that both 3'-AMP and 5'-AMP are direct products from the enzymatic hydrolysis of 3',5'-cyAMP. The enzyme apparently is able to cleave both ester linkages.

Properties of the cyNPDE Enzyme

Time Course and Enzyme Concentration.--With the standard assay procedure and 10 µg of protein of the enzyme preparation, the progress curve of cyAMP breakdown by pea cyNPDE (Figure 10) showed that the amount of P_i released maintained a linear rate up to 2 hr with either 2',3'-cyAMP or 3',5'-cyAMP as substrate. In addition, the amount of cyAMP hydrolyzed was a linear function of protein concentration over a wide range under standard incubation conditions (Figure 11). The rate of hydrolysis of 2',3'-cyAMP was about twice that of 3',5'-cyAMP.

Effect of pH on Enzyme Activity and Stability.--As shown in Figure 12, cyNPDE had a relatively sharp pH optimum at 5.4-6.0 toward either 2',3'-cyAMP or 3',5'-cyAMP. These

Figure 10.--Time course of cyNMP breakdown by pea cyNPDE.

The reaction mixture containing 60 µg of protein, 2 mM substrate, 0.1 M K-acetate buffer, pH 5.4, and distilled water in a final volume of 3.0 ml was incubated at 37°. 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'-cyAMP, ⊙----⊙; 3',5'-cyAMP, ●——●.

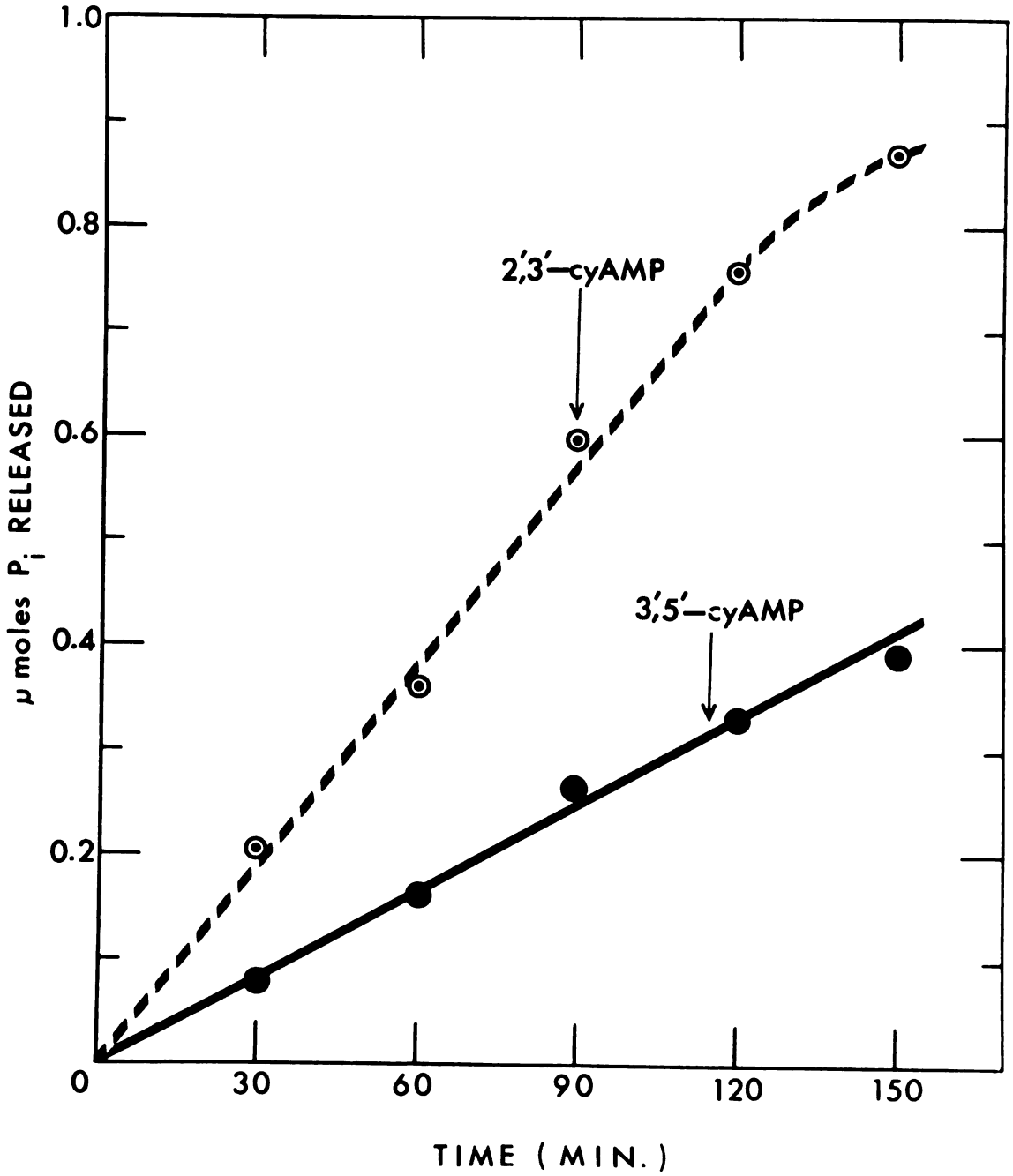

1.0
0.8
0.6
0.4
0.2
0
μ moles P_i RELEASED
2',3'—cyAMP
3',5'—cyAMP
30
60
90
120
150
TIME (MIN.)

Figure 11.--Activity of pea cyNPDE 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'-cyAMP, ⊙----⊙; 3',5'-cyAMP, ●——●.

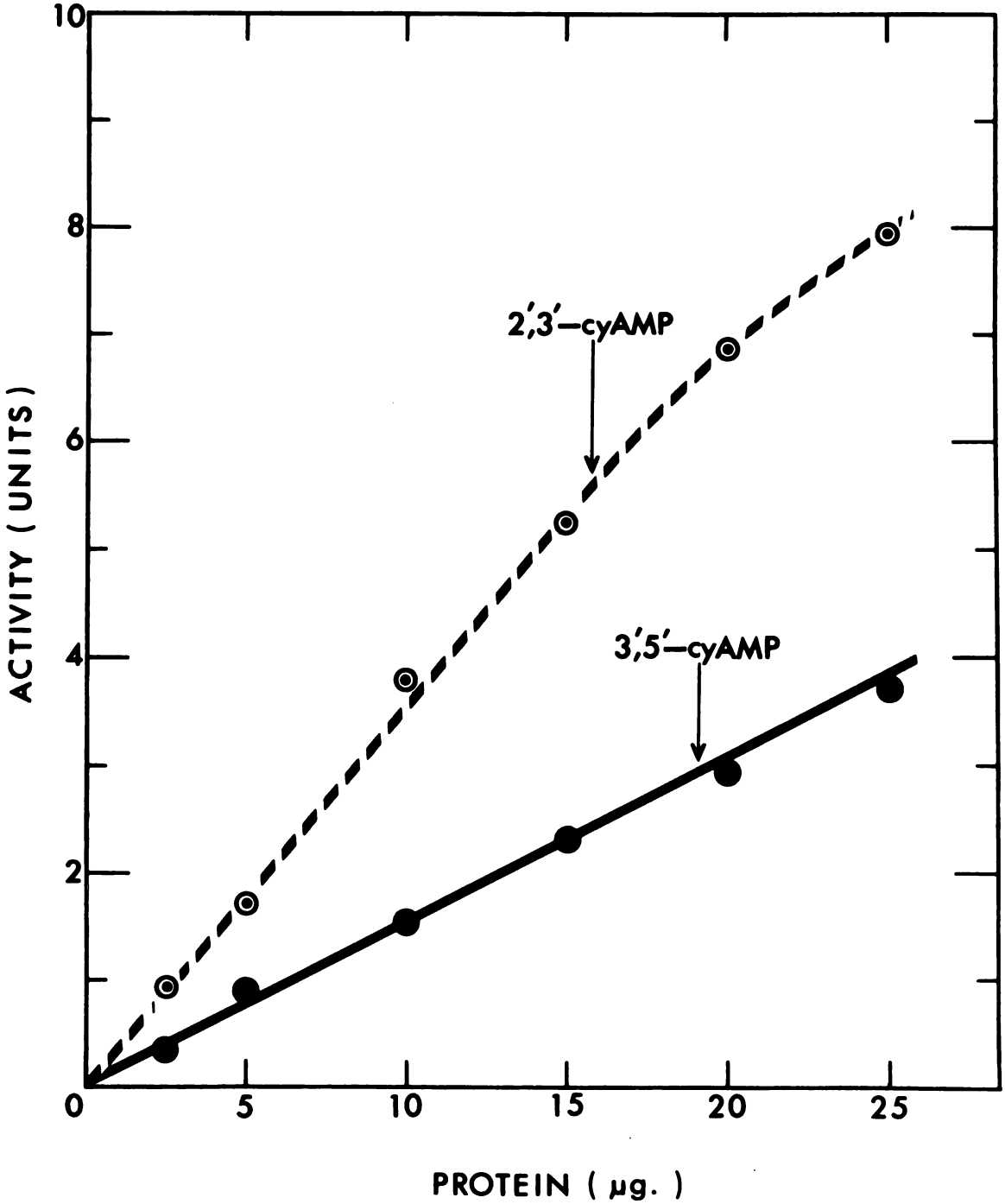
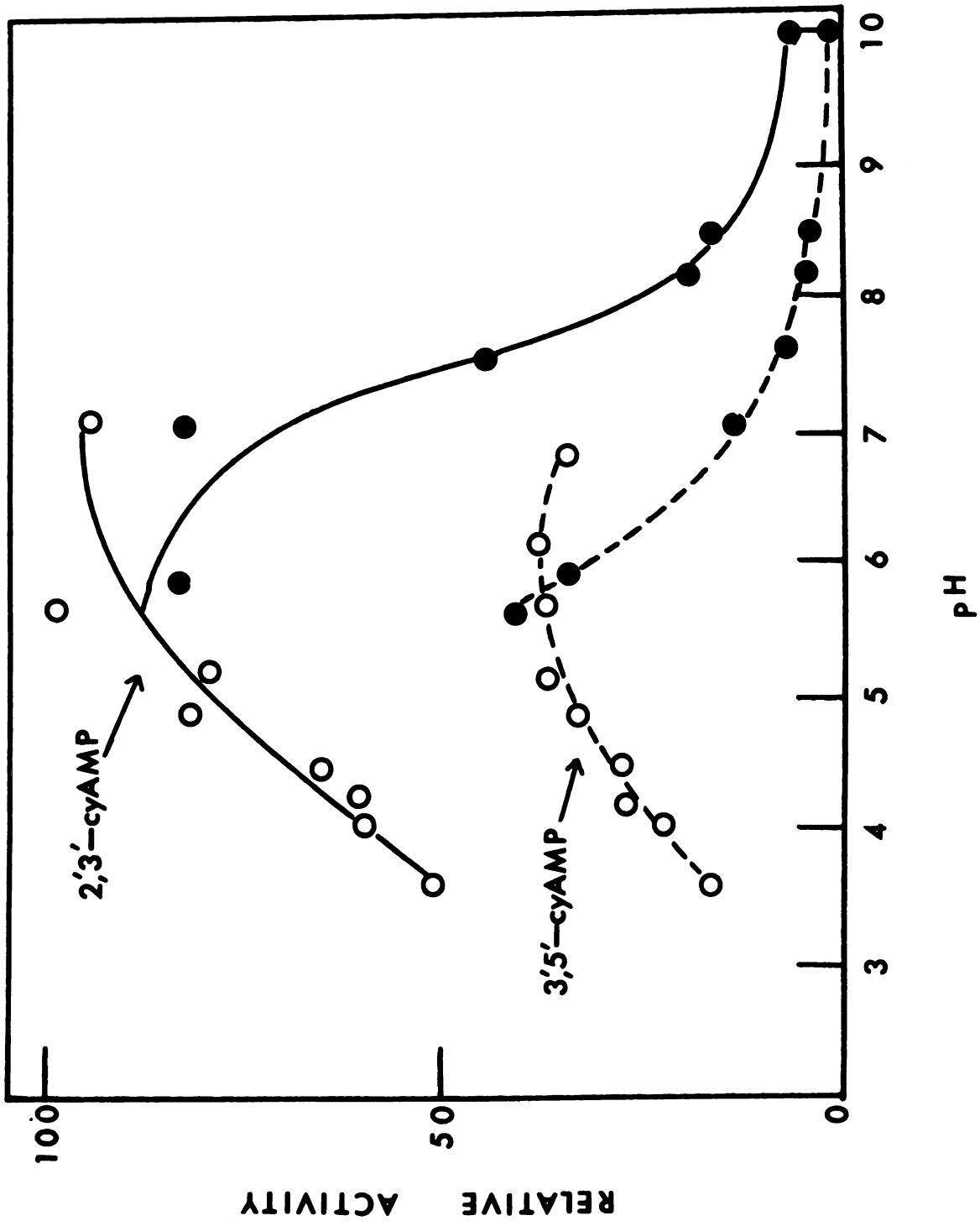

10
8
6
4
2
0
5
10
15
20
25
ACTIVITY (UNITS)
PROTEIN (µg.)
2',3'—cyAMP
3',5'—cyAMP

Figure 12.--Effect of pH on the activity of pea cyNPDE.

The reaction mixture and experimental procedures were as described for the standard assay. Buffer used: 0.01 M K-acetate, O; 0.1 M Tris-acetate, ●. Substrate used: 2',3'-cyAMP, ———; 3',5'-cyAMP, ------.


RELATIVE ACTIVITY
100
50
0
3
4
5
6
7
8
9
10
pH
2',3'-cyAMP
3',5'-cyAMP

enzyme activities were quite stable under acidic pH at 4° or -20°. The optimal pH of pea cyNPDE was different from that of similar enzymes in microorganisms (38, 39) and animal systems (33-35) for which the enzyme has been shown to have a maximal activity in alkaline pH region, pH 7.5 to 8.0.

Influence of Various Metal Ions.--"Assay method-1" and 3'-nucleotidase II were used in the assay of the hydrolysis of 2',3'-cyAMP in the presence of various metal ions. For the assay of the influence of metal ions on enzyme activity toward 3',5'-cyAMP, purified ^{3}H-3',5'-cyAMP and "Assay Method-2" were used.

The effect of various metal ions on enzyme activity is shown in Table 4. It is evident that both activities behaved quite similarly in response to the presence of metal ions concerned. A slight increase in both enzyme activities was observed in the presence of Mn^{++}, Co^{++}, and Zn^{++} at concentrations of 0.1-1.0 mM. NaF at 1 mM showed a 45% of inhibition on enzyme activity toward 3',5'-cyAMP with no apparent effect on the enzymatic hydrolysis of 2',3'-cyAMP. The EDTA at the concentration of 0.1 mM or 1.0 mM appeared to have no effect on either activity as also observed in Serratia marcescens (38).

Effect of Various Concentration of Sulfhydryl Compounds and NaF.--Because sulfhydryl compounds such as cysteine and dithiothreitol do not inhibit the activity of

TABLE 4.--Effect of inorganic ions on the activity of cyclic nucleotide phosphodiesterase.

Compound	Cyclic Nucleoside Monophosphate Assayed	
	2',3'-cyAMP[a]	3',5'-cyAMP[b]
	Activity Remaining (%)	
None	100	100
$MgCl_2$, 1×10^{-3}M	101	100
1×10^{-4}M	101	101
$MnCl_2$, 1×10^{-3}M	112	138
1×10^{-4}M	110	137
$CoCl_2$, 1×10^{-3}M	110	132
1×10^{-4}M	105	117
$ZnCl_2$, 1×10^{-3}M	118	130
1×10^{-4}M	108	114
$(NH_4)_2SO_4$, 1×10^{-3}M	107	110
1×10^{-4}M	100	101
KCN, 1×10^{-3}M	105	107
1×10^{-4}M	100	101
KCl, 1×10^{-3}M	108	110
1×10^{-4}M	110	119
NaCl, 1×10^{-3}M	100	101
1×10^{-4}M	101	100
NaF, 1×10^{-3}M	117	55
1×10^{-4}M	110	78
EDTA, 1×10^{-3}M	100	100
1×10^{-4}M	100	100

[a]With 2 mM 2',3'-cyAMP as substrate, the standard assay was performed under the condition that 3'-nucleoditase was not rate limiting.

[b]With 2 mM ^{3}H-3',5'-cyAMP as substrate, the details are described in the text.

pea 3'-nucleotidase I as described in the previous section, pea 3'-nucleotidase I was used in a coupled enzyme assay system for the study of the effect of reducing reagents on the activity of pea cyNPDE. As shown in Table 5, 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 35-45%. The NaF inhibited the hydrolysis of 3',5'-cyAMP, but not 2',3'-cyAMP.

Effect of Urea on Enzyme Activity.--Both activities decreased in parallel in the presence of various concentration of urea (Figure 13). However, points of 50% activity were at 6 M urea for activity toward 3',5'-cyAMP, and 8 M urea for the hydrolysis of 2',3'-cyAMP. It was also observed that both activities decreased simiarly with respect to various times of preincubation in 6.5 M urea (Figure 14).

Optimum Temperature and Heat Stability of Enzyme Activity.--As seen in Figure 15, both activities had an optimum temperature at 40° under the standard assay condition. It is evident that heat inactivation began appreciably at 50°. Activity toward 2',3'-cyAMP was apparently more sensitive to temperature than that toward 3',5'-cyAMP. An Arrhenius plot of data taken from Figure 15 displayed a change in slope at 40° as shown in Figure 16. With the integrated form of the Arrhenius equation,

$$E_a = \frac{2.303\ R\ T_1T_2\ (\log k_2 - \log K_1)}{T_2 - T_1},$$

TABLE 5.--Effect of various concentration of reducing reagents and NaF on the activity of pea cyclic nucleotide phosphadiesterase.

Addition[a]	Substrate Assayed	
	2',3'-cyAMP	3',5'-cyAMP
	Activity Remaining (%)	
None		
Cysteine, 4×10^{-3}M	100	100
2×10^{-3}M	134	144
4×10^{-4}M	119	109
2×10^{-4}M	129	121
4×10^{-5}M	95	105
Dithiothreitol,		
4×10^{-3}M	133	113
2×10^{-3}M	129	110
4×10^{-4}M	126	134
2×10^{-4}M	108	105
4×10^{-5}M	120	98
NaF, 4×10^{-3}M	111	37
2×10^{-3}M	116	38
4×10^{-4}M	121	43
2×10^{-4}M	114	79
1×10^{-4}M	110	79

[a]All reagents were added at zero time with the standard reaction mixture. The 3'-nucleotidase in excess amount was used for the coupled assay system as described under "Methods."

Figure 13.--Effect of the concentration of urea on the activity of pea cyNPDE.

The standard reaction mixtures containing 20 µg protein were incubated with various concentration of urea at 37° for 1 hr. After incubation, excess 3'-nucleotidase was added and the inorganic phosphate released was determined as described under "Methods." Substrate used: 2',3'-cyAMP, ⊙----⊙; 3',5'-cyAMP, ●——●.

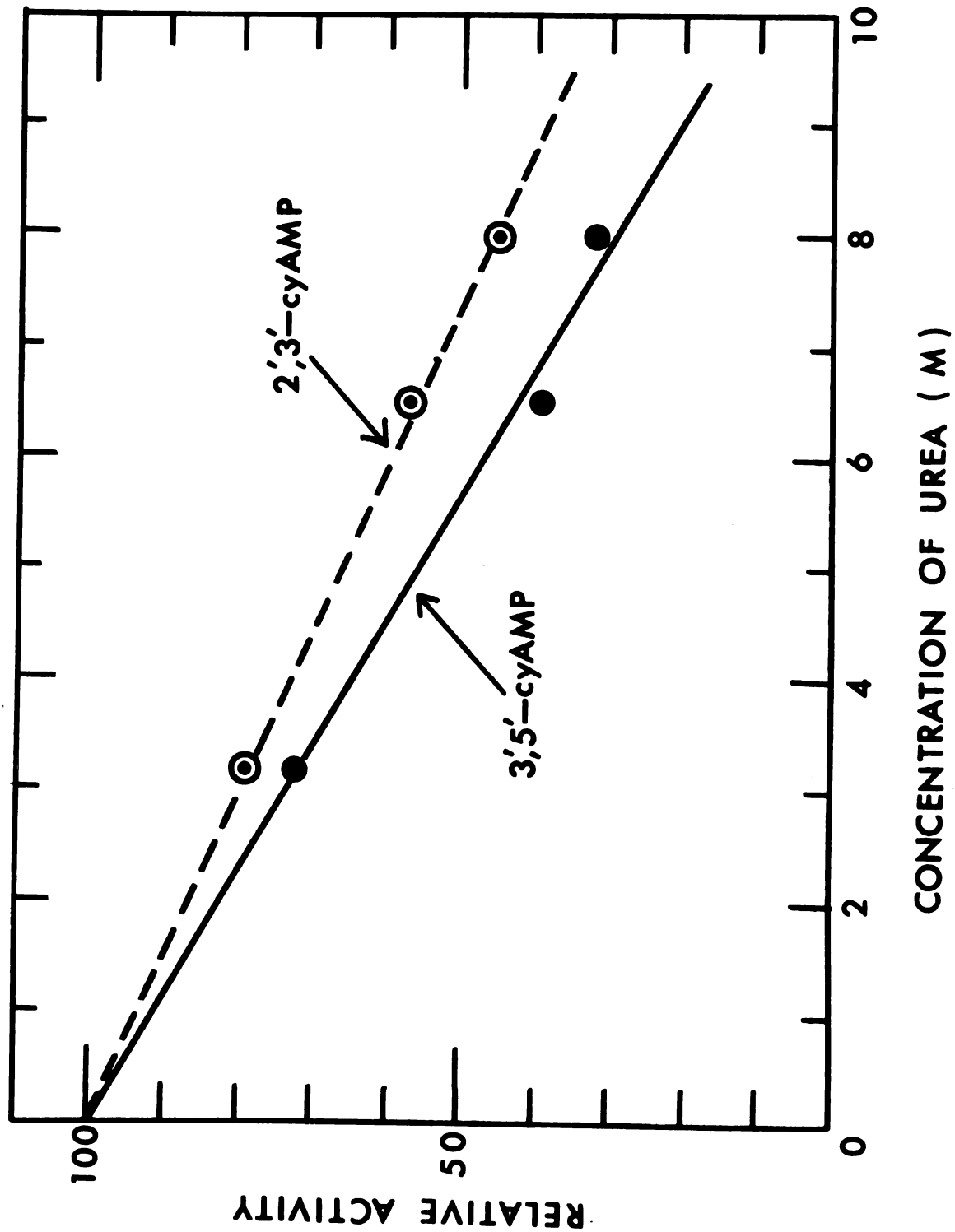

RELATIVE ACTIVITY
100
50
0
2',3'-cyAMP
3',5'-cyAMP
0
2
4
6
8
10
CONCENTRATION OF UREA (M)

Figure 14.--Time course of the effect of urea on cyNPDE activity.

The enzyme (20 µg of protein) was preincubated with 6.5 M urea in a volume of 0.2 ml at 37° for various times. After preincubation, the standard reaction mixture was added to give a final volume of 2.0 ml. The assay mixture was incubated further at 37° for 1 hr. Excess 3'-nucleotidase was used for the coupled assay system as shown under "Methods." Substrates used: 2',3'-cyAMP, ⊙----⊙; 3',5'-cyAMP, ●——●.

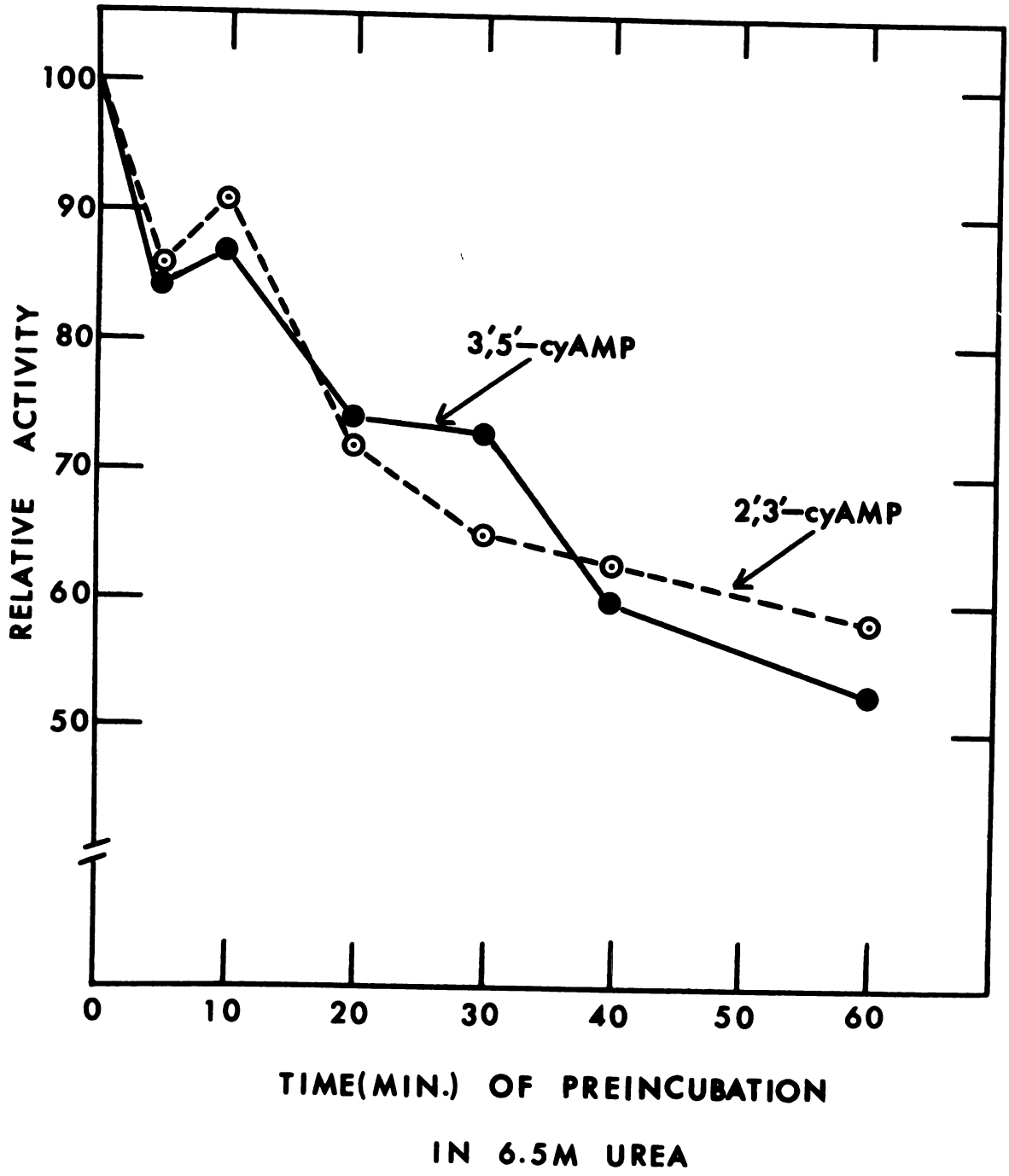

100
90
80
70
60
50
RELATIVE ACTIVITY
3',5'-cyAMP
2',3'-cyAMP
0
10
20
30
40
50
60
TIME(MIN.) OF PREINCUBATION
IN 6.5M UREA

Figure 15.--Temperature-activity profile for pea cyNPDE.

Standard reaction mixture containing 20 µg protein was used for this study. Excess 3'-nucleotidase was added after 1 hr incubation at various temperature. For further details, refer to the standard methods. Substrate used: 2',3'-cyAMP, ⊙----⊙; 3',5'-cyAMP, ●——●.

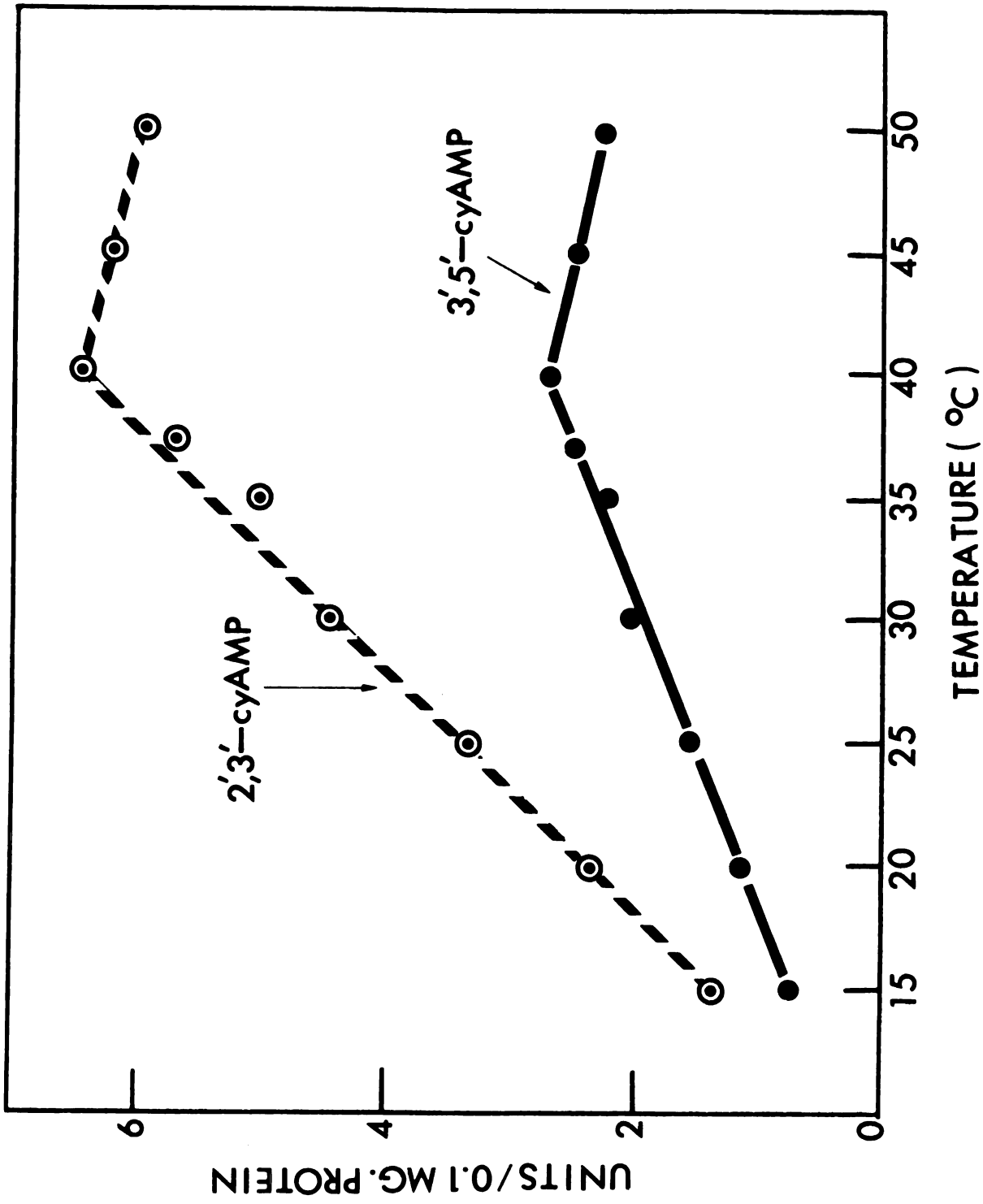
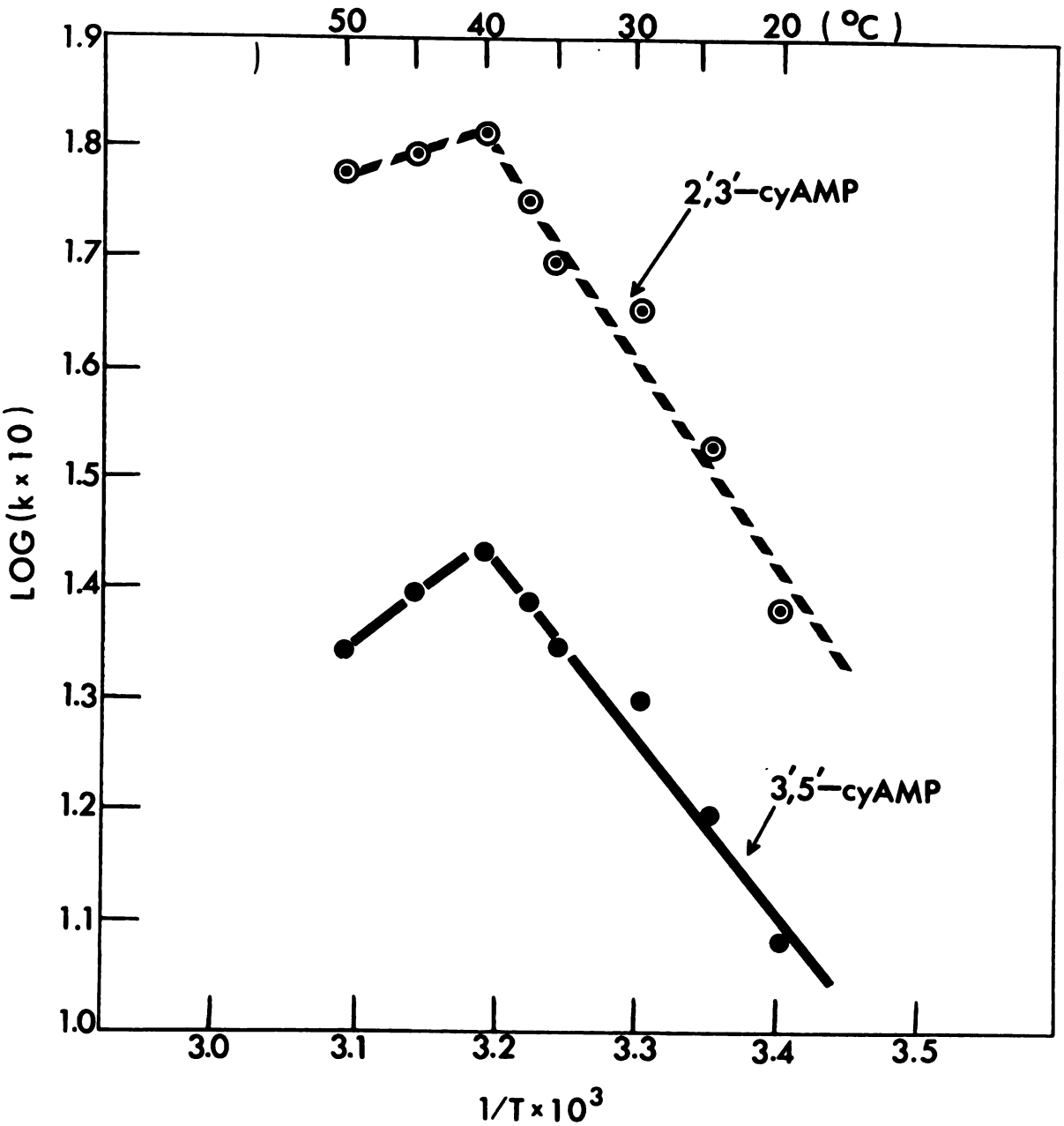

UNITS/0.1 MG. PROTEIN
0
2
4
6
TEMPERATURE (°C)
15
20
25
30
35
40
45
50
2',3'-cyAMP
3',5'-cyAMP

Figure 16.--Arrhenius plot for the determination of the activation energy.

Data are taken from Figure 15. Detailed experimental conditions are described in Figure 15. Substrates used: 2',3'-cyAMP, 0----0; 3',5'-cyAMP, ●——●.


50
40
30
20 (°C)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
LOG (k × 10)
2',3'–cyAMP
3',5'–cyAMP
3.0
3.1
3.2
3.3
3.4
3.5
1/T × 10³

the activation energy was calculated to be 8.6 Kcal/mole for hydrolysis of 2',3'-cyAMP and 7.2 Kcal/mole for hydrolysis of 3',5'-cyAMP between the temperature range from 20° to 40°. The value for the hydrolysis of 3',5'-cyAMP is close to the value of 7.5 Kcal/mole reported by Cheung on rat brain enzyme (35), but quite different from the 19 Kcal/mole reported by Nair on dog heart enzyme (34). The two activities showed a similarity in response to heat inactivation as shown in Figure 17.

Effect of Organic Compounds on Enzyme Activity.--It has been reported that methylxanthines such as caffeine and theophylline inhibit animal 3',5'-cyclic nucleotide phosphodiesterase (33-36, 43) and the microbial enzymes of S. marcascens (38) and P. polycephalum (39), but not the microbial enzyme from E. coli (13) and the enzyme from slime mold of D. discoideum (37). In addition, nucleoside and nucleotide derivatives inhibit the animal enzyme (35). Do such organic compounds inhibit pea cyNPDE activity?

Using "Assay Method-2," activity was determined in a 0.02 ml of aliquot from a 0.25 ml of reaction mixture containing 2 mM ^{3}H-3',5'-cyAMP (2.5×10^{5} cpm/mM), 0.04 M K-acetate buffer, pH 5.4, 10 µg of protein and the organic compound at the concentration indicated. The incubation was at 37° for 1 hr. The relative activities are shown in Table 6. Caffeine and theophylline at a concentration of either 0.1 mM or 4.0 mM showed no appreciable effect on pea

Figure 17.--Heat stability of pea cyNPDE.

The standard reaction mixtures containing 20 μg of protein were preincubated at the various temperature for 5 min. After preincubation, the tubes were chilled in an ice bath and 2 mM substrate was added and incubated for further 1 hr at 37°. In the presence of excess of 3'-nucleotidase, inorganic phosphate released was determined as described under "Methods." Substrates used: 2',3'-cyAMP, O----O; 3',5'-cyAMP, ●——●.

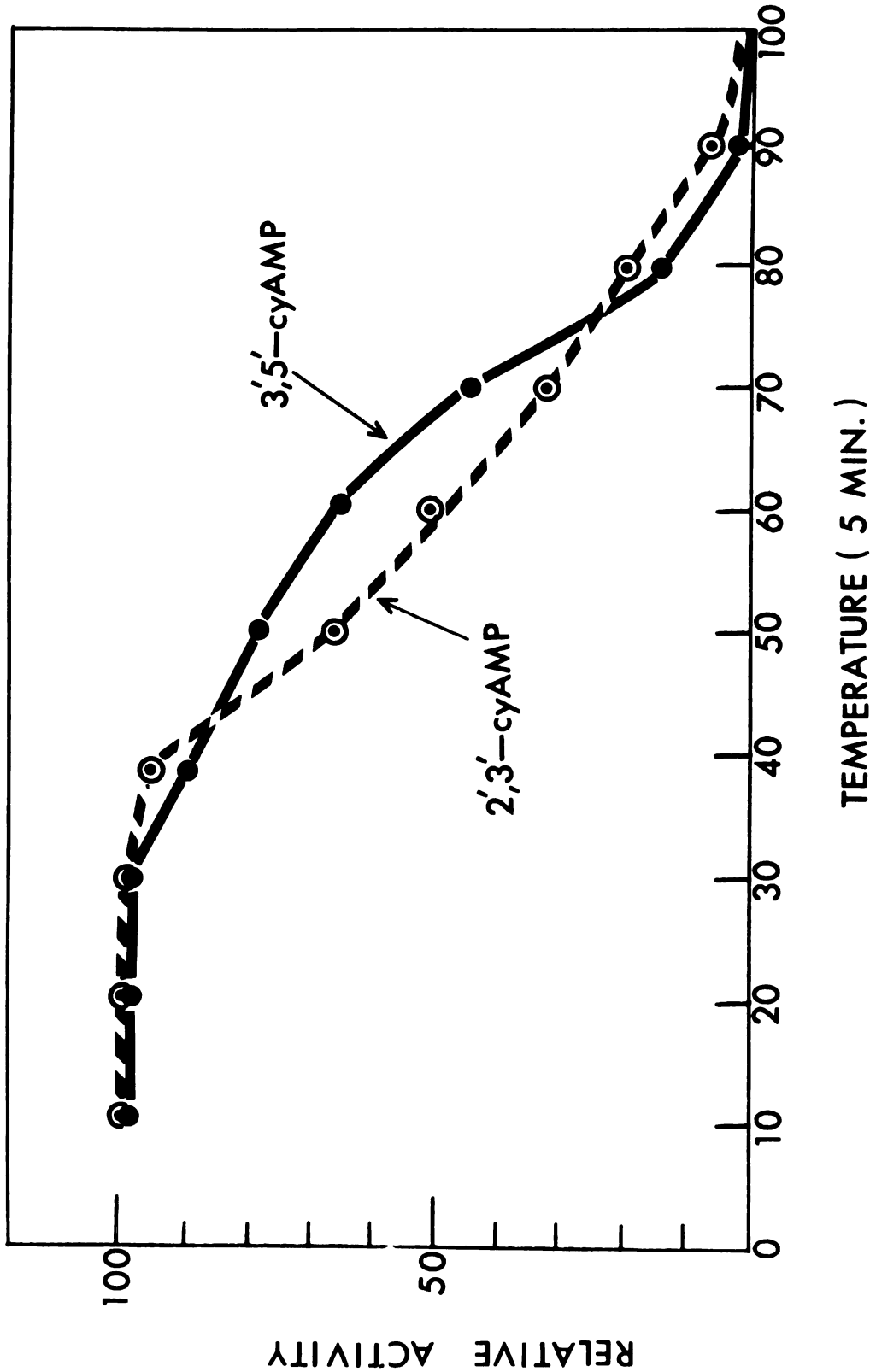

RELATIVE ACTIVITY
100
50
0
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (5 MIN.)
3',5'—cyAMP
2',3'—cyAMP

TABLE 6.--Effect of organic compounds on the activity of pea cyNPDE using ^{3}H-3',5'-cyAMP as substrate.[a]

Compound Added	Concentration of Compound Added	
	4×10^{-3}M	1×10^{-4}M
	Activity Remaining (%)	
None	100	100
3',5'-cyGMP	41	78
3',5'-cyCMP	28	81
2',3'-cyAMP	27	95
2',3'-cyUMP	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

[a]A 0.25 ml reaction mixture containing 2 mM ^{3}H-3',5'-cyAMP (1×10^{7} cpm), 0.04 M K-acetate buffer, pH 5.4, 10 units of enzyme and organic compound at the concentration indicated was incubated at 37° for 1 hr and the radioactive end products from a 0.02 ml aliquot were determined as described under "Methods."

cyNPDE. However, the nucleoside and nucleotide derivatives except adenosine, P_i, and PP_i showed varied inhibition of the enzyme activity toward 3',5'-cyAMP. Imidazole at the concentration of 4 mM has been shown to activate phosphodiesterase in various animal tissues (33, 34, 44-47), but it has no effect on the pea enzyme.

Rate of Hydrolysis of Cyclic Nucleoside Monophosphates.--The relative rates of hydrolysis of cyclic nucleoside monophosphates were studied. With 2 mM substrate, pea cyNPDE showed no particular specificity for any 2',3'-cyNMP or 3',5'-cyNMP (Table 7). The relative rates of hydrolysis of the substrates in decreasing order is as follows: 2',3'-cyUMP > 2',3'-cyAMP > 3',5'-cyUMP > 2',3'-cyGMP > 3',5'-cyIMP > 3',5'-cyAMP > 2',3'-cyCMP, 3',5'-cyGMP, 3',5'-cyTMP > 3',5'-cyCMP. It is interesting that 2,6-dibutyryl 3',5'-cyAMP, an analog of 3',5'-cyAMP which is insensitive to the animal enzyme (2), was hydrolyzed with at a rate similar to that of 3',5'-cyAMP in pea system. In general, the enzymes so far isolated and partially purified from animal tissues have been shown to be specific for hydrolysis of 3',5'-cyAMP and 3',5'-cyGMP (2, 49). Although the diesterase from rabbit brain seems to have activity toward 2',3'-cyAMP, it may be due to the contamination of a separate enzyme (48).

TABLE 7.--Relative cyNPDE activities toward cyclic nucleoside monophosphates.[a]

Substrate Assayed	Relative Activity (%)
2',3'-cyUMP	100
2',3'-cyAMP	83
2',3'-cyGMP	51
2',3'-cyCMP	26
3',5'-cyUMP	68
3',5'-cyIMP	45
3',5'-cyAMP	41
3',5'-cyGMP	26
3',5'-cyTMP	26
3',5'-cyCMP	23

[a]Assays were carried out as described under "Methods." Two mM substrates were used for assays.

Determination of Michaelis Constant (Km).--The Km values for 2',3'-cyNMP and 3',5'-cyNMP were calculated from the experimental data obtained by incubating several dilutions of the substrate with 10 μg of protein on cyNPDE preparation at 37° for 1 hr. The reaction was stopped by heating to 100° for 3 min. The reaction mixture was cooled, excess of 3'-nucleotidase was added and P_i was measured as described under "Methods." The double reciprocal plots of 1/S vs. 1/V for various substrates by the method of Lineweaver and Burk (50) are shown in Figure 18 and Figure 19. It is apparent that the affinity constant (1/Km) of the respective substrate decreased in the order as follows: 3',5'-cyUMP > 2',3'-cyUMP > 2',3'-cyAMP > 3',5'-cyAMP > 3',5'-cyGMP > 2',3'-cyGMP > 3',5'-cyCMP > 2',3'-cyCMP. A summary of the properties of the well-characterized 3',5'-cyclic nucleotide phosphodiesterase and the pea cyNPDE is shown in Table 8.

Activity Toward Other Organic Phosphates.--Although the pea cyNPDE from the Sephadex G-200 contained activities toward RNA, DNA, and other organic phosphates as indicated in Figure 20, such activities were probably due to the contamination of specific or nonspecific phosphatases rather than cyNPDE itself having such activities. For instance, evidence for the contamination of RNase and 3'-nucleotidase in cyNPDE preparation was demonstrated in the study of gel electrophoresis as the result shown in Figure 3. Furthermore,

Figure 18.--Effect of substrate (2',3'-cyNMP) concentration on the activity of pea cyclic nucleotide phosphodiesterase.

The reaction mixture contained 10 µg of protein and various concentrations of 2',3'-cyAMP (●), 2',3'-cyGMP (O), 2',3'-cyUMP (Δ), or 2',3'-cyCMP (▲) as the substrate. Enzyme assays were performed as described under "Methods." Left, the plot of enzyme activity (V) versus substrate concentration (S). Right, the Lineweaver-Burk plot of 1/V cs. 1/S. The Km for each substrate is shown.

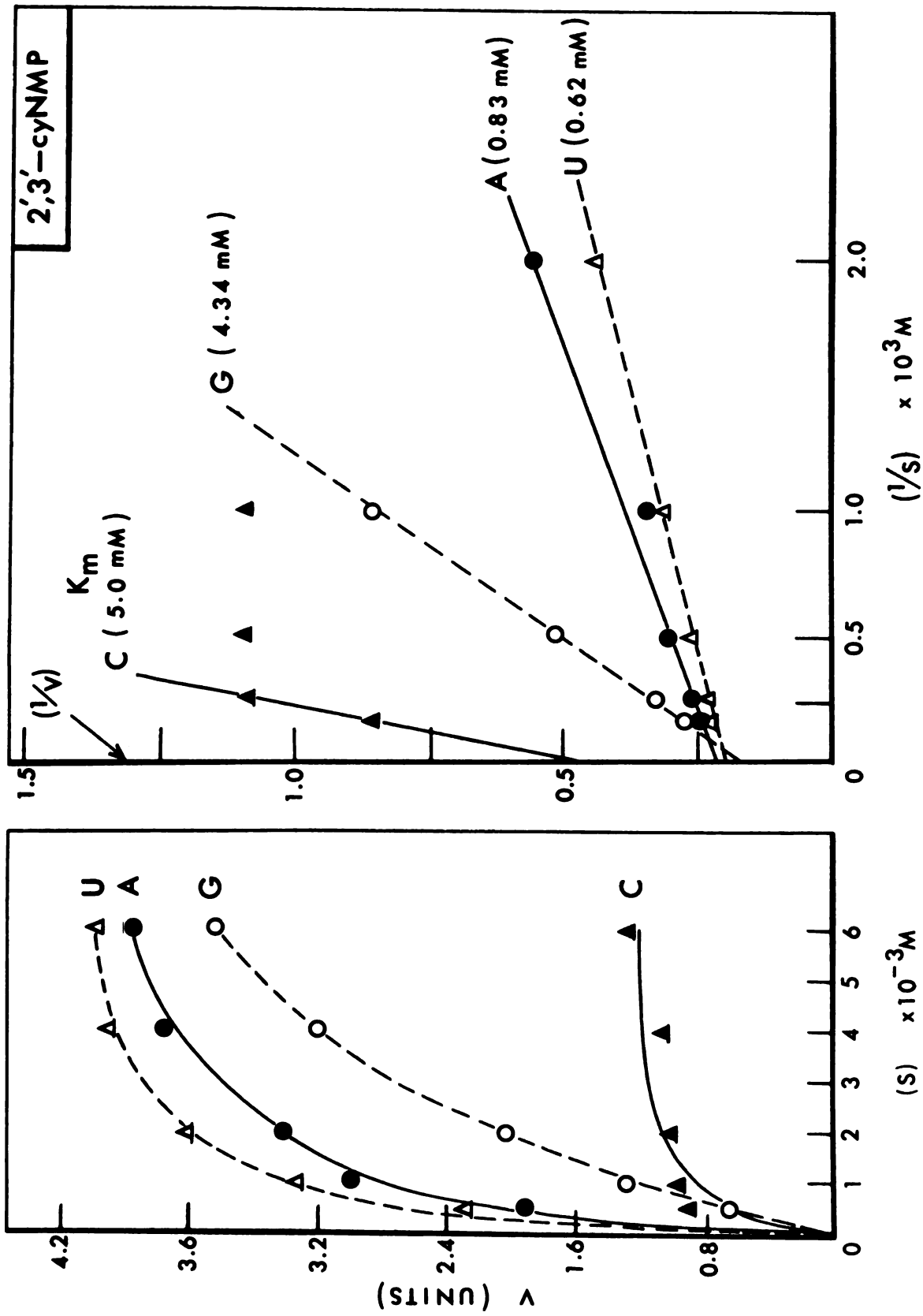

2',3'—cyNMP
A (0.83 mM)
U (0.62 mM)
G (4.34 mM)
C (5.0 mM)
K_m
(1/v)
(1/s) x 10^3M
v (UNITS)
(S) x10^{-3}M
U
A
G
C

Figure 19.--Effect of substrate (3',5'-cyNMP) concentration on the activity of pea cyclic nucleotide phosphodiesterase.

The reaction mixture contained 10 µg of protein and various concentrations of 3',5'-cyAMP (●), 3',5'-cyGMP (O), 3',5'-cyUMP (Δ), or 3',5'-cyCMP (▲) as the substrate. Detailed procedures and the inorganic phosphate assay were performed as described under "Methods." Left, the plot of enzyme activity (V) versus substrate concentrations. Right, the Lineweaver-Burk plot of 1/V vs. 1/S. The Km for each substrate is shown.

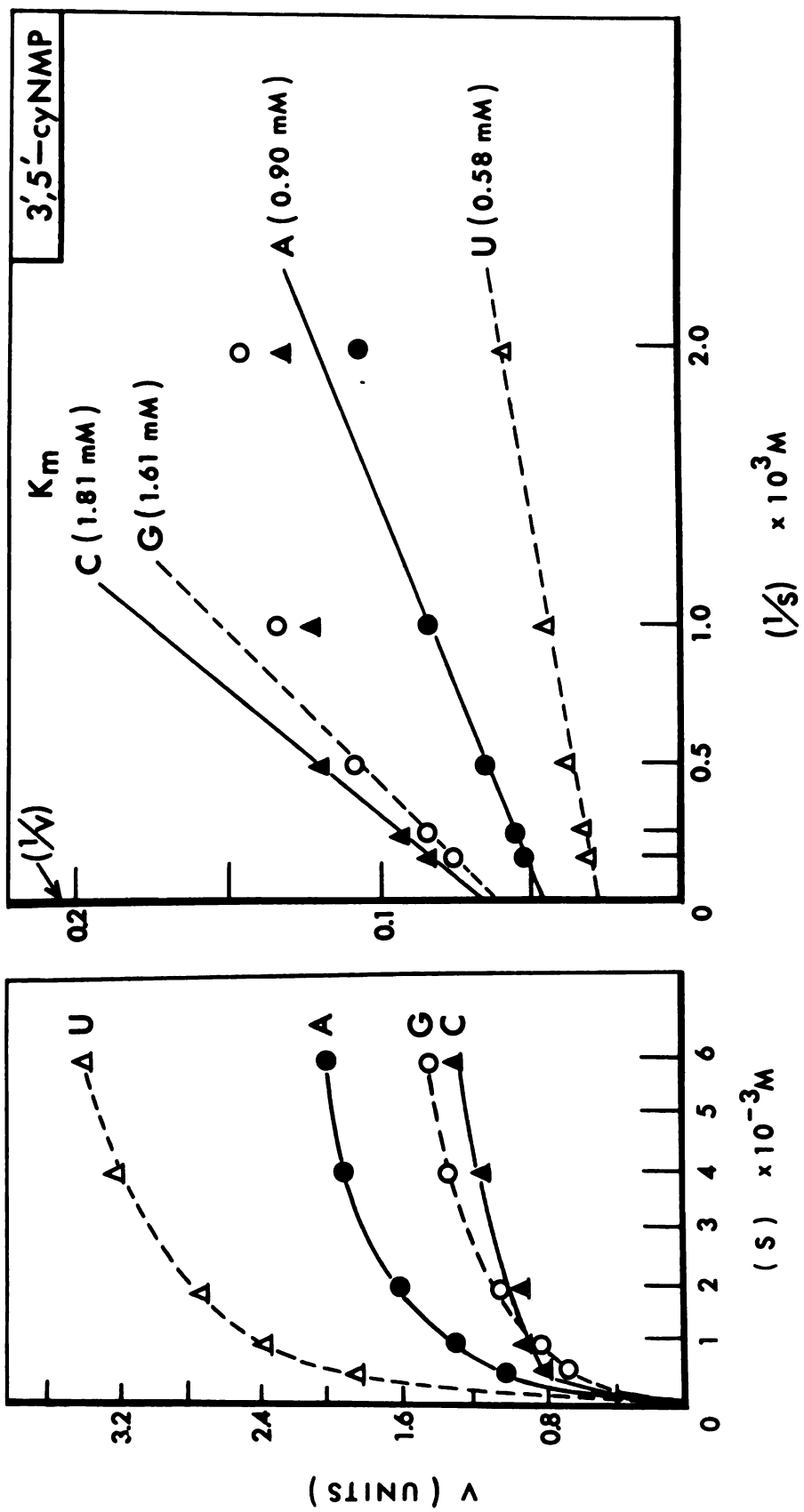

3',5'-cyNMP
Km
C (1.81 mM)
G (1.61 mM)
A (0.90 mM)
U (0.58 mM)
(1/v)
0.2
0.1
0
0.5
1.0
2.0
(1/s) x 10^3 M
U
A
G
C
3.2
2.4
1.6
0.8
0
1
2
3
4
5
6
(s) x 10^-3 M
v (UNITS)

Figure 20.--The elution profiles of enzyme activities from Sephadex G-200 column chromatography.

All experimental conditions are the same as described in Figure 1. Enzyme activities for various substrates were assayed according to "Methods." Upper, each solid curve represents the enzyme activity for one specific substrate as indicated by the arrow. Dashed line shows the activity toward p-nitrophenol phosphate (PNP) as measured by the absorbance at 410 mμ. Each fraction corresponds to the number shown in the lower part of the figure. Lower, solid curve represents protein concentration as measured by the absorbance at 280 mμ. Enzyme activities (in units/ml) on 2',3'-cyAMP (●——●), 3',5'-cyAMP (0——0), 3'-AMP (pH 8.0) (X----X), RNA (▲----▲), and DNA (Δ----Δ) were determined as described under "Methods."

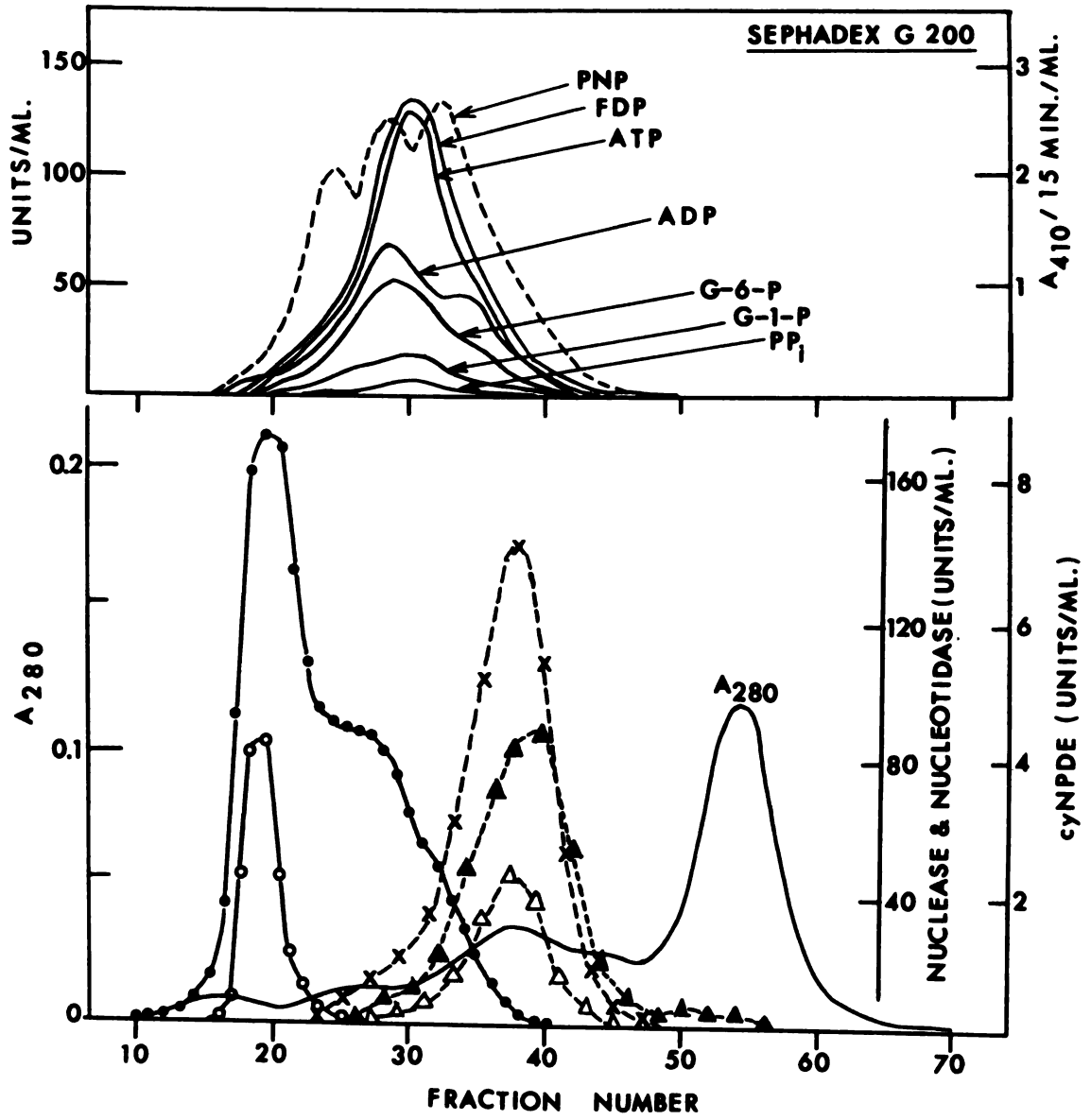

SEPHADEX G 200
PNP
FDP
ATP
ADP
G-6-P
G-1-P
PPi
UNITS/ML.
A410/15 MIN./ML.
A280
NUCLEASE & NUCLEOTIDASE (UNITS/ML.)
cyNPDE (UNITS/ML.)
FRACTION NUMBER

TABLE 8.--Summary of well-characterized 3',5'-cyclic nucleotide phosphodiesterases.

Source	Sp. Act.[a]	pH	$K_{m(M)}$	Mg^{++}[b]	Methyl-Xanthine	End Product	Mol. Wt.	$E_{a(Kcal/mol)}$	Reference
Beef Heart	8.50	7.9	$6\text{-}10\times10^{-5}$	+	Inhibition	5'-AMP			Butcher et al. (1962)
Rabbit Brain	0.04	7.5	---	+	Inhibition	5'-AMP			Drummond et al. (1961)
Dog Heart	0.95	8.5 9.2	4.9×10^{-4}	+	Inhibition	5'-AMP		19.0	Nair (1966)
Rat Brain	0.07	8.0	$1\text{-}3\times10^{-4}$	+	Inhibition	5'-AMP		7.5	Cheung (1967)
Rat Liver	0.41	7.5	6.2×10^{-5}	+	Inhibition	5'-AMP	>200,000		Menahan et al. (1969)
Trout Brain	0.05	9.0	9×10^{-5}	+	Inhibition	5'-AMP			Yamamoyo et al. (1969)
D. discoideum	0.80	7.5	2×10^{-3}	+	No effect	5'-AMP	300,000		Chang (1968)
E. coli	0.03	8.5	7.7×10^{-4}	?	No effect	5'-AMP	>200,000		Mondarde et al. (1969)
S. marcescens	268.0	8.0	5.2×10^{-4}	-	Inhibition	5'-AMP	51,000		Okabayashi et al. (1970)
P. polycephalum	0.80	7.5	0.5×10^{-3}	+	Inhibition	5'-AMP			Murray et al. (1971)
Pea seedling	0.27	5-6	9.0×10^{-4}	-	No effect	$\frac{3'\text{-AMP}}{5'\text{-AMP}}=7$	350,000	7.2	This study

[a]Specific Activity (Sp. Act.): μmoles 3',5'-cyAMP hydrolyzed/min/mg of protein.

[b]+ denotes enzyme required the presence of Mg^{++} for maximal activity. - represents no requirement.

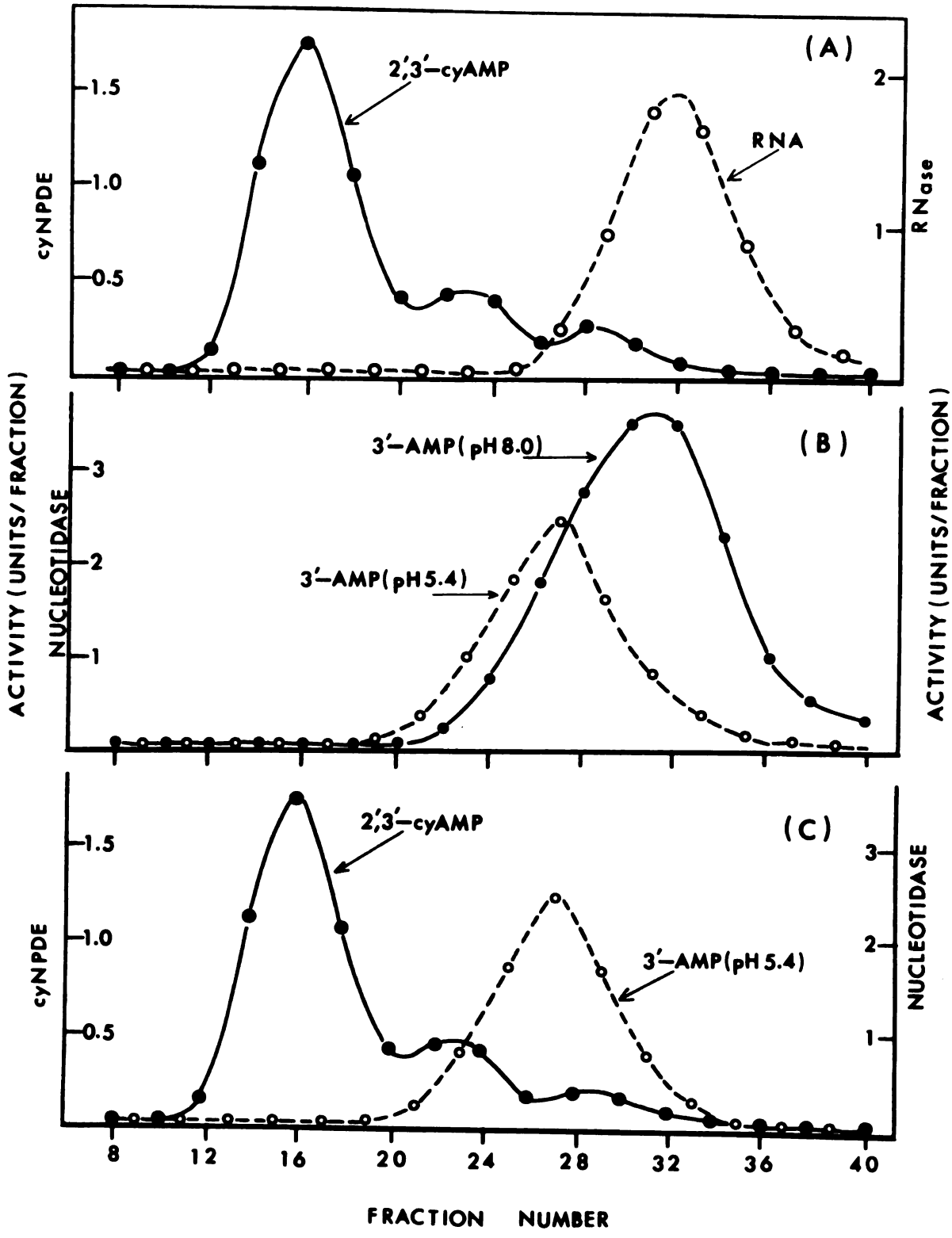
Figure 21.--The elution profiles of cyNPDE, RNase, and 3'-nucleotidases from sucrose density gradient centrifugation.

For detailed experimental conditions, refer to the description in Figure 2-A.

(A) The elution profile of cyNPDE activities toward 2',3'-cyAMP (●———●) and RNase (O----).

(B) The elution profile of 3'-nucleotidases activities. Substrates and buffers used: 3'-AMP, K-acetate 0.1 M, pH 5.4 (O----O); 3'-AMP, Tris-acetate 0.1 M, pH 8.0 (●———●).

(C) The elution profile of cyNPDE activity toward 2',3'-cyAMP (●———●) and 3'-nucleotidase activity toward 3'-AMP (O----O). 0.1 M K-acetate buffer, pH 5.4 was used for assays.


(A)
2',3'-cyAMP
RNA
cyNPDE
RNase
1.5
1.0
0.5
2
1
(B)
3'-AMP(pH8.0)
3'-AMP(pH5.4)
ACTIVITY (UNITS/ FRACTION)
NUCLEOTIDASE
ACTIVITY (UNITS/FRACTION)
3
2
1
(C)
2',3'-cyAMP
3'-AMP(pH 5.4)
cyNPDE
NUCLEOTIDASE
1.5
1.0
0.5
3
2
1
8
12
16
20
24
28
32
36
40
FRACTION NUMBER

the sucrose density gradient centrifugation provided evidence that pea cyNPDE had no significant activity toward either RNA or 3'-nucleotides as the result shown in Figure 21 (the experimental procedures were the same as described in Figure 2).

Discussion

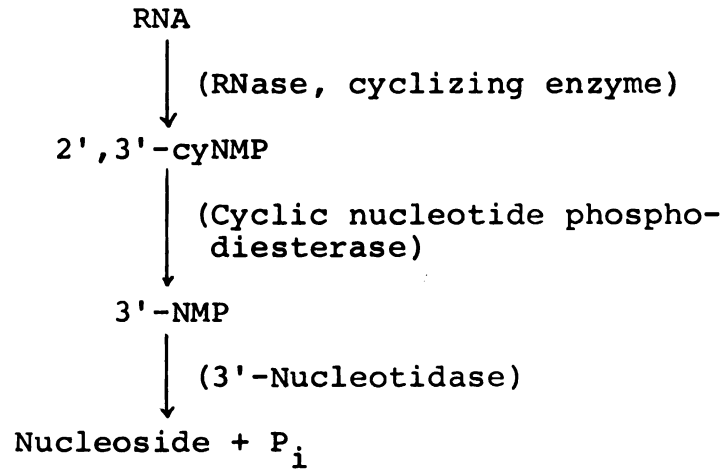
An enzyme from Alaska pea seedlings hydrolyzes both 2',3'-cyNMP and 3',5'-cyNMP. This cyclic nucleotide phosphodiesterase was purified approximately 218-fold with a recovery of about 8% of the total activity toward 2',3'-cyAMP.

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 cyNPDE itself. Evidence for this is as follows:

1. Pea cyNPDE activity can be separated completely from the enzyme activities toward nucleotides, RNA, DNA, and various organic phosphates by means of sucrose density gradient centrifugation, and polyacrylamide gel electrophoresis.
2. Compared to the properties of pea RNase (3'-nucleotidase II) as described in the first section, pea cyNPDE 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, if not all, of the RNases so far characterized from higher plants are cyclizing enzymes that yield 2',3'-cyNMP with little further activity toward 2',3'-cyNMP, the pea cyNPDE described here may play a crucial role in the degradation of RNA. I suggest that RNA degradation in higher plants may not follow the scheme that is generally accepted in which RNase (cyclizing enzyme) hydrolyzes both RNA and 2',3'-cyNMP (8, 9). I propose that the degradation of RNA in higher plants is as follows:



Because pea cyNPDE catalyzes the formation of 3'-AMP exclusively from the hydrolysis of 2',3'-cyAMP, the 3'-NMP formed from the study of RNase in higher plants as described in the first section strongly suggest that the contamination of cyNPDE in the RNase preparation. In barley seedlings also the 2',3'-cyNPDE activity is different from RNase.

With respect to the hydrolysis of 3',5'-cyAMP, the pea cyNPDE was purified approximately 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 of NaF is not clear.

The pea enzyme catalyzes the formation of 3'-AMP mainly from 3',5'-cyAMP rather than 5'-AMP which is the exclusive product in the animal system. The pea enzyme catalyzes the formation not only of 3'-AMP but also of 5'-AMP with a ratio of 3'-AMP:5'-AMP of about 7:1. Both products were formed directly from 3',5'-cyAMP; there was no interconversion of these two nucleotides under the assay conditions.

The data suggest that the formation of two products from one substrate is probably due to a single enzyme. Evidence for this is based on the following:

1. The formation of the two nucleotides was parallel with a constant ratio throughout the whole time course of the hydrolysis of 3',5'-cyAMP.

2. The ratio of the two nucleotides was constant throughout the fractions of the sucrose density gradient.
3. Both 3'-AMP and 5'-AMP showed a similar degree of inhibition of the enzyme activity toward 3',5'-cyAMP.

The activities of enzyme toward 2',3'-cyAMP and 3',5'-cyAMP were maintained in a rather constant ratio throughout the purification procedures and were quite similar with respect to pH optima, metal ions effect, 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.

Since attempts to demonstrate the presence of adenyl cyclase and the incorporation of radioactive adenine and adenosine into 3',5'-cyNMP in either the pea or the barley system have been unsuccessful, the possible biological significance of the presence of an enzyme with activity toward 3',5'-cyNMP in higher plants is not known.

Summary

An enzyme able to hydrolyze both 2',3'-cyNMP and 3',5'-cyNMP has been purified about 200-fold from germinating pea seedlings.

The enzyme shows maximal activity at pH 5.4-6.0 with a Km of 0.62 mM for 2',3'-cyUMP, 0.83 mM for 2',3'-cyAMP, 4.34 mM for 2',3'-cyGMP, 5.0 mM for 2',3'-cyCMP, 0.58 mM for 3',5'-cyUMP, 0.90 mM for 3',5'-cyAMP, 1.61 mM for 3',5'-cyGMP, and 1.81 mM for 3',5'-cyCMP. There is no apparent requirement of metal ions for full activity.

The enzyme catalyzes the formation of 3'-AMP exclusively from 2',3'-cyAMP and the formation of 3'-AMP and 5'-AMP with a ratio of 3'-AMP:5'-AMP of about 7:1 from 3',5'-cyAMP. There is no interconversion of 3'-AMP and 5'-AMP. The formation of the two products from one substrate is probably not due to the presence of two different enzymes.

The activities of the enzyme toward 2',3'-cyAMP and 3',5'-cyAMP were quite similar with respect to pH optima, effect of metal ions, effect of sulfhydryl reagents, heat stability, temperature optima, and sensitivity of treatment with urea. Furthermore, the two activities had identical physical properties. It is, therefore, suggested that the two activities reside on a single protein molecule.

With gel filtration and sucrose density gradient, three enzyme activities toward 2',3'-cyAMP were found. Only

one of these (molecular weight about 350,000) had a preferential activity toward 3',5'-cyAMP. Methylxanthines showed no effect on enzyme activity. Activation energy for hydrolysis of 2',3'-cyAMP was 8.6 Kcal/mole and for 3',5'-cyAMP was 7.2 Kcal/mole. Isoelectric points of the three activities were at pH 4.3, 4.6, and 4.8. Because the cyNPDE does not hydrolyze RNA, a new mode of RNA degradation in higher plants has been proposed.

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