AN ABSTRACT OF THE THESIS OF

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Title _PURIFICATION AND CHARACTERIZATION OF A CYTOPLASMIC APYRASE_

FROM PLUMULES OF PISUM SATIVUM

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The 105,000 x g supernatant fluid (S_{HS}) from extracts of pea seed-lings (Pisum sativum, var. Alaska) etiolated for 60 hours yielded a very potent adenosine triphosphatase activity. This activity was designated as an apyrase when the reaction stoichiometry indicated that adenosine triphosphate (ATP) was hydrolyzed to adenosine monophosphate (AMP) and two moles of inorganic phosphate (P_i).

The apyrase has been purified 88-fold with 40% recovery from acetone powders of the S_{HS}. This 6-day procedure involved filtration of the acetone powder solutions through Sephadex G-200 and G-75 followed by protamine sulfate treatment to remove polyanionic material. After film dialysis the enzyme activity was chromatographed on carboxymethyl cellulose (CMC) with a gradient NaCl elution.

A second more rapid purification scheme was used to purify extracts of acetone powders to 79- to 100-fold with overall yield of 67% to >100%. This 15-hour scheme involved protamine treatment of the acetone powder extracts, followed by ammonium sulfate fractionation in the presence of 0.01 M ethylenediaminetetraacetic acid (EDTA) with pH being continuously maintained at 7.8 during salt dissolution. The precipitate obtained between 2.7 to 4.0 molal ammonium sulfate was film

dialyzed and then chromatographed on CMC with NaCl gradient elution.

With ATP as substrate the apyrase (ATPase activity) showed a temperature optimum at $30^{\circ}C$ and an optimum of the apparent V_m at pH 6.1. With ADP as the substrate the enzyme (ADPase activity) showed an optimum of the apparent V_m at pH 7.1. The double-reciprocal plots of the ATPase activity showed an upward deviation at high substrate concentration. However, the ADPase reaction showed an upward deviation in these plots at low substrate concentration. The ATPase reaction differed from the ADPase by showing diminished activity in maleate and imidazole buffers.

Divalent cations were required for both reactions; the most effective were $Mn^{++} > Ca^{++} > Mg^{++}$. No phosphoesterase or inorganic pyrophosphatase activity were detected. Triphosphates of guanosine, cytidine, inosine, and uridine were hydrolyzed faster than ATP.

In contrast to other apyrases in the literature, the pea seedling enzyme was seen to hydrolyze ADP faster than ATP. Co-purification and non-additivity of the two activities indicated that they were due to one enzyme.

PURIFICATION AND CHARACTERIZATION OF A CYTOPLASMIC APYRASE
FROM PLUMULES OF PISUM SATIVUM

by

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Typed by Jerry Hilliard

To SUMI, for sustaining my spirit and encouraging my labors...and for the advice: "--it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!"

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PURIFICATION AND CHARACTERIZATION OF A CYTOPLASMIC APYRASE
FROM PLUMULES OF PISUM SATIVUM

I. INTRODUCTION

Otto Myerhof (45) first applied the term "apyrase" to those adeno-
sine nucleotide hydrolases which did not distinguish between adenosine
triphosphate (ATP) and adenosine diphosphate (ADP) as substrates, e.g.,
the potato adenylpyrophosphatase (a general term for ATPase and/or
ADPase) reported by Kalckar (30). The terms ATPase and ADPase were re-
served for those enzymes which specifically catalyzed the one phosphate
removal from ATP and ADP respectively. Kalckar's study of the apyrase
from potato extracts had revealed that the enzyme catalyzes the hydro-
lysis of ATP to one mole of adenylic acid (AMP) and two moles of inor-
ganic phosphate (P_i). Data suggested that the hydrolysis of ATP and
ADP was due to one enzyme (30). The Enzyme Commission classification
Number 3.6.1.5. and the systematic name ATP diphosphohydrolase apply
to this activity (15, p. 758). Although the trivial name of apyrase
describes an enzyme which distinctly catalyzes two reactions sequen-
tially, this activity is often obscured in the literature by being re-
ferred to as an ATPase, which term has been imprecisely used and
which encompasses a very ubiquitous activity.

A further definition of an apyrase might include a description of
its metabolic role in vivo. But at present no experimental data are
available on this point. In contrast to apyrases, most ATPases can be
functionally defined. The ATPases induced in mitochondria by dinitro-
phenol can be explained as an artifactual function resulting from an

uncoupling of phosphorylation processes from those of oxidation (56).

The ATPase associated with the contractile proteins of muscle apparent-

ly takes part in transforming chemical energy into mechanical motion

(47). Still another function with which certain ATPases are identified

is that of ion transport across membranes (9). An exception to the

neat functional relations of the preceding enzymes is the association

of ATPases (and other nucleotidases) with microsomes (16). In contrast

to mitochondria, there is no evidence yet that these minute vesicles,

which presumably are evolved from the disruption of the endoplasmic

reticulum, participate in energy generation or in active transport

(17). Hence, there appears no obvious function with which the micro-

somal ATPases may be associated.

Except for the activity found in insect flight muscle sarcosomes

(22), information is lacking for most apyrases with respect to their

association with a cellular organelle or with a metabolic function

(35, 54, 58). For want of some suggestion, it may be proposed that the

apyrases function in regulation of nucleotide levels in the cell or in

certain compartments in the cell (55). This type of function can be

ascribed easily to an exzyme which catalyzes two reactions in sequence

or to a one-step enzyme (ATPase). By contrast, the exclusive one-step

character is to be noted for the ATPase-associated functions which have

been described above.

Assuming that the two-step catalytic nature of the apyrase com-

pletely precludes this enzyme from the types of function attributed to

other ATPases, an operational definition is proposed for this enzyme.

The apyrase is a soluble enzyme which catalyzes the conversion of ATP

to AMP and two P_i and which does not originate from the mitochondrion. It may be associated with the microsomal fraction of the cell and unless shown otherwise, the enzyme does not participate in biological transport.

A search through the literature for an apyrase activity so defined encounters not a little confusion. Depending on the extent of characterization or purification, the activity has been reported as an apyrase, an ATPase, or a phosphatase. Also there is a wide variation in the care exercised by various workers in the way the activities have been extracted from tissues. Hence, in many papers there is no way for the reader to ascertain the intracellular origin of the activity. Where soluble apyrase activities have been reported we can only assume that these enzymes fit the definitions detailed above. It will be useful to review a few papers here to get an idea of the variety of apyrases thus far known.

One of the more curious reports is that of Sugiyama and Dack, which described an apyrase activity that was associated with a purified enterotoxin from a staphylococcus (58). In addition to ATP and ADP, the enzyme could liberate P_i from AMP and nicotinamide adenine dinucleotide (NAD), although the esterase activity (the hydrolysis of phosphate ester bonds) was about 1/10 of the pyrophosphatase activity (the hydrolysis of phosphoric anhydride bonds). An exogenous cation source was not required; however, added Mg^{++}, Co^{++}, and Ca^{++} were seen to activate. Two mM KF was found to be completely inhibitory. A sharp pH optimum at 8.0-8.1 was noted with ATP as substrate. The presence of 0.028 M KCl was seen to enhance the activity. An inhibition of

activity was evident at high substrate concentration with either ATP or ADP. ATP saturated the enzyme at lower concentration than ADP.

Although no extensive purification was undertaken, Mazelis was able to show apyrase activities in the washed particles (23,000 x g pellet) and in the cytoplasmic supernatant (the 23,000 x g supernatant fluid) obtained from 0.5 M sucrose extracts of cabbage leaves (43). The broad substrate specificities shown by both particulate and soluble systems are due no doubt to a mixture of enzymes. Mazelis found it difficult to conceive a role for the presence of apyrase in both soluble and particulate fractions of the cell. But in view of the crudeness of his preparations, any questions bearing on the multiplicity of apyrase activities in the cell must be deferred.

The most prominent of the apyrases is that obtained from potatoes (30). At least four major attempts have been made to purify the activity (34, 39, 46, 62). The more recent work wherein the apyrase was purified by chromatography on ion-exchange celluloses indicates that more than one type of apyrase is obtainable. Both Molnar and Lorand (46) and Liebecq, et al. (39) have isolated two types of apyrases (called A and B) from potato extracts. Apyrase A seems to hydrolyze ATP faster than ADP. The type B seems to hydrolyze ATP and ADP at comparable rates. Liebecq, et al. have shown the hydrolytic specificity to extend to four other nucleoside tri- and diphosphates, all of which are catalyzed at a lower rate than the adenine nucleotides. Because of the broad nucleotide specificity, these workers have been inclined to call the enzymes a nucleoside triphosphate phosphohydrolase or diphosphohydrolase. The most recent work on the potato enzymes has

been reported by Cori's laboratory (11, 62). These workers have shown the identity of the ATP and ADP hydrolysis reactions by demonstrating the two activities to be associated with one molecular weight, the rate of gamma-ray inactivation to be the same for both activities, and the inhibition by ATP of the release of radioactivity from β-P^{32} labelled ADP.

The literature on apyrases from pea seedlings appears to be non-existent. However, some papers dealing with soluble phosphatases from pea seedlings can provide a background to this subject. Forti, et al. (20) have reported a 200-fold purified nucleotide phosphatase from pea leaves which hydrolyzes NADP, CoA, NADPH, PP_i, ATP, ADP, AMP, and other organic phosphates. The broad substrate specificity indicates the presence of esterases as well as pyrophosphatases. However, the ratio of the NADP-phosphatase (the esterase activity) to the ATPase (the pyrophosphatase activity) is constant over a 200-fold purification through four steps. The competitive inhibition between any two of the substrates argues rather strongly for a single enzyme. The esterase activity seems to be limited to phosphate esters of secondary alcoholic groups. The intracellular origin of these activities is not clearly discernable due to the hypotonic extraction medium used to extract the enzymes.

Young and Varner in 1959 (65) reported a study of the change with time of the levels of three enzymes in germinating tissues of Alaska pea seedlings. One of the activities followed was that of a phosphatase which they noted to be very prominent in the microsomal fraction and in the 105,000 x g supernatant fraction. The enzyme was purified

three-fold and characterized. The pH optimum was about 6.5 for the ATP

substrate. Divalent cations activated the enzyme reaction in the order

of $Cd^{++}>Mn^{++}>Mg^{++}>Ca^{++}>Co^{++}>Zn^{++}$. A study of the substrate specificity

revealed that ATP and ADP were by far the best substrates for the

enzyme; however these workers did not report the activity as an apy-

rase. On close examination their data reveal that ADP may be hydro-

lyzed faster than ATP.

Recently Raacke (54, 55) reported the presence of four distinct

nucleoside triphosphatases in the ribosomal (microsomal) and superna-

tant fractions of pea seedling extracts. These enzymes can be extract-

ed from ribosomes with 0.5 M KCl and further purified on diethylamino-

ethylcellulose (DEAE). They are accompanied by nucleoside diphospha-

tase activities but will not hydrolyze the monophosphates. The acti-

vities show different pH optima and patterns of divalent metal-ion

activation for each of the nucleotides. The hydrolysis of diphos-

phates and triphosphates apparently shows similar patterns of activa-

tion. This is taken to suggest that both activities reside on one

enzyme. We may then interpret that four varieties of apyrase are pre-

sent, but more definitive information is required for such assignments

to hold.

The work on apyrases presented in this thesis rose out of several

attempts to isolate amino-acyl RNA synthetases from homogenates of

pea seedling etiolated for 60 hours. The predominance of a powerful

ATPase activity in these tissue extracts over the amino acid activat-

ing reactions required that this enzyme be isolated and characterized.

The stoichiometry of the ATP hydrolysis catalyzed by this enzyme very

early in the work led to the designation of the activity as an apyrase.
Further interest in this enzyme was promoted by the finding that ADP is
hydrolyzed faster than ATP, in contrast to apyrases from other sources
(22, 46).

II. MATERIALS

Pea seeds. The seeds of <u>Pisum</u> <u>sativum,</u> var. Alaska, were ob-
tained from the Jenks-White Seed Co., Salem, Oregon.

Substrates. ATP, ADP, AMP, inosine triphosphate (ITP), cytidine
triphosphate (CTP), guanosine triphosphate (GTP), and uridine triphos-
phate (UTP) were sodium salts obtained from muscle and were products
of the Sigma Chemical Co. (Sigma). Potassium glucose 1-phosphate and
sodium glucose 6-phosphate and fructose 1,6-diphosphate were also ob-
tained from Sigma. Sodium pyrophosphate was from the J. T. Baker Co.
(Baker) and was reagent grade. Adenosine was from Sigma.

Buffers. Malonic acid, glycine, and imidazole were obtained from
Nutritional Biochemicals Corp. (NBCo). Tris(hydroxymethyl-)-amino-
methane (Tris) was a product of Sigma (Trizma Base). Maleic anhydride
was a product of Matheson, Coleman and Bell (MCB).

Standards. Bovine serum albumin was obtained from the Armour Co.
and also from Sigma. High purity (>98%) ovalbumin was a product of
Pentex, Inc. D-ribose was from Sigma.

Miscellaneous Reagents. Ammonium sulfate obtained from Baker was
reagent grade. Ethylenediaminetetraacetic acid (EDTA) also from Baker
was recrystallized from ethanol and used as the sodium salt. Mercap-
toethanol was purchased from Calbiochem. Isobutyric acid from MCB had
to be decolorized by shaking with charcoal and redistilled before use.
Without these measures, the blank paper chromatography strips (controls)

which were developed always showed a high absorbance in the ultra-violet. Acetone and benzene were CP grade and were redistilled before use. Acetone for protein desiccation was stored tightly stoppered in the freezer (-15°C) after distillation. All other acids, bases, and salts used were reagent grade chemicals. Protamine sulfate (from Salmon sperm) was obtained from Sigma.

Chromatography Materials. Diethylaminoethyl cellulose (DEAE), carboxymethyl cellulose (CMC), and phospho-cellulose (P-cel) were obtained from Sigma. Bio-Rex 70, which is finely graded Duolite CS-121, is a product of Bio-Rad Laboratories. Sephadex gel beads (dextran) are a product of the Pharmacia, Inc.

Miscellaneous Materials. Aquacide II is a product of Calbio-chem. Carbowax 20 M (a polyethylene glycol) was obtained through the courtesy of the Union Carbide and Chemical Co. Polyvinylpyr-ollidone (PVP-40) was obtained in U.S.P. grade from Sigma.

III. METHODOLOGY

A. General Procedures

1. Phosphate Assay

Method a. The bulk of the orthophosphate determinations in this work was done by a modified Martin and Doty procedure (42). Samples containing about 0.5 μmole of phosphate in one ml volume of water were placed on ice (to minimize hydrolysis of nucleotides). To these was added one ml of acid-molybdate (Reagent A" - 5% ammonium molybdate in 2.5 N H_2SO_4) (5) and the mixture was thoroughly agitated. Five ml of isobutanol/benzene (50:50, v/v) was added, and the mixture was vigorously agitated for 15 seconds with a vibrating test tube mixer. The tubes were set on a rack at room temperature, and after phase separation 0.5 to 2.0 ml of the organic layer was transferred to a clean tube. The organic aliquot was made up to 2.0 ml with isobutanol/benzene solution and diluted by the addition of 4 ml of acid-ethanol (8 ml concentrated H_2SO_4 + 242 ml 95% ethanol) reagent. The resulting solutions were treated with 0.5 ml of the $SnCl_2$ reagent (10% $SnCl_2.2H_2O$ in concentrated HCl; diluted 1 to 100 with 0.5 N H_2SO_4 just before use) to develop the intense blue color which was read at 710 mμ after 5 minutes. The standard phosphate curve was 0 to 0.5 μmole and the corresponding OD_{710} typically ranged 0 to 0.650.

Method b. Phosphate assays by Method a, while being very sensitive and accurate, were found to be very time consuming and especially burdensome when many analyses were to be conducted in a short period

of time. Hence a more rapid assay was devised. Samples containing less than 0.5 µmole phosphate in 1 ml volume were mixed thoroughly with 1 ml of acid-molybdate (Reagent A* - 1% ammonium molybdate in 2.5 N H_2SO_4). After five minutes, 3 ml of acid ethanol (see above) were added and thoroughly mixed in. Color was developed by adding 0.5 ml of the $SnCl_2$ reagent (see above) and mixing quickly. Optical density was determined at 660 mµ. The standard phosphate curve from 0 to 0.5 µmole yielded a linear color response to 1.31 OD. The blue color developed in about 5 minutes at room temperature and was stable for at least 12 hours. The presence of ATP was seen to affect the color yield, but this could easily be corrected for by an ATP control. Since unhydrolyzed nucleotides were present in the final color-developed so-lution, the samples were read in the shortest period of time possible after color development.

2. Protein Assay

Proteins were estimated by the method of Lowry, et al. (41) using bovine serum albumin as protein standard. When it was available, the high purity (>98%) ovalbumin from Pentex, Inc. was found much superior to bovine serum albumin as the protein standard.

3. Ribonucleic Acid Assay

RNA was estimated by the copper-orcinol method of Ceriotti (8). D-ribose was used as the color standard. Ceriotti's factor of 3.76 was used to obtain the weight of RNA (i.e., ribose wt. x 3.76 = RNA wt.).

4. Chloride Determination

Chloride content in sodium chloride gradients was determined potentiometrically with silver nitrate according to the procedure developed by the Radiometer Co. (72 Emdrupvej, Copenhagen NV, Denmark) for their Type TTT1 Titrator system. Their method refers to work by Müller (48) and Lehmann (38).

5. Nucleotide Chromatography

Purine and pyrimidine nucleotides were chromatographed on 4 x 56 cm strips of Whatman No. 1 paper. Amounts up to 200 nanomoles of a given nucleotide were applied on a narrow line six cm from one end of the paper strip. Chromatographic separation was by descending elution of the solvent—isobutyric acid:ammonia:water (66:1:33) with pH about 3.7. (Solvent I, (51)). The ultraviolet (UV) light absorbing spots were quantitated by cutting them out, extracting the nucleotide with an acetic acid solvent (71.9 ml glacial acetic acid + 5.06 ml concentrated NH_4OH + water to 250 ml) and measuring the OD (at suitable wavelength) of the extract against appropriate blanks.

6. Enzyme Activity

One unit of enzyme activity is defined as that amount which liberates one μmole of phosphate in one minute. Specific activity is expressed as units of enzyme per milligram protein (14, p. 16).

Typically the incubation mixtures contained 2 μmole of substrate, 10 μmole of divalent cation, and 100 μmole of buffer in a one ml volume. Incubation was for 15 minutes at $30^{o}C$. The enzyme reaction was

stopped by adding 1 ml of 1 M trichloroacetic acid/sodium acetate (pH ≃ 2). Suitable aliquots of the resulting solution were then used for phosphate assay. Values reported are the averages of duplicate or triplicate samples. All buffers used in this study were prepared using hydrochloric acid or sodium hydroxide to obtain the desired pH.

B.   Techniques in Enzyme Purification

1.   Pea Seed Germination

The actual hardware for the germination of peas was changed as technique was improved. Usually about 500 g of dry pea seeds were washed in 500 ml of 0.5% sodium hypochlorite solution for 10 minutes. This was followed by two rinses in 500 ml of distilled water and by imbibtion in distilled water at $30^{o}C$ for 6-10 hours. The imbibed seeds were layered between pieces of moist cheese cloth and placed in a chamber which was aerated by moist air. Germination was for 60 hours in the dark. Improved incubation methods involved using vermiculite moistened with 0.004 M $Ca(NO_3)_2$ solution as the supporting medium and using regulated flow (5 ft^3 per hr) of base and acid washed air.

The germinated seeds at 60 hours exhibited plumules about 1 cm in length and radicles about 4-5 cm in length. The out-germinated tissue (plumules and radicles) was harvested by clipping it off from the seed at the base.

2.   Desiccation With Acetone

The general approach to acetone powder preparation was adapted

from the recommendations of Nason (49, p. 62-3). The harvested tissue was placed in a chilled mortar with TEM extracting medium (0.4 M tris-(hydroxymethyl-)aminomethane (Tris), 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.05 M mercaptoethanol, pH 7.8), (about 0.4 ml of TEM per gram tissue). The mixture was ground with a pestle into a fine pulp which was then filtered through cloth to remove the non-extractable debris. The filtrate was centrifuged at 30,000 x g for 20 minutes to remove mitochondria and similarly sized organelles. In earlier preparations this mitochondria free supernatant (S_{MF}) was used directly to make the acetone powder. But subsequent extracts of such acetone powders required centrifugation at 105,000 x g to remove insoluble material. This step could be eliminated by preparing acetone powders from a high speed supernatant (S_{HS}). Either procedure gave acetone powders which when redissolved in buffer gave the same soluble enzyme activities. The S_{HS} was obtained by centrifuging the S_{MF} at 105,000 x g for 75 minutes. The manipulations up to this point were carried out at 0-4°C in a cold room. Treatment of the S_{HS} with organic solvents was done at -15°C in a freezer. About 100 ml of S_{HS} was added dropwise over the course of one minute to 200 ml of ethanol which was being vigorously stirred. After about a minute, 1 liter of acetone (at -15° C) was added to the stirring mixture and stirring was continued for another 10 minutes, after which the cream-white flocculent precipitate was allowed to settle for 30 minutes. The residue was collected by filtration on a Büchner funnel and resuspended in 300 ml of fresh acetone (-15°C). After about another 15 minutes of mixing, the residue was collected by filtration and dried overnight _in vacuo_ over fresh P_2O_5.

The residue while being collected on the Büchner funnel tended to be very hygroscopic, hence it had to be handled quickly. After complete desiccation, the light powder finally obtained did not exhibit marked hygroscopic properties.

3. Gel Filtration

Dextran gels (Sephadex) obtained in bead form were used to prepare columns for the molecular sieving of enzyme preparations. The dry Sephadex beads were usually sieved through standard mesh screens to obtain particles sized in a narrow diameter range. This assures a more uniform packing of the column and optimal flow rate (25). In general the handling of Sephadex materials was according to the recommendations of Flodin (19). The imbibed gel particles were washed in 0.1 M Na_3PO_4, distilled water, 0.1 N HCl, distilled water, 0.01 M EDTA, and distilled water. Either water or equilibrating buffer slurries of the gel particles was used to pack columns. At the suggestion of a representative from Pharmacia, Inc., tall columns of high porosity Sephadex (G-75, G-200) were prepared so that the distance between the miniscus of the buffer in the column and the effluent orifice was kept as close as was practical to about 20 cm. This tended to relieve the hydrostatic compression of the gel beads at the lower end of the column which led to diminished flow rates. Typical flow rates in usable columns were 5 to 8 ml per hr.

In addition to enzyme fractionation, Sephadex gels of low porosity (G-25) were used to desalt enzyme solutions. Typically columns of 0.85 cm diameter with gel bed heights of 20 cm were used.

4. Ammonium Sulfate Fractionation

In general, the procedures for salt fractionation of the enzyme solution were according to the guidelines recommended by Dixon and Webb (14). To avoid the nuisances inherent in working with salt concentration in terms of percent saturation, it was more practical to obtain concentrations in molalities—the amount of solute added to a given starting weight of solvent. Salt fractionations were conducted in a double-walled glass vessel (titrating vessel) which was cooled by pumping ice water through it. This apparatus permitted good regulation of temperature at $2^{O}C$ for all salt fractionations.

Method $\underline{a}$. Half a gram of acetone powder was suspended in 20 ml of 0.05 M Tris buffer at pH 7.8 with the aid of a Potter-Elvehjem homogenizer. The resulting solution was centrifuged, if necessary, at 105,000 x $\underline{g}$ for 30 minutes (see III, B, 2). After removing suitable aliquots for checking the enzyme activity, the volume was adjusted to 25 ml with the 0.05 M Tris pH 7.8 buffer. The solution was placed in the titrating vessel and stirred magnetically. For the first fractionation (0-2.4 molal salt), 7.95 g $(NH_4)_2SO_4$ was added slowly. After dissolution of the salt, the mixture was allowed to stand for 20 minutes with occasional stirring. The precipitate was removed by centrifugation at 16,000 x $\underline{g}$ for 10 minutes. The supernatant fluid was retained and further fractionated by adding to it 5.3 g of $(NH_4)_2SO_4$ (2.4-4.0 molal salt). The precipitate was collected after 20 minutes by centrifugation at 16,000 x $\underline{g}$ for 10 minutes.

Method b. This procedure differs from the foregoing in two ways:
a) the pH of the enzyme solution is continuously monitored through
every salt addition and adjusted to pH 7.8 by the addition of 10 M
NaOH when necessary and b) the enzyme solution includes EDTA at 0.01
molar. For example, an enzyme solution which had been treated with
protamine sulfate (see III, B, 5) was made 0.01 molar in EDTA and vol-
ume adjusted to 20 ml. Using the handling procedures described in
Method a, three ammonium sulfate fractionations were done: 1st (0-2.0
molal), 2nd (2.0-2.7 molal), and 3rd (2.7-4.0 molal). The protein pre-
cipitate from the third fractionation contained the enzyme activity.

5. Protamine Sulfate Treatment

The strong cationic nature as well as its apparent specificity
(18) makes this substance (from salmon sperm) a very potent agent for
precipitating nucleic acids and other anionic biopolymers. Since this
treatment is rather harsh, the precipitation is carried out with the
enzyme solution in an ice bath.

Method a. The protamine sulfate solution was prepared by dis-
solving the dry powder in 0.01 M maleate pH 6.5 to a concentration of
20 mg/ml, and the pH was adjusted to 6.5 with NaOH. The solution was
turbid and had to be used at room temperature since cooling caused the
protamine to separate out as an oil. Typically one ml of this solu-
tion was added to a cold enzyme solution (2 to 6 ml), which had been
obtained by redissolving the precipitate obtained from ammonium sul-
fate fractionation (e.g. III, B, 4, Method a). After about 20 minutes

the rapidly formed precipitate was removed by centrifuging at 16,000 x

g for 10 minutes.

Method b. It was observed that when protamine sulfate is dis-

solved in water, the resulting solution has a pH of 2.2. A solution

was prepared by dissolving the dry powder in distilled water to a con-

centration of 30 mg/ml. The solution was titrated to pH 6.5 with 10

M NaOH. At this pH the solution became very turbid, and the precipitate

was removed by centrifuging at 15,000 x g for 10 minutes at room tem-

perature. The clear supernatant was used to treat enzyme solutions.

When this solution was stored in the cold, the protamine separated out

as an oil, hence it was necessary to warm it up and dispense it at

room temperature. A typical application was as follows: 0.5 g of

acetone powder was dissolved in 15 ml of 0.05 M maleate pH 6.5 buffer

which was 0.1 M in NaCl. This solution was treated with 3.2 ml

(determined to be sufficient) of the protamine solution and mixed thor-

oughly. The precipitate was removed after 30 minutes by centrifuging

at 15,000 x g for 10 minutes. This preparation was called S'_{HS}.

6. Film Dialysis

The techniques developed here are essentially based on the theory

and practice of dialysis by Craig and King (13). An 18 cm section

of moistened 22/32 inch Visking cellophane tubing was knotted at one

end, and the open end was drawn a few millimeters over one end of a

glass collar 4 cm long with 17.3 mm O.D. and 14.4 mm I.D. Two to 3

ml of an enzyme solution was placed in this bag. This was followed by

a glass plunger insert (made from test tube 14 mm O.D.) which caused

the enzyme solution to be spread over a wide area of the Visking bag. This arrangement was suspended in the dialyzing buffer. The dialysis rate could be enhancedby rotating the glass plunger insert by a motor drive, but rotatory motion of the plunger often tended to shear the Visking bag off the glass collar. When it worked, this set-up could dialyze out 97% of a small ion (phosphate) initially present in the bag in 30 minutes. The system was improved by changing the plunger motion to a reciprocating one (e.g. with a Cenco-Motsinger Recipro-cating Stirrer #18860—a vacuum operated automobile windshield wiper motor). Also a series of grooves (rings) were blown into the glass plunger perpendicular to the cylindrical axis. These grooves fa-cillitated mixing by causing eddy currents with each displacement of the plunger. A Tygon-coated wire frame was devised to afix the closed end of the Visking bag and to prevent it from following the plunger movements. Foaming could be prevented by proper adjustment of the reciprocation rate. While the procedure took two hours compared with only 30 minutes for G-25 Sephadex filtration, it was found de-sirable because it did not cause the several-fold dilution of the enzyme solution that inevitably resulted on gel filtration.

7. Ion-exchange Column Chromatography

The general procedures for handling and working with cellulosic ion-exchangers have been derived from the work of Peterson and Sober (52). The concepts and principles of elution and displacement analysis of solid-liquid adsorption chromatography by Hagdahl (24) served as a very helpful background in the development of the present

work on column chromatography.

Diethylaminoethyl cellulose (DEAE). A coarse mesh grade of this
exchanger with capacity of 0.9 meq/dry gram was regenerated by washing
in 1 M NaOH and distilled water. The exchanger was suspended in 0.05
M Tris pH 7.8 buffer to form a slurry which was continuously stirred.
A glass tube, which was plugged at its lower end with a wad of fiber
glass wool and whose effluent orifice was closed, was filled with dis-
crete volumes of the DEAE slurry. After the formation of a cm or so
of the exchange bed the effluent orifice was eased open and slow drain-
age was permitted. After a few centimeters of bed had formed by gravity
the additional volumes of slurry were packed in under 5 p.s.i. of
nitrogen pressure. This procedure yielded a tight, mechanically
stable column. Discrete sample aliquots of the constantly stirring
slurry were taken and dried down to determine the dry weight content
of cellulosic exchanger. The 0.85 cm diameter by 30 cm column contain-
ed 2.31   dry grams of DEAE cellulose.

Carboxymethyl cellulose (CMC). This exchanger, which was a
coarse mesh grade, with capacity of 0.7 meq/dry gram, was regenerated
by washing in 0.5 M NaOH, distilled water, 0.1 M HCl, distilled water,
0.5 M NaOH and distilled water. A glass tube was packed using a pro-
cedure similar to that for packing the DEAE column. A column with
bed dimensions of 0.9 cm x 40 cm was obtained which contained 3.8 dry
grams of CM-cellulose.

For re-use the CMC column was eluted with 0.1 or 0.2 M Na_3PO_4,
rinsed with distilled water, and finally eluted with ca. 2 μmoles EDTA

and the equilibrating buffer. All the cellulosic exchangers yielded columns with high flow rates which did not diminish on re-use. Therefore, the effluent flow rate had to be attenuated to permit proper equilibration of the components being chromatographed.

Phospho-cellulose (P-cel). This material was hydrated by adding 4 g of the dry powder to 250 ml of 0.1 M NaCl in 0.05 M glycine buffer at pH 10 and stirring the slurry for two hours. After a 15 minute settling time, the fines were decanted off. The exchanger was suspended in water and decanted to remove fines four more times. The P-cel was then washed in 0.1 M Na_3PO_4 followed by washing with distilled water until the pH of the rinse water $\simeq$ 7.0. Methods used previously for DEAE were followed to pack the P-cel column whose final bed dimensions were 0.85 cm x 20 cm. The column was then eluted with ca. 2 μmoles EDTA and 0.01 M maleate pH 6.5 buffer.

Elution Techniques. Linear gradient elutions of cellulosic ion-exchange columns were performed according to the recommendations of Peterson and Sober (52, p.16-18). The beakers (usually 150 ml capacity) of identical dimensions were mounted at the same level and interconnected with a tubing which was pinched off in the middle. One vessel, designated the reservoir, was filled with the 0.01 M eluent buffer (100 ml) containing 1 M NaCl. The second vessel, designated the mixer, was filled with 0.01 M eluent buffer (same pH as that in the reservoir). The latter was stirred magnetically and was also connected with tubing to the ion-exchange column. The whole gradient apparatus was situated some 100 cm above the head of the chromatography

column. After sample application, the column was usually eluted for several milliliters with only the 0.01 M buffer, then the mixer vessel volume was adjusted (to 100 ml) and the gradient started by releasing the inter-vessel pinchcock.

Bio-Rex 70 (BR-70). This is a poly-acrylic resin with carboxylic exchange groups (capacity: 100 meq/dry gram; 4.8 meq/ml resin bed; actual wet-mesh: #100-200 (U.S. standard screens)). The moist powder was twice suspended in water, settled, and decanted to remove fines. A glass column plugged with fiber glass was filled with a slurry of the resin which was allowed to pack under gravity. The column was equilibrated in the cold room and washed there overnight with distilled water. Dimensions of resin bed were 0.85 x 20 cm—the height fluctuated with the ionic strength of the eluent. Since BR-70 is essentially the same substance as Amberlite IRC-50, the recommendations of Hirs (28, p. 113-125) were followed. Procedures described by Hirs suggested that BR-70 would be a strong binding agent for RNAase A, α-chymotrypsin and egg white lysozyme. Hence, in contrast to the 0.01 M buffers used when sorbing proteins to CMC, the buffer for BR-70 elution was made 0.2 molar. The BR-70 column was washed with EDTA followed by distilled water, then equilibrated by overnight washing with 0.2 M pH 6.7 maleate buffer. After application of the sample, the column was eluted with the equilibration buffer.

8. Miscellaneous

Effluents from chromatography columns were monitored by

ultra-violet (UV) light by passing through a Vanguard Model 1056-A

Ultra-Violet Analyzer (flow monitor). The reference cell was always

filled with the buffer used in elution. Where linear NaCl gradients

were involved, the chloride-less buffer was placed in the reference

cell, and the optical density (OD) contribution by the salt was con-

sidered negligible. The use of maleic acid buffers presented a special

problem due to their relatively high UV absorbance. But with the

monitor light source set at 285 mμ, proteins were detectable due to

the broad and large extinction coefficient of tryptophan (63). When

the flow monitor failed to function, aliquots from each of the fract-

ions collected were read manually in the Beckman DB at a suitable UV

wavelength.

The column effluent upon passing through the flow monitor was

directed to the LKB 3400 B RadiRac fraction collector. Siphons of

suitable volume dimensions were used to determine the fraction size

collected.

The concentration of protein solutions was accomplished by

osmotic removal of the solvent (57, p. 78-79). The enzyme solution

was held in a cellophane dialysis bag (Visking tubing) which was then

coated with a hydrophilic agent (usually dry). Some of the water and

buffer ion withdrawing materials used were Aquacide II (a carboxy-

methyl cellulose derivative), polyvinylpyrollidone, 40,000 mol wt

(PVP-40) (32) and Carbowax 20 M (a polyethylene glycol, est. mol wt =

15,000-20,000). The Carbowax 20 M was the most rapid in effecting

water withdrawal, but due to its relatively low molecular weight,

counterdialysis (i.e. dialysis of materials against the movement of

water across the membrane) may be a problem sufficient to dissuade its indiscriminate use as a dessicant (29). Aquacide II and PVP-40 took several hours to a day to effect about a six-fold concentration of the protein.

IV. RESULTS

A. Enzyme Purification

1. Desiccation with Acetone

The use of ethanol in our process for desiccation of enzyme solutions represents a departure from the usual procedures. This particular step was devised to avoid the formation of the gummy, intractable residue which inevitably formed when the extract supernatants were added directly to acetone. Typically 500 g of dry seeds yielded about 150 g of shoots, which in turn resulted in about 120 ml of S_{HS} from which was obtained about 5 g of acetone powder. No explicit study of the stability of enzyme activity in acetone powders was made. However, one estimate obtained from data of different experiments indicated that over a three month interval about 7% of the activity present at the start of the period was lost per month. This is suggestive of a relatively labile enzyme, but this estimation of the activity had not rigorously taken into account the parameters bearing on optimal storage conditions for acetone powders.

In contrast to the activity decline noted during storage of the acetone powder, an increase in activity was noted upon the acetone desiccation of S_{HS}. Although this was not explicitly studied, data were available where the S_{HS} obtained by dissolving acetone powders exhibited 125% of the activity present in S_{HS} preparations which had not undergone acetone treatment.

In general, acetone powders offered a great convenience in

accumulating a stockpile of crude enzymes in concentrated form.
Powders were readily soluble in buffers and provided a convenient
starting point in the purification program.

2. Salt Fractionation

Early experiments indicated that the highest ATPase activity
could be precipitated at the higher ammonium sulfate concentrations,
especially above 2.87 molal. Hence, the first thorough attempt at
ammonium sulfate fractionation was to examine the precipitate ob-
tained between 2.87 and 3.64 molal salt. Table I gives the results
of a number of fractionation steps done over a period of time em-
ploying Method a (see III, B, 4). The salt concentration limits
(cuts) were adjusted in hopes of improving recovery while main-
taining a high purification factor. The ammonium sulfate purified
enzyme was called AS. The variability of recovery and degree of
purification in the several experiments are hard to explain. The
trend of results predicated the need of more improved techniques.
A change to phosphate salts was considered but not tried, since
some early experiments had met with handling difficulties. The
precipitates obtained after centrifugation of phosphate treated
solutions were very light and tended to disrupt very easily and
become dispersed.

Using the enzyme solution S'_{HS} described in III, B, 5, Method
b, a careful restudy of ammonium sulfate fraction was made along
the lines of the Method b described in III, B, 4. The results
are depicted on Table II. The starting solution, S'_{HS}, contained

TABLE I. ENZYME PURIFICATION BY METHOD a AMMONIUM SULFATE FRACTIONATION

Expt. No.	Enzyme source	$(NH_4)_2SO_4$ fractionation pattern	Units start	Units result	Yield	S.A.[4] start	S.A.[4] result	Purification
AY-1	14-Jn-63[1] powder	(0-2.87) (2.87-3.64)	89^2	21.6^2	24%	0.76	4.56	6.0 x
AY-2	14-Jn-63 powder	(0-2.4) (2.4-4.0)	70^2	44.5^2	64%	0.56	3.04	5.4 x
AY-10	16-Jn-64 supernatant	(0-1.0) (1.0-2.0) (2.0-3.5)	117^3	57.2^3	49%	0.63	0.89	1.4 x
AY-11	16-Jn-64 powder	(0-1.2) (1.2-2.4) (2.4-4.0)	290^3	79.7^3	28%	0.56	1.16	2.1 x

1 Enzyme preparations are coded by date; Jn = June.
2 Assay notes: 0.1 M Tris pH 7.8, 0.01 M ATP, 0.01 M $MgCl_2$, 37°C.
3 Assay notes: 0.1 M Tris pH 7.8, 0.002 M ATP, 0.002 M $MgCl_2$, 37°C.
4 S. A. = Specific Activity

TABLE II. AMMONIUM SULFATE FRACTIONATION OF APYRASE

Fraction	E_1	E_2	E_3	E_4	E_5	E_6	E_7	E_8	E_9	S_{HS}
Salt range (molal)	0–1.5	1.5–2.0	2.0–2.5	2.5–3.0	3.0–3.5	3.5–4.0	4.0–4.5	4.5–5.0	5.0^+	---
Activity/ml	0.53	0.4	0.93	6.2	29.3	20.6	1.33	0.4	2.0	9.6
Protein mg/ml	2.9	4.1	6.4	5.8	5.2	3.5	2.6	2.5	11.5	7.46
S.A. (U/mg)	0.183	0.098	0.145	1.07	5.62	5.88	0.51	0.16	0.174	1.29
Purification				0.83x	4.37x	4.58x	0.4x			---

167 units of activity. The sum of the three most active fractions (E_4, E_5, E_6) totalled 112 units of activity. The recovery turned out to be 67.3% while the best average purification was about 4.5 fold.

For simplicity the fractionation procedure was reduced to three steps in Method b (III, B, 4). Results from the use of this procedure are shown in Table III. The high recoveries as well as the

TABLE III. AMMONIUM SULFATE FRACTIONATION OF APYRASE USING
            IMPROVED METHOD

Expt.	Units starting	Units recovered	Yield	S.A. starting	S.A. resulting	Purifi- cation
AY-16	108	99	92%	0.7	4.42	6.3 x
Ay-16.1	106	108	102%	0.61	3.4	5.6 x

Units = μmole phosphate released per min
S.A. = specific activity, units per mg protein

degree of purification attained speak strongly for the importance of constantly maintaining pH near neutrality and of using chelators (EDTA) to bind traces of heavy metals during ammonium sulfate fractionation. The purified enzymes were designated AS' since protamine sulfate had been used in part of the purification.

3. Ion-exchange Chromatography

Enzyme preparations which had been purified only by ammonium sulfate fractionation (AS) followed by desalting were found not to adsorb to either DEAE or CMC columns. Therefore an attempt was

made to determine the isoelectric point (pI) of the enzyme protein

by filtration through a mixed bed of DEAE and CMC. A bed of 1 cm

(diameter) by 10 cm (height) was prepared. Aqvist and Anfinsen (4)

report a procedure using this principle for determining isoelec-

tric points. Water slurries of hydroxyl and hydrogen forms of the

respective exchangers are mixed in 1:1 dry weight equivalent ratio

(clumping forms immediately) then packed into a glass chromatography

tube under N_2 pressure (5 p.s.i.). A dialyzed preparation of the

ATPase was applied to the column and eluted with distilled water.

No protein was washed out after a day of eluting. Next, the col-

umn was washed with several volumes of 0.05 M Tris at pH 7.8 but

no activity emerged. Finally, 0.05 M Tris buffer which contained

0.4 M NaCl eluted the activity from the column. Unfortunately,

the necessity of using strong solvents precluded the isoelectric

point determination.

The cationic nature of the enzyme protein was eventually sug-

gested by the study involving protamine sulfate treatment of the

enzyme preparations (see IV, A, 4). This finding predicated the use

of CMC as the column sorbant. When the protamine sulfate treated

enzyme preparation (called PS) was chromatographed on CMC pre-

equilibrated at pH 7.8 (Tris), the elution pattern recorded (in

the UV) on the Vanguard flow monitor closely resembled that of

the experiment wherein AS preparations (or non-protamine treated

enzyme) were directly eluted through this column. It was conjec-

tured that if the pI of the enzyme were in the vicinity of pH 8,

then the CMC column equilibrated at pH 7.8 may be only poorly

effective in binding the protein. When the CMC column was equili-
brated with 0.01 M maleate buffer at pH 6.5, the enzyme activity
readily sorbed onto the exchanger. Elution of the column with a
linear NaCl gradient in the maleate buffer revealed a new UV-ab-
sorbing peak on the Vanguard flow monitor trace (see Fig. 1).
However a fraction by fraction check for the enzyme activity re-
vealed no coincidence of this new protein peak with enzyme activi-
ty. The enzyme activity was found to be located in two regions.
The bulk of the activity emerged at the void volume (V_o) of the
column (peak A) but another region of activity was seen at V_e (elu-
tion volume) $\simeq 4\ V_o$ (peak B). Although only about 23% of the to-
tal activity was found in peak B, the specific activity under this
peak was eight times greater than that in peak A. These results
having confirmed our notions regarding the cationic nature of the
enzyme protein, we sought to improve the yield of the enzyme in
peak B. The increased conversion of the "A" enzyme to "B" enzyme
involved changes in the pretreatment of the enzyme before the CMC
chromatography, hence it will be discussed in a later section.
The recovery of enzyme after CMC chromatography was 52% of the
activity present in AS.

In addition to changes in the pretreatment of the enzyme for
the enhancement of purity, chromatography on the other cation ex-
changers was tried. The results of apyrase chromatography on phos-
pho-cellulose are shown on Fig. 2. In general, the OD_{280} pattern
and the enzyme activity pattern resemble that of CMC elution pat-
terns. The stronger basicity of phospho-anion of the exchanger
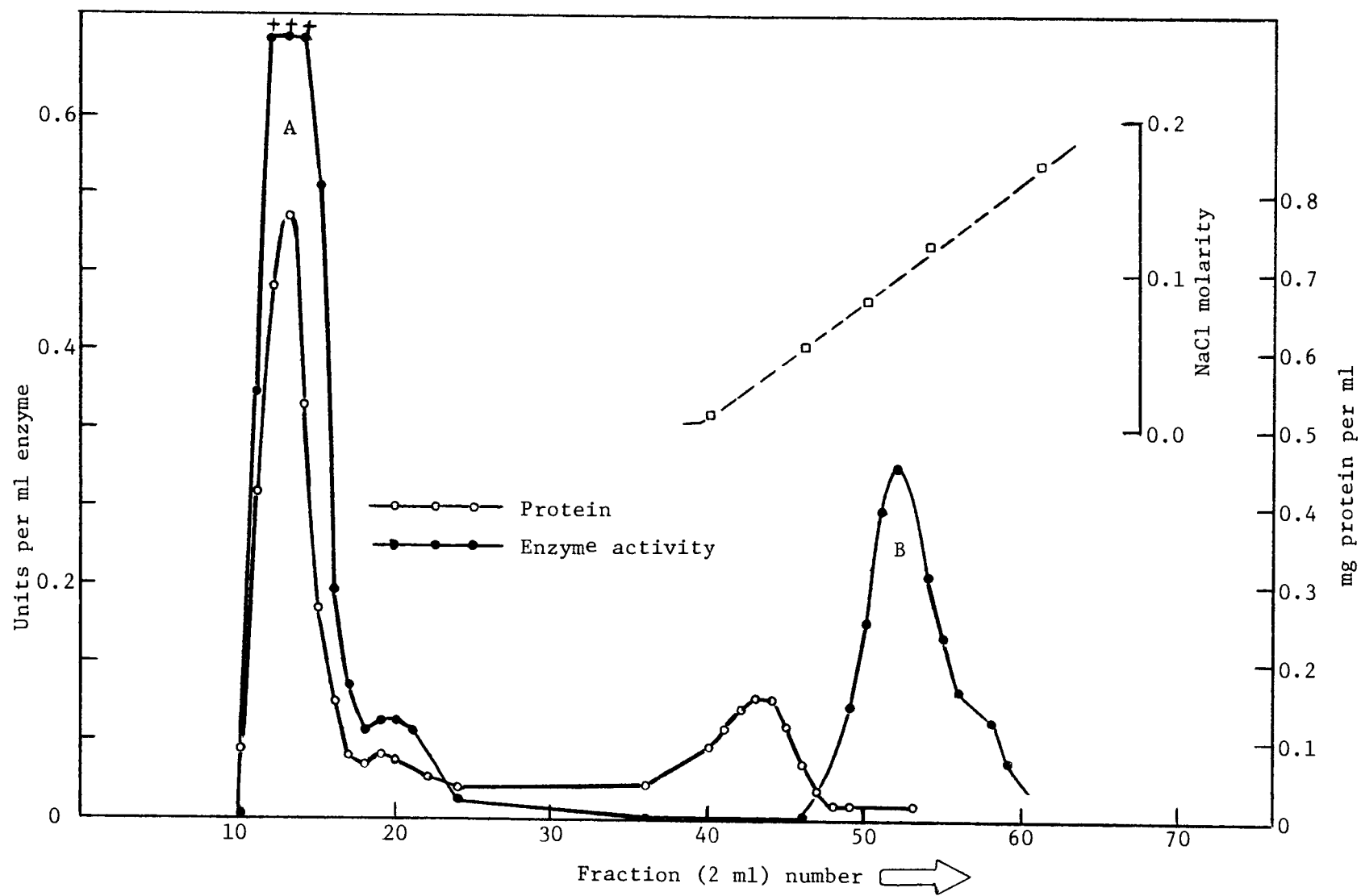
Figure 1. CMC chromatography of protamine treated apyrase.
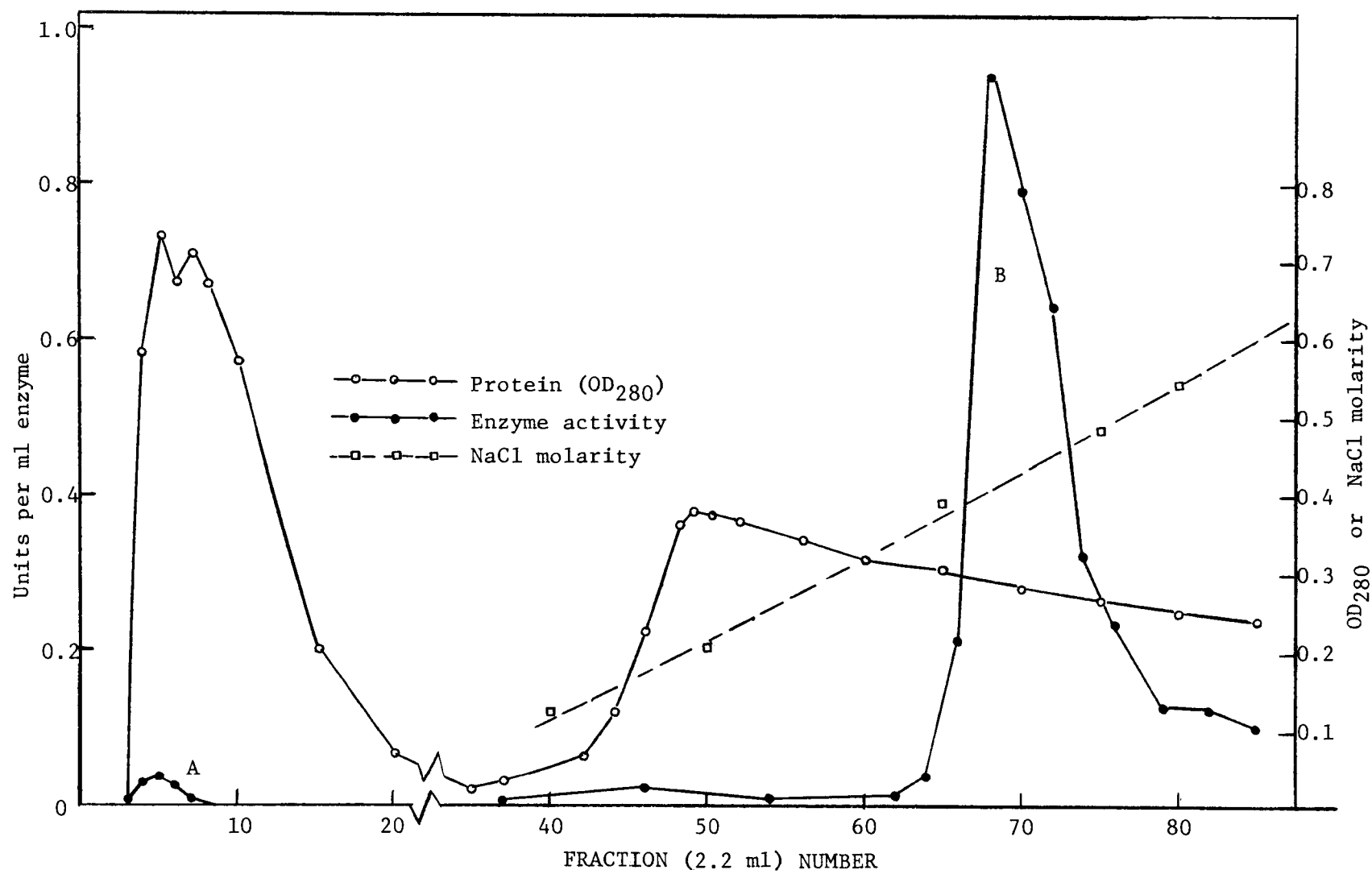
Figure 2. Chromatography of apyrase on phospho-cellulose.

is reflected in the greater retention of the enzyme protein and
in the higher NaCl concentration required for its elution. With
the lack of a markedly different elution pattern from CMC, chroma-
tography on P-cel is somewhat disadvantageous, since it consumes
more time.

Chromatography of the apyrase on BR-70 is shown on Fig. 3. The
activity pattern reveals a slight separation of the enzyme activity
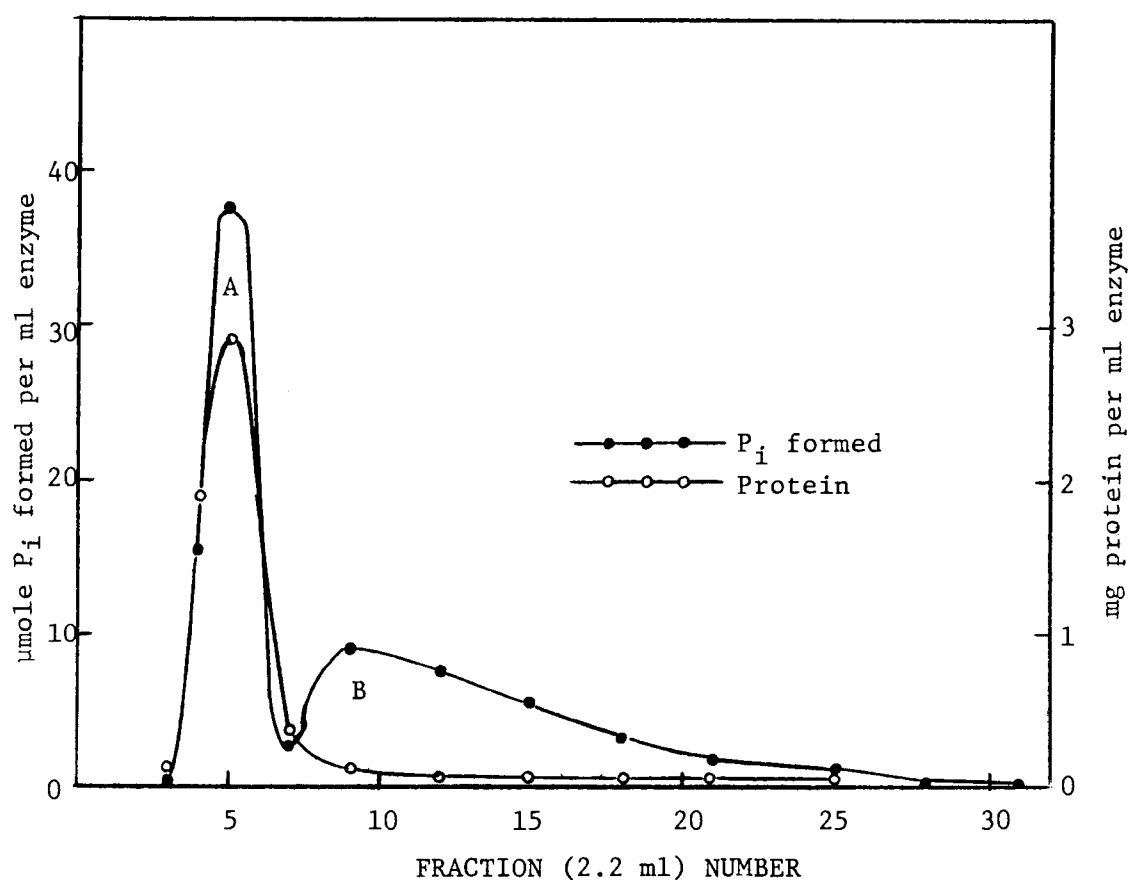
Enzyme assay notes: 0.1 M Tris pH 7.8, 0.002 M ATP, 0.002 M
MgCl$_2$. Incubation for 15 min at 37°C.

Figure 3. Chromatography of apyrase on Bio-Rex 70.

into two peaks. Even though the specific activity in peak B is 6.7 times that in peak A, the closeness of the two peaks and the extremely long "tailing" of peak B made this process unfavorable. It may be possible, however, to find better eluent compositions for improved apyrase purification. The "tailing" problem is noted to be always greater in straight elution situations than where gradients of increasing solvent power are used (24).

4. Protamine Sulfate Treatment

The dilemma presented by the apparently neutral behavior of the AS preparations (see III, B, 4, a), which passed directly through DEAE and CMC columns, was resolved by the addition of protamine sulfate to these enzyme solutions. On mixing the two solutions, copious amounts of flocculent precipitate were formed. Removal of these sediments by centrifugation yielded a supernatant fluid which possessed all of the activity present in samples which had not been treated with protamine. Enzyme preparations before and after protamine treatment were assayed for RNA content. A fair amount of material which reacts like the nucleic acid was observed in the preparations (Table IV). A correlation appears to exist between the amount of RNA lost upon protamine treatment and the amount of purified apyrase protein obtained on CMC chromatography. Methods to improve the purification of the apyrase on CMC involved finding conditions for more effective precipitation of the anionic polymers. First, the amount of protamine sulfate added to a given amount of AS was quadrupled. Secondly, the AS was film dialyzed against 0.01 M maleate pH 6.5 buffer preceeding the

TABLE IV.   RNA ANALYSIS OF PROTAMINE TREATED ENZYME PREPARATIONS

Expt.	Purification step	Total RNA	Δ RNA	Total protein in CMC purified enzyme
AY-9	AS (dialyzed)	2.96 mg		
	PS	1.96 mg	1.0 mg	1. mg
AY-11	AS (dialyzed)	13.4 mg		
	PS	10.0 mg	3.4 mg	4.3 mg

protamine treatment. The results are reflected in Fig. 4 where

the conversion of the A enzyme peak to the B enzyme peak has been

improved such that B possesses 95% of the activity recoverable from

the CMC chromatography.

Protamine sulfate was also used to precipitate anionic poly-

mers from S_{HS} type solutions (Method b; III, B, 5), which were

then designated S'_{HS}. The method for determining the amount of

protamine to be used in S'_{HS} preparations should be elaborated

here. The Warburg and Christian method (37) for the estimation of

nucleic acid and protein by the OD_{280}/OD_{260} ratio was of no use

with the acetone powder solutions since the ratio never rose above

0.574 with increased protamine treatment of the solutions. Hence

a sort of titration curve was set up to estimate the amount of

protamine necessary for the precipitation reaction. Ten tubes

were set up containing from zero to 0.1 ml of protamine solution

(Method b; III, B, 5) and the volumes were all adjusted to 0.1 ml

with distilled water. Then about 12.5 mg of protein (in 0.5 ml)

from an acetone powder solution was added to each tube. After a
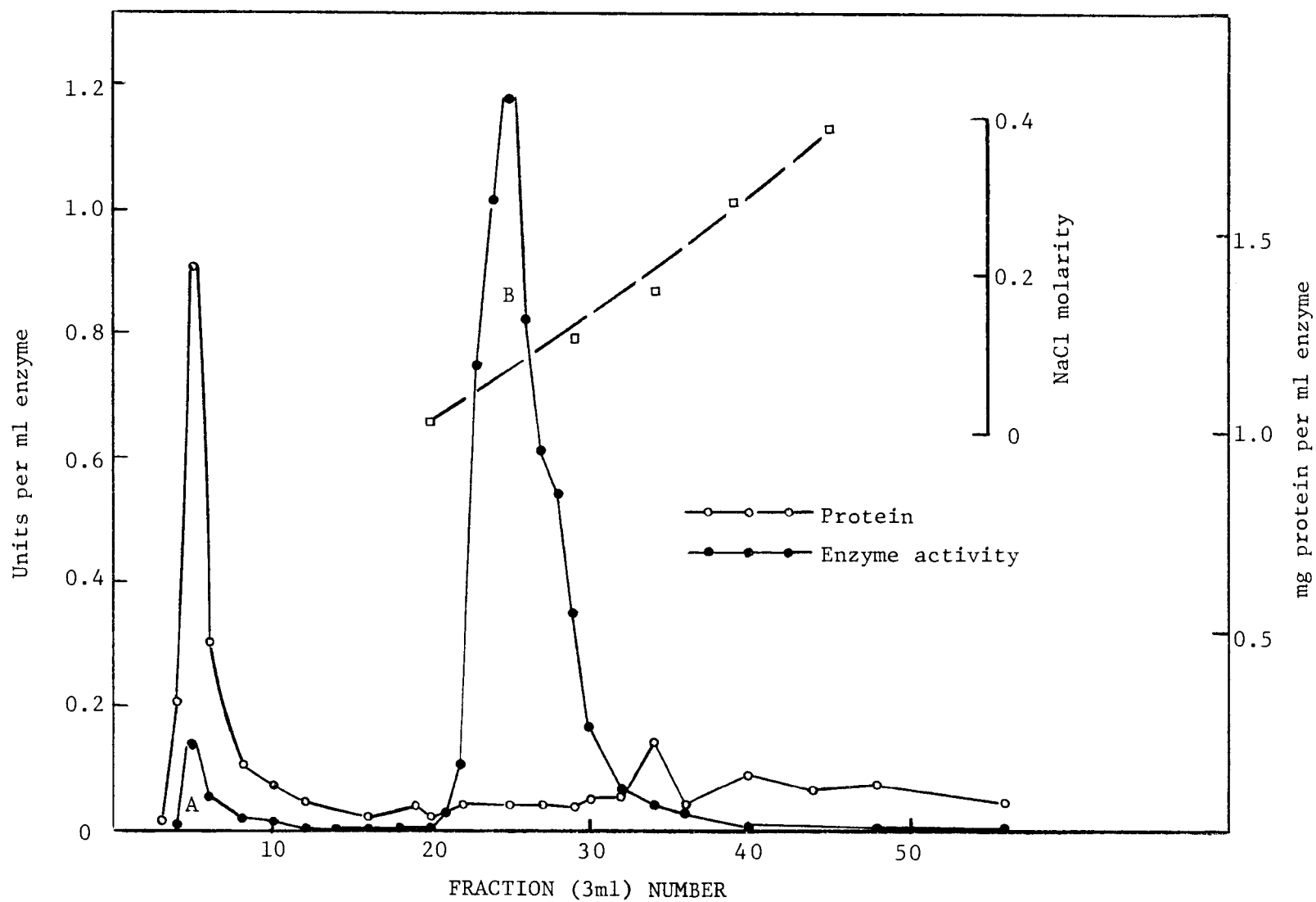
Figure 4.   CMC chromatography of apyrase with increased yield in zone B.

15 min wait and removal of precipitates by centrifugation, 100 µl

aliquots of the water-clear supernatant were treated with 10 µl of

the protamine solution. The tube containing the smallest total

amount of protamine which did not give a turbid reaction on the

second protamine treatment was considered the equivalence point.

5. Gel Filtration

Early attempts to purify the ATPase by filtration through G-200

Sephadex revealed an interesting conversion of the enzyme activity

from a fast moving (large molecular weight) to a slower moving

(smaller molecular weight) peak when 0.25 M NaCl was present in

the eluting buffer (see Fig. 5a, b). This suggested a purification

scheme using only gel and CMC columns avoiding the ammonium sulfate

fractionation which was giving poor recoveries (see Table I).

Table V, gives the result of one of the studies. While the overall

recovery is favorable, as is the high degree of purification,

the disadvantage was that the process took six days.

6. Purification Schemes

The summaries of three purification schemes are presented in

Tables VI, VII, and VIII. The three schemes represent a progres-

sion in the improvement of overall yield and degree of purifica-

tion. The summary on Table VI depicts rather low recoveries which

may be due to the fact that the chromatography at pH 6.5 was pre-

ceded by an extra chromatography step at pH 7.8, which was of

course, not germane to the procedure. These procedures can be

FRACTION (3ml) NUMBER
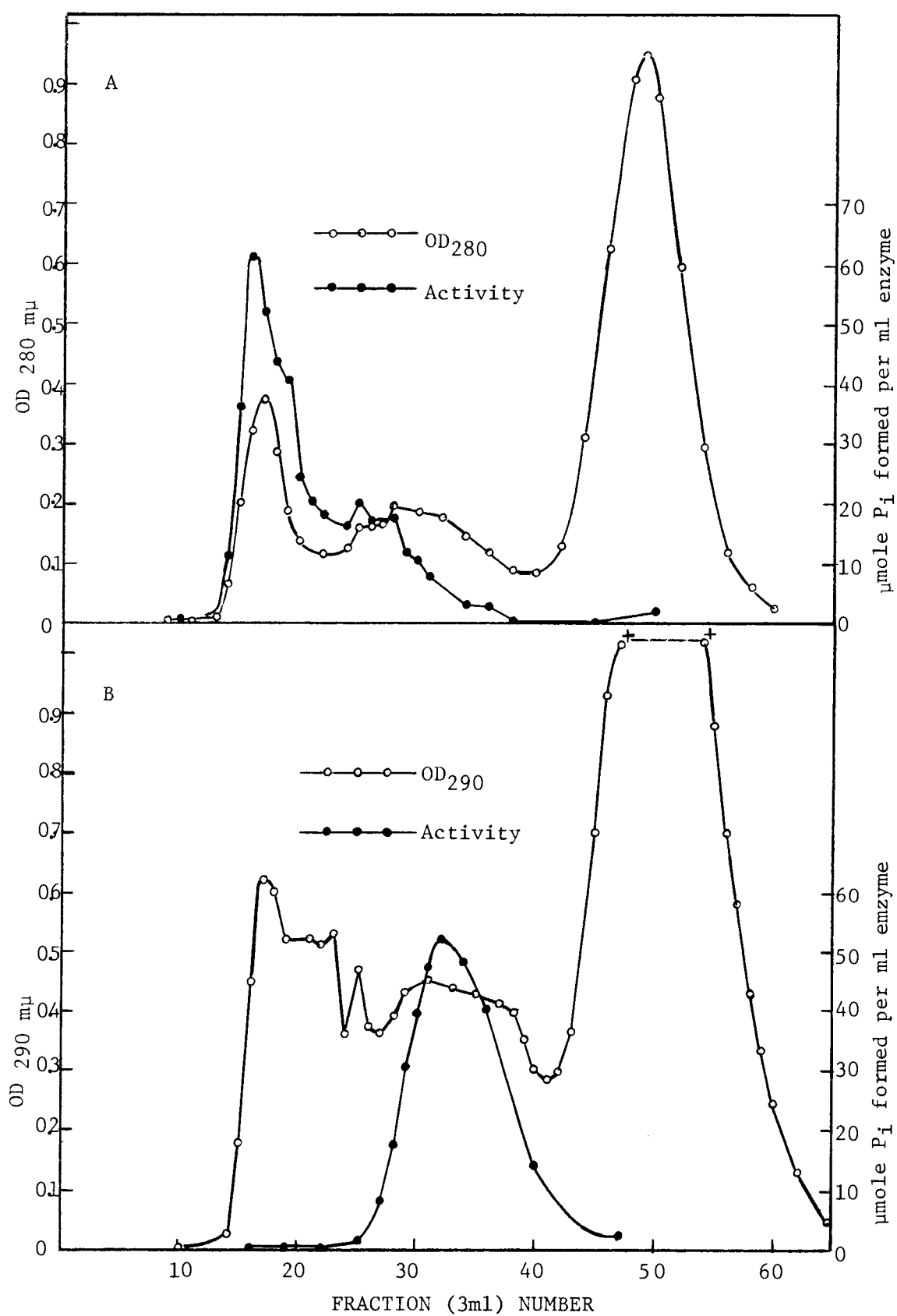
Figure 5. Sephadex G-200 filtration of apyrase.

    a) Elution buffer was 0.05 M Tris pH 7.8 and

0.01 M mercaptoethanol. Flow rate about 14 ml/hr

(avg.). Hydrostatic head = 0.8 meter. Bed dimen-

sions = 1.6 cm (diam.) by 61 cm (height).

    b) Elution buffer was 0.05 M Tris pH 7.8 ,

0.01 M mercaptoethanol, and 0.25 M NaCl. Flow

rate about 10 ml/hr (avg.). Hydrostatic head =

1 meter. Bed dimensions = 1.6 cm by 62 cm.

    Assay notes: 0.1 M Tris pH 7.8, 0.01 M ATP,

0.01 M $MgCl_2$ and 0.3 ml aliquot of enzyme from each

fraction tube. Total volume of one ml incubated

15 min at 37°C.

TABLE V. SUMMARY OF APYRASE PURIFICATION WITH GEL FILTRATION AND CMC CHROMATOGRAPHY

Procedure	Volume (ml)	Activity per ml	Total activity	Protein per ml	Specific activity	Yield (%)	Purification
Acetone powder solution	11.6	7.46	88.6 U	13.7 mg	0.56 $\frac{U}{mg}$	100	----
G-200 filtration	74.	1.29	96.0	10.2	1.27	108	2.3 x
Concentration; G-75 filtration	17.5	3.72	65.2	1.72	2.16	74	3.9 x
Concentration; Protamine	5.75	8.66	49.8	4.09	2.12	56	3.8 x
CMC chromatography	22.6	1.58	35.6	0.032	49.2	40	88. x

Assay conditions: 0.1 M malonate pH 5.5, 0.002 M ATP, 0.01 M CaCl$_2$ in 1 ml incubation volume.

A buffer which was 0.05 M Tris pH 7.85 and 0.25 M NaCl was used to suspend the acetone powder and to elute the G-200 and G-75 Sephadex columns. Enzyme solutions were concentrated by using Aquacide II. Protamine prepared according to Method b was used for precipitation. CMC chromatography as in Fig. 4.

TABLE VI. SUMMARY OF APYRASE PURIFICATION INCLUDING PROTAMINE TREATMENT

Procedure	Volume (ml)	Activity per ml	Total activity	Protein per ml	Specific activity	Yield	Purification
Acetone powder solution, centrifuged 30 min at 105,000 x _g_	18.8	64.9	1219	6.75 mg	9.6	100%	---
Method _a_ ammonium sulfate fraction, 2.4-4.0 molal	10.	69.8	698	1.37 "	51.	57%	5.3 x
a) 5.5 mg protamine added; b) $(NH_4)_2SO_4$ to 4.0 molal to ppt. activity; desalt on G-25 Sephadex c) CMC chromatography at pH 7.8 Tris d) CMC chromatography at pH 6.5 maleate; peak A	30	6.1^1	198	0.224^1 "	27.	16%	2.8 x
peak B	44	1.34^1	59	0.013^1 "	103.	5%	10.7 x

1 These are average values from pooled fractions.
Assay notes: 0.1 M Tris pH 7.8, 0.01 M ATP, 0.01 M $MgCl_2$ and suitable enzyme aliquots.

TABLE VII. SUMMARY OF APYRASE PURIFICATION INCLUDING IMPROVED PROTAMINE TREATMENT

Procedure	Volume (ml)	Activity per ml	Total activity	Protein per ml	Specific activity	Yield	Purifi- cation
High speed supernatant (S_{HS}) of acetone powder solution	19	2.54	48.2	6.7 mg	0.374 $\frac{U}{mg}$	100%	---
2.4-4.0 molal $(NH_4)_2SO_4$ ppt. Method a;	1.5	14.3	21.5	12.7 "	1.09 "	45%	2.9 x
Film dialysis, protamine sulfate treatment	2.4	7.94	19.1	8.34 "	0.955[1]"	40%	---
CMC chromatography, peak B pooled;	16.6	0.874[2]	14.5	0.06[2]"	14.5 "	30%	39. x

1 Drop in specific activity may be due Lowry color formation by protamine sulfate.
2 These are average values since a pool has been used.
Assay notes: 0.1 M Tris pH 7.8 0.002 M ATP, 0.002 M $MgCl_2$ and appropriate enzyme aliquots.

TABLE VIII. SUMMARY OF APYRASE PURIFICATION WITH IMPROVED AMMONIUM SULFATE FRACTIONATION
AND CMC CHROMATOGRAPHY

Procedure	Volume (ml)	Activity per ml	Total activity	Protein per ml	Specific activity	Yield	Purification
Solubilized acetone powder; plus protamine; plus EDTA (S'$_{HS}$)	17.2	6.28 U	108 U	8.95 mg	0.7 $\frac{U}{mg}$	100%	----
Ammonium sulfate fraction, 2.7-4.0 molal (Method b)	4.0	24.9	99	5.63	4.42	91.6	6.3 x
Film dialysis; CMC chromatography: peak A	6.	1.26	7.56	-----	-----	7.01	---
peak B	24.	3.02	72.4	0.055	55.	67.1	78.6 x

    Assay conditions: 0.1 M malonate pH 5.5, 0.002 M ATP, 0.01 M CaCl$_2$, and appropriate aliquots of enzyme such that only about 20% of the maximum available phosphate is released.

worked in about a day (15 hours). The purification summary on Table VIII represents the methods of choice in the preparation of a purified enzyme.

B. Characterization

1. Time Course of the Reaction

The apyrase activity is seen to release phosphate from ATP as a linear function of time through 15 minutes of incubation time (Fig. 6). Similarly a linear response of phosphate release with time was noted for the apyrase reaction with ADPase substrate (Fig. 7).

2. Effect of Enzyme Concentration

Under defined concentrations of substrates in the incubation volume the release of phosphate from ATP was seen to be a linear function of the enzyme concentration (Fig. 8). This linearity persisted even where more than 50% of the potentially available phosphate was released. With ADP as substrate the apyrase release phosphate as a linear function of enzyme through 75% hydrolysis of available phosphate (Fig. 9).

3. Enzyme Stability

In general the purified apyrase activity seems to be rather stable. The ammonium sulfate fractionated preparation (AS) lost less than 5% activity when it was frozen and thawed three times

Assay notes:
0.1 M acetate pH 5.2
0.002 M ATP
0.002 M $MgCl_2$
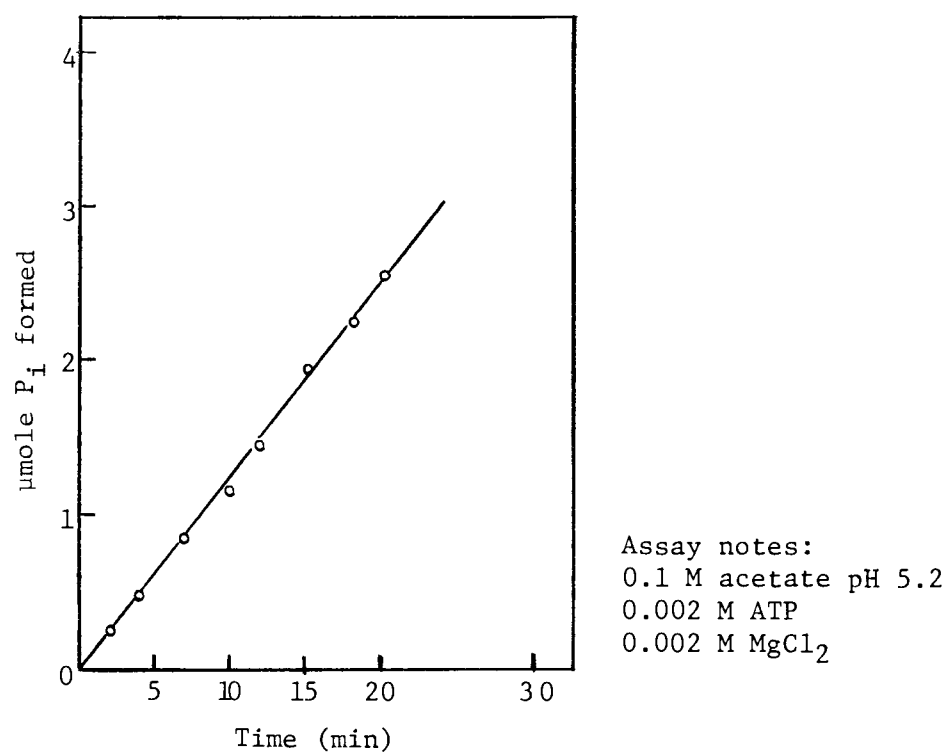
Figure 6. Time course of ATP hydrolysis.

Assay notes:
0.1 M Tris-maleate pH 7.4
0.004 M ADP
0.01 M $CaCl_2$
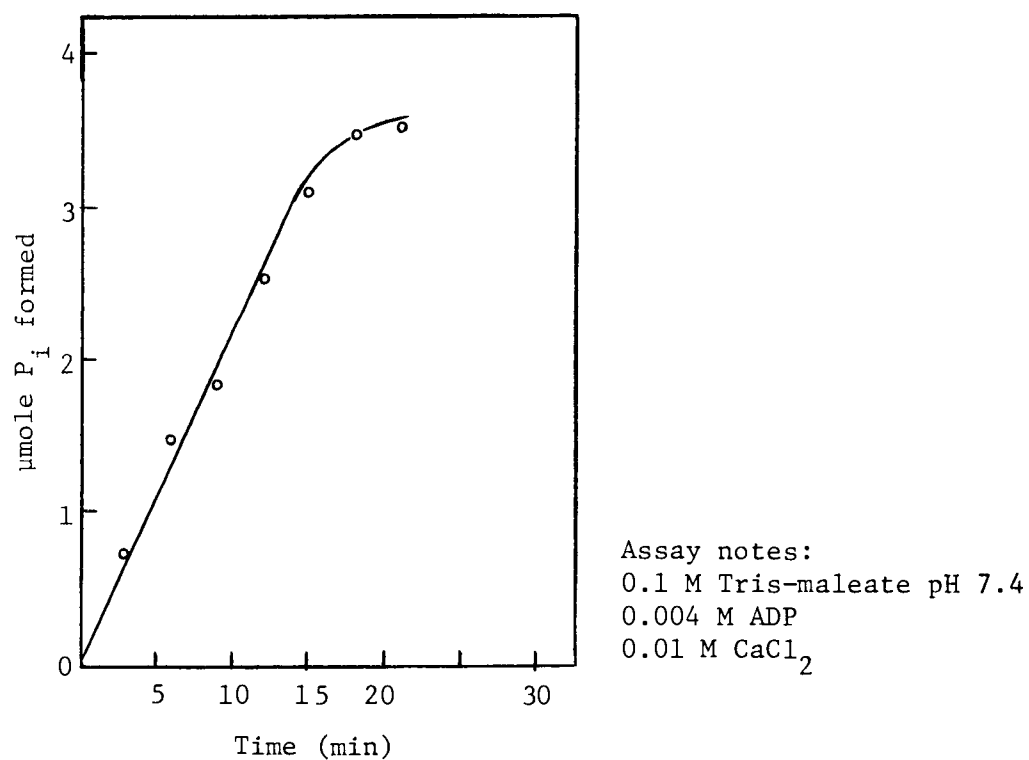
Figure 7. Time course of ADP hydrolysis.
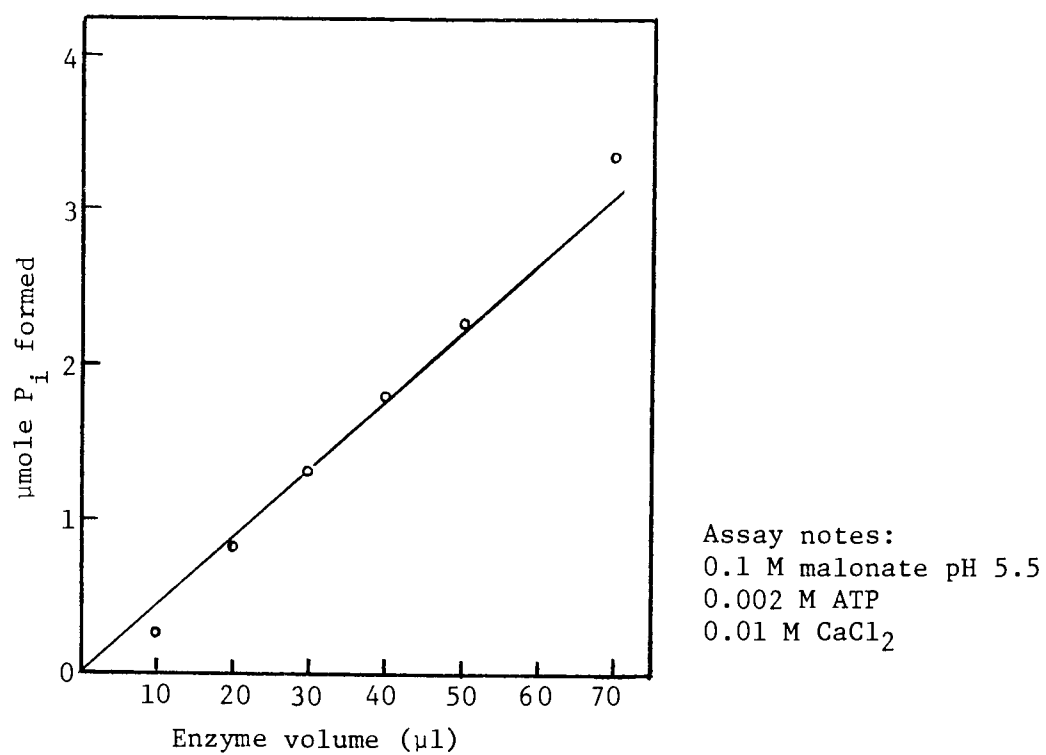
Figure 8. ATP hydrolysis as function of enzyme concentration.

Assay notes:
0.1 M malonate pH 5.5
0.002 M ATP
0.01 M $CaCl_2$
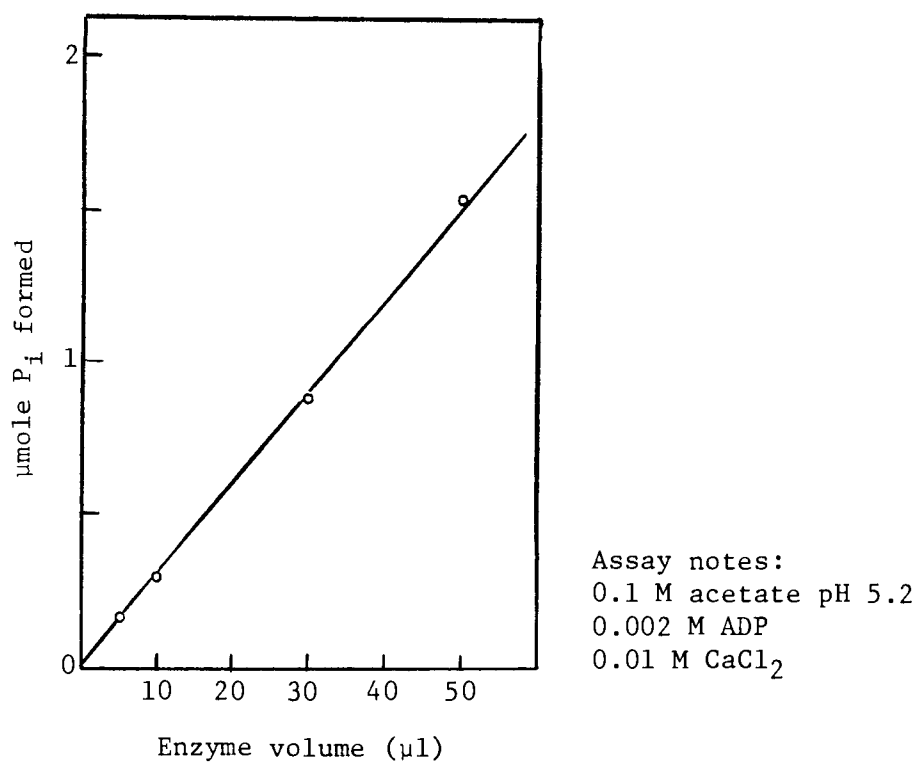
Figure 9. ADP hydrolysis as function of enzyme concentration.

Assay notes:
0.1 M acetate pH 5.2
0.002 M ADP
0.01 M $CaCl_2$

over a 90 hr period. The CMC purified enzyme was frozen (to -67°C) and thawed both slowly and rapidly, but no significant pattern of inactivation was discernable. With the availability of an ultra-deep freeze, all enzyme preparations upon purification were stored at -67°C. It has been observed that with the most highly purified enzymes (>80-fold) one month storage at -67°C wherein the enzyme stock was frozen and thawed twice (for sample removal) virtually no activity was lost.

4. Effect of Temperature

The rate of phosphate release from ATP was studied from 20°C to 50°C. The apyrase exhibited a curve with a broad peak and an optimum at 30°C.

5. Effect of pH

When the initial velocity of the apyrase reaction was studied as a function of pH using ATP as the substrate, a maximum was observed in the region about pH 5.8 (Fig. 10). With ADP as the substrate, the apyrase reaction revealed a pH optimum at about pH 6.8 (see Fig. 11). The particular rate value for a given pH fluctuated considerably from experiment to experiment as well as between replicate samples in a given experiment. Hence the best fitting line is drawn through Figures 10 and 11 to reflect the "true" pH dependency of the activity.

The study of reaction rates as a function of pH also revealed distinct patterns of activity which depended on the type of
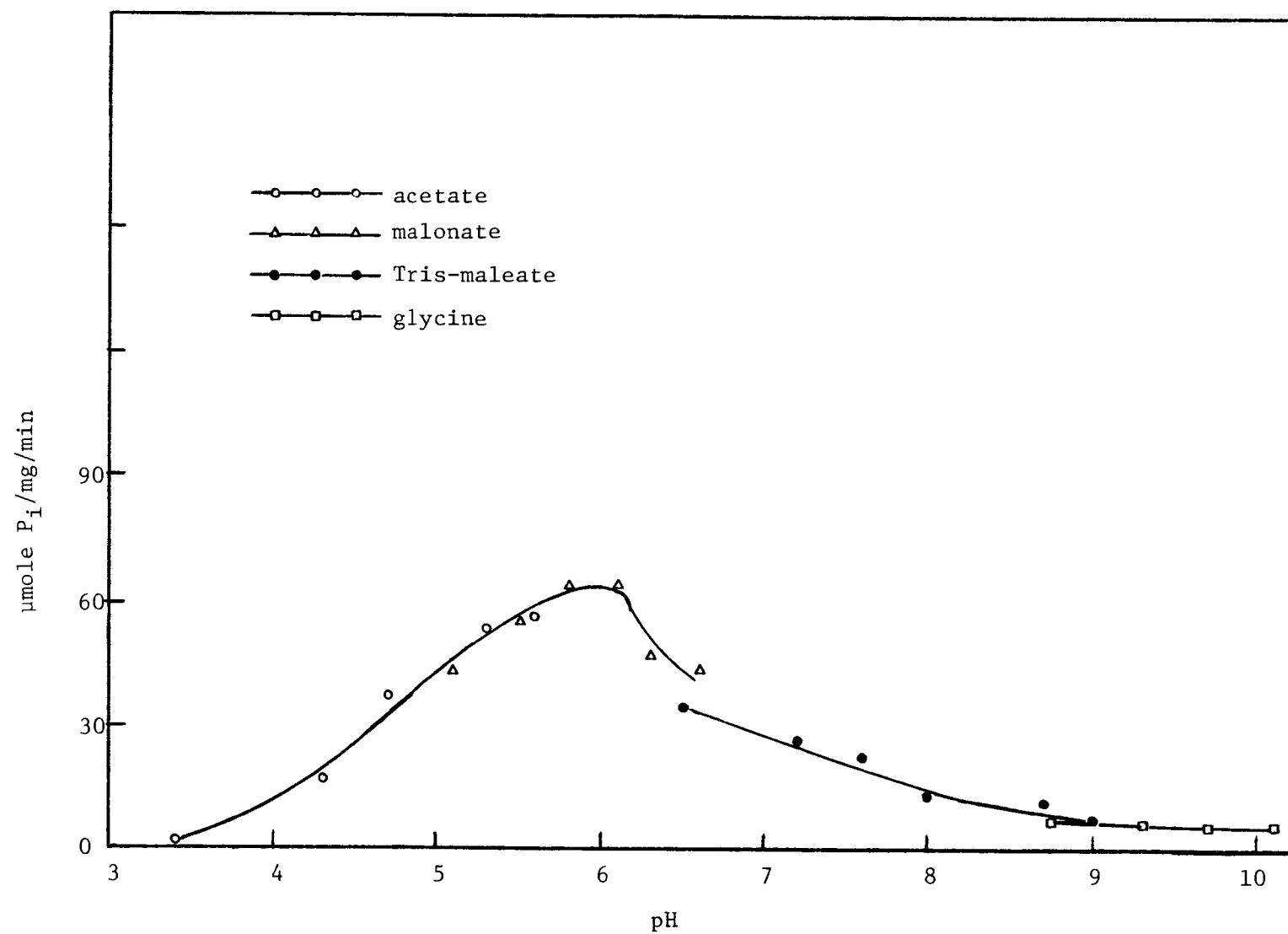
Figure 10. ATP hydrolysis as a function of pH.
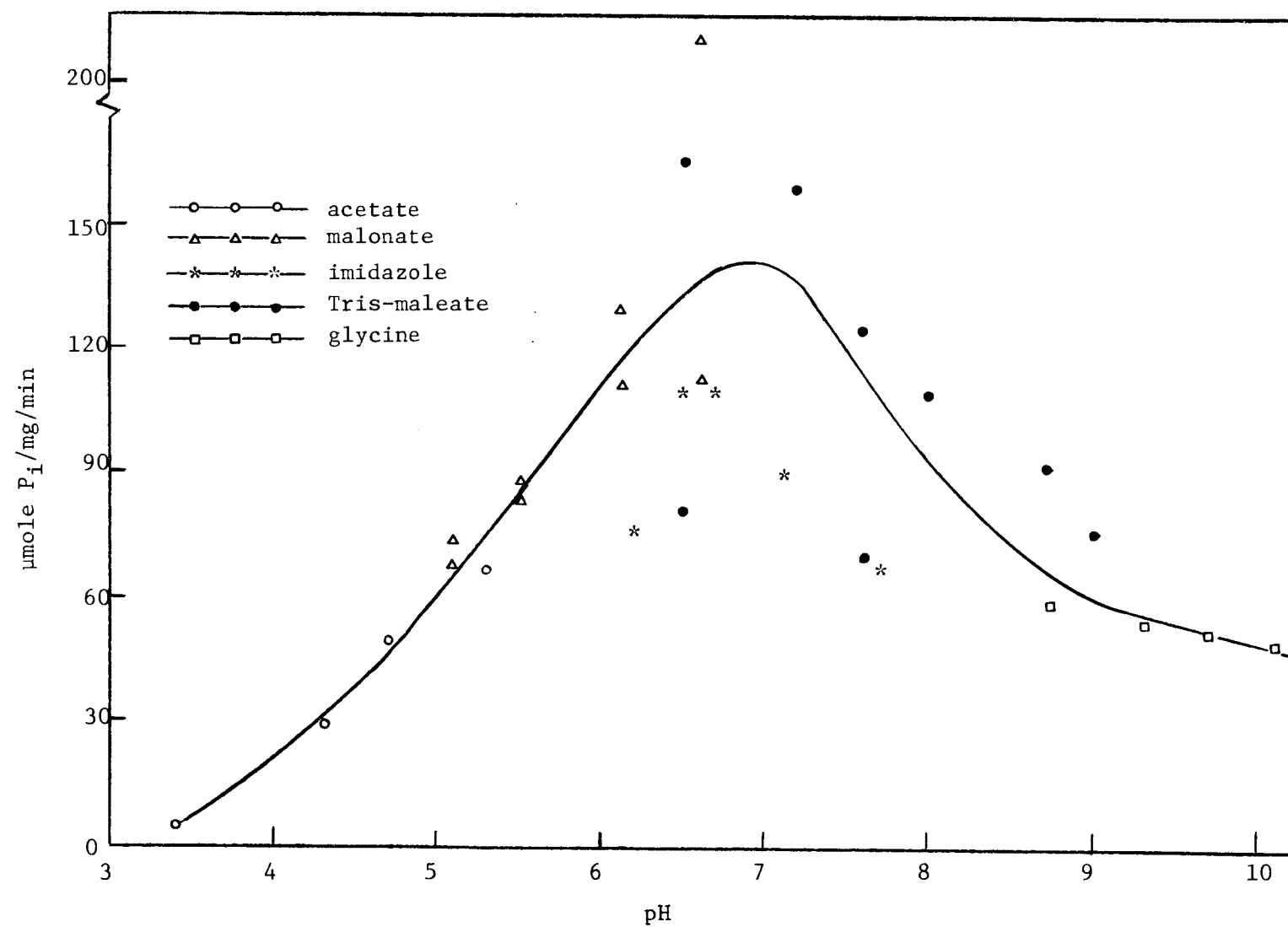
Figure 11.   ADP hydrolysis as a function of pH.

buffering ion that was used to support the hydrolytic reaction.
When ATP is used as the substrate the activity vs. pH curve can be
drawn smoothly through pH regions maintained by acetate and malon-
ate anions (see Fig. 10). A discontinuity is observed on changing
to maleate, Tris-maleate or imidazole (not shown but data super-
imposes on Tris-maleate) buffers. This discontinuity is more dis-
tinctly shown in Fig. 12 where a 50:50 molar ratio of acetate to
maleate anions results in a pH curve exactly intermediate between
the individual acetate and maleate curves. A slightly different
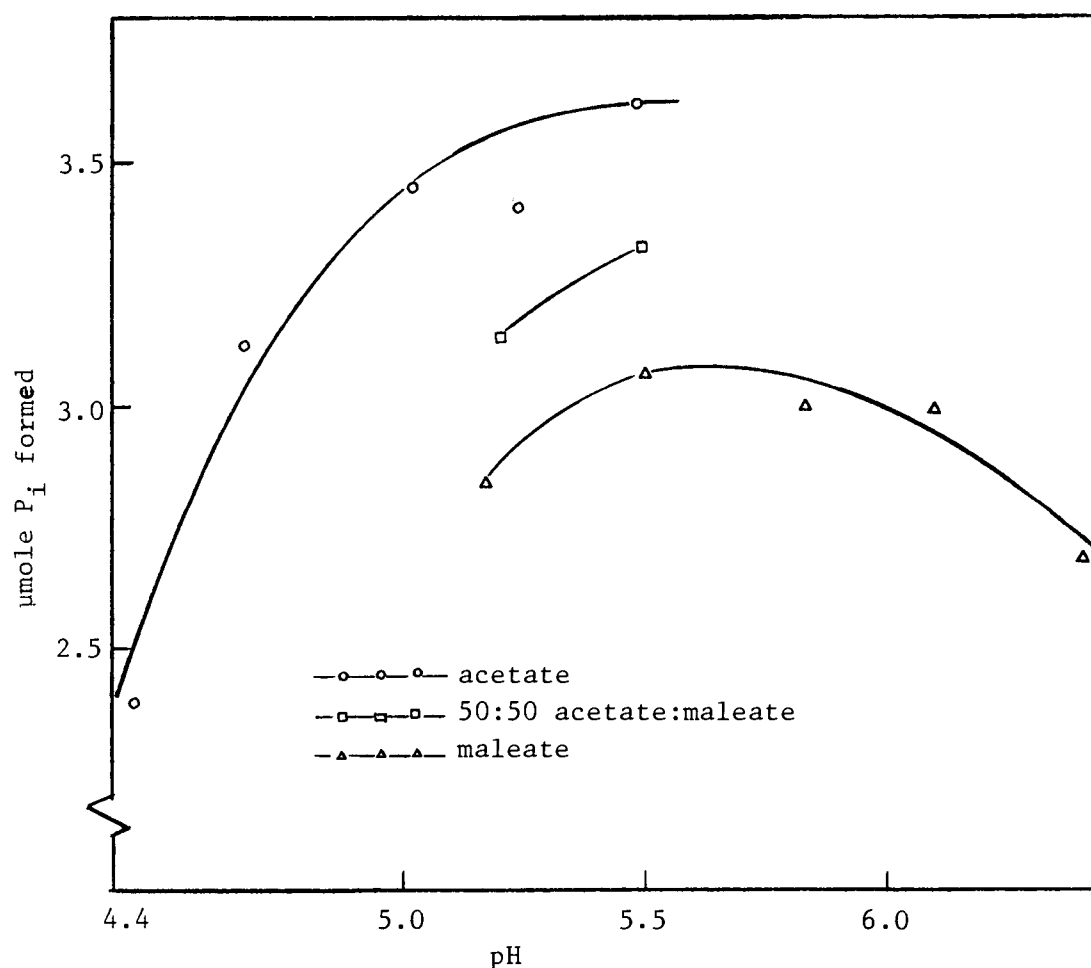
Figure 12.   Buffer effect on ATP hydrolysis.

pattern is noted when ADP is used as the substrate. The plot in

Fig. 11 reveals a somewhat continuous curve through the acetate,

malonate, imidazole and Tris-maleate buffered regions.

6. Kinetic Constants

The maximum velocity (V_m) and Michaelis constant (K_m), as de-

fined by Briggs-Haldane theory (15, p. 92-94), were determined by the

double-reciprocal plot method of Lineweaver and Burk (40). With

ATP as the substrate, the apyrase exhibited at pH 6.1 a double-

reciprocal plot with an upward curvature at low $1/(S)$ as shown in

Fig. 13. Extrapolations yielded K_m(app) = 1.54 mM and V_m(app)

= 222 μmole P_i/min/mg protein. When ADP served as substrate at

pH 7.1, the double-reciprocal plot revealed an upward deviation

at high $1/(S)$ values (see Fig. 14). Extrapolations yielded

K_m(app) = 0.725 mM and V_m(app) = 179 μmole P_i/min/mg protein.

Because of the curvilinear deviations present in these plots the

K_m and V_m must be designated as "apparent" values.

7. Kinetic Constants as Affected by pH

As a check on the pH dependency of the initial velocity, the

V_m(app) was studied as a function of pH. This was done by obtain-

ing data for a double-reciprocal plot at each of the different pH

values that were studied and extrapolating for the apparent con-

stants. Fig. 15 (ATP curve) shows the change in the V_m (app) with

pH for the ATP hydrolysis reaction. The largest value is seen at

pH 6.1. For the reaction with ADP as substrate, the V_m(app) to pH
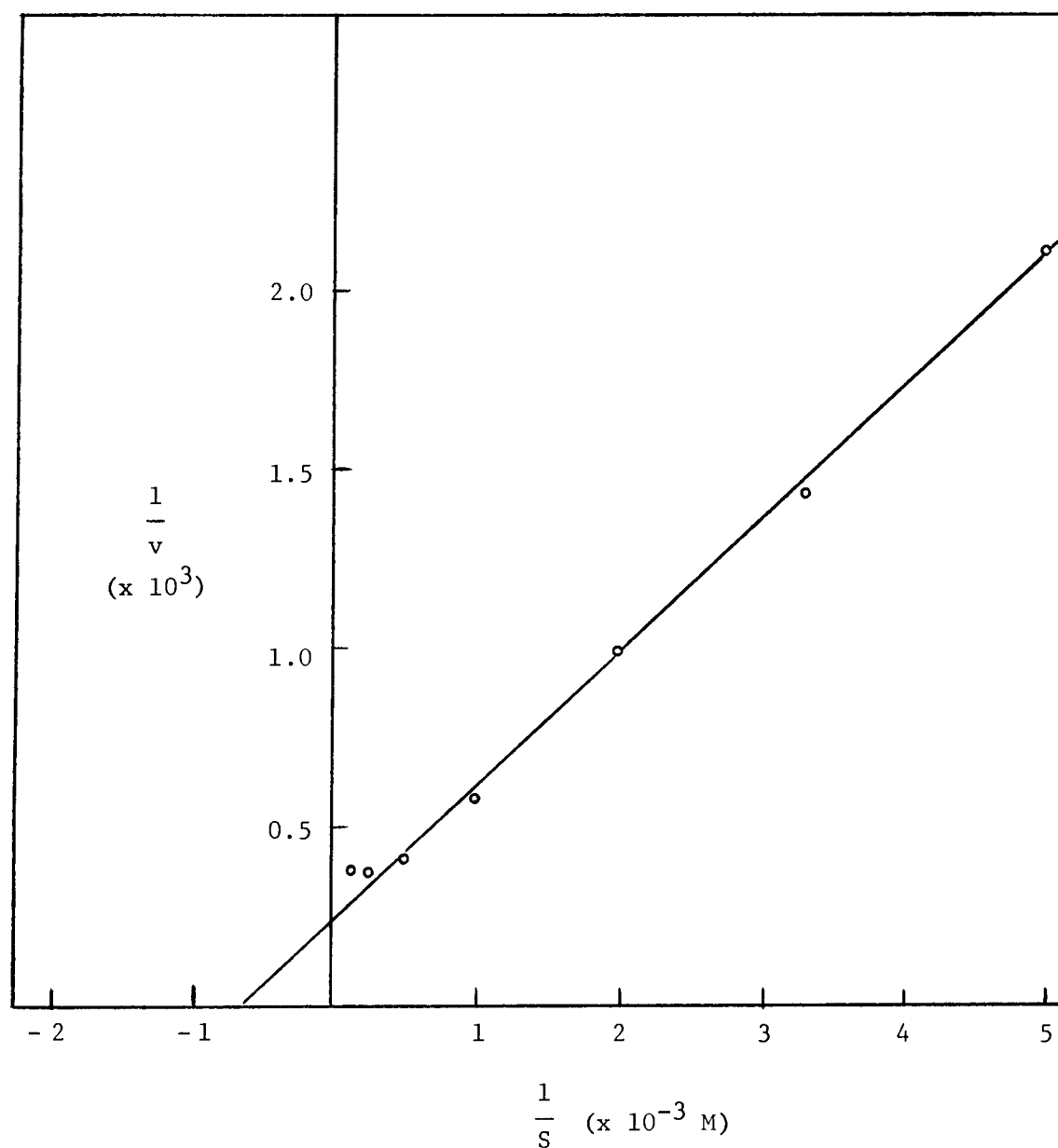
Figure 13. Double-reciprocal plot of ATP hydrolysis.

Assay notes: 0.1 M malonate pH 6.1, 0.01 M $CaCl_2$, 0.2 to 8.0 M ATP
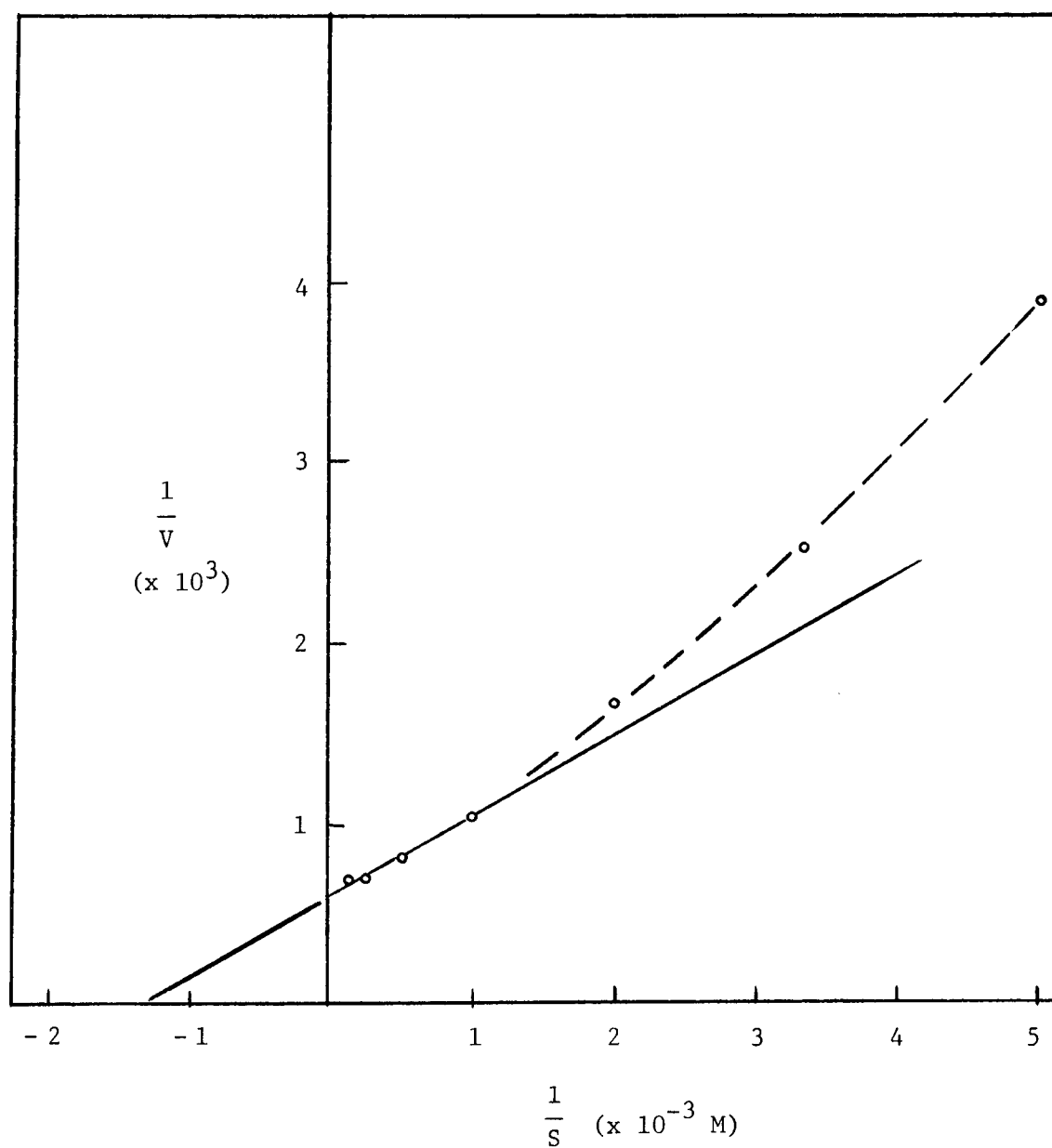
Figure 14. Double-reciprocal plot of ADP hydrolysis.

Assay notes: 0.1 M imidazole pH 7.1, 0.01 M $CaCl_2$, 0.2 to 8.0 M ADP
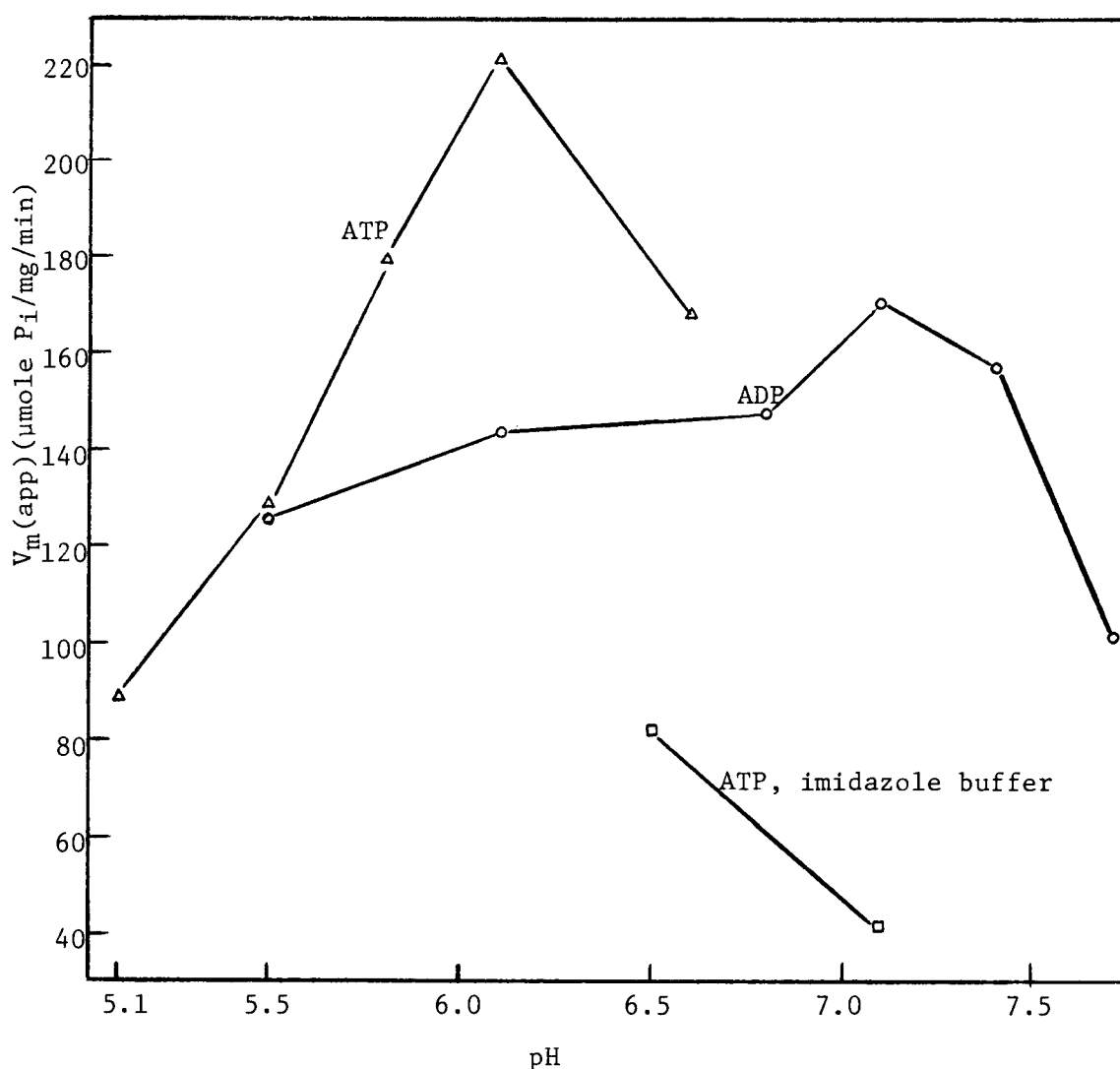
Figure 15. V_m(app) as a function of pH.

relation is shown on Fig. 15 (ADP curve), where the largest V_m(app) is seen at pH 7.1.

8. Effect of Divalent Cations

Hydrolysis of ATP by the apyrase enzyme was found to be markedly activated by added divalent cations. Table IX shows the relative effectiveness of several. For the ATP reaction, the sample

without added cations was only about 1/19 as effective as the sample with Ca^{++} added and about 1/31 as effective as when Mn^{++} was added. In contrast to this example, other enzyme preparations have shown no hydrolysis of ATP without added divalent cations. With added EDTA the hydrolysis of ATP and ADP was reduced to zero for all practical purposes. The order of effectiveness in activation was the same for both the ATP and the ADP reactions: $Mn^{++} > Ca^{++} > Mg^{++} > Co^{++} > Zn^{++} > Ni^{++}$ except for the $Zn^{++} > Co^{++}$ in the ADP reaction.

TABLE IX.   EFFECT OF DIVALENT CATIONS ON THE APYRASE REACTION

Additions	μmole P_i from ATP	μmole P_i from ADP
$CaCl_2$	1.79	2.08
$MnCl_2$	2.98	2.95
$MgCl_2$	1.40	1.92
$CoCl_2$	0.490	0.74
$Zn(acetate)_2$	0.398	0.85
$NiCl_2$	0.085	0.11
EDTA	0.04	0.0
none	0.095	----

Assay conditions. ATP reactions: 0.002 M substrate, 0.1 M malonate pH 5.5, 7.2 μg enzyme protein. ADP reactions: 0.004 M substrate, 0.1 M Tris-malonate pH 7.4, 4.3 μg enzyme protein. All additions were 0.002 M.

9. Substrate Specificity

The apyrase was examined for its ability to catalyze the hydrolysis of various phosphate compounds. Under conditions identical to those in which 2.3 µmole of P_i could be released from ATP, orthophosphate was not detected from sodium pyrophosphate, adenosine 5'-monophosphate, glucose 1-phosphate, glucose 6-phosphate, or fructose 1,6-diphosphate. A survey has revealed high activities for uridine triphosphate (UTP), inosine triphosphate (ITP), cytosine triphosphate (CTP), and guanosine triphosphate (GTP). These nucleotides were all better substrates than ATP (Table X).

TABLE X. COMPARISON OF VARIOUS NUCLEOTIDES AS SUBSTRATES FOR APYRASE

	ATP	UTP	ITP	CTP	GTP
µmole P_i formed	1.18	1.99	2.14	2.45	1.56

Assay notes: 0.002 M XTP, 0.01 M $CaCl_2$, 0.1 M malonate pH 5.8, and 0.86 µg of enzyme protein all in one ml. Enzyme stock solution contained 0.2 mg/ml of ovalbumin to help stabilize solution.

10. Compounds Which Affect ATP Hydrolysis

A brief survey was made of various agents that might affect the apyrase action on ATP. Table XI shows some comparisons. Only the SDS exhibited dramatic effects on the activity by completely abolishing it. The degree of inhibition (or stimulation) manifested by other agents was smaller.

TABLE XI.   EFFECT OF VARIOUS AGENTS ON THE APYRASE REACTION

Additions	μmole P_i formed	Inhibition (%)
None	1.80	----
2,4-dinitrophenol (DNP)	1.64	9.
oleic acid	1.59	12.
NaF	1.88	-4.
Sodium dodecyl sulfate (SDS)	0.00	100.
2,4-dichlorobenzoic acid (DCB)	1.78	1.8
2,4-dichlorophenoxy- acetic acid (2,4-D)	1.73	4.3
Adenosine-5'-phosphate (AMP)	1.48	18.
Adenosine	1.62	10.

Assay notes: 0.1 M acetate pH 5.2, 0.01 M $CaCl_2$, 0.002 M ATP, 0.001 M of added agent. and 2.6 μg enzyme protein in one ml. Values are averages of duplicates.

11. ADP Equilibration

An approach to ascertaining the presence of an adenylate kinase in the apyrase preparations was to attempt the conversion of ADP to ATP with the apyrase solution. At pH 7.1 in 0.1 M imidazole buffer and 0.01 M $CaCl_2$, 4.0 μmole of ADP was acted upon by 0.43 μg of the apyrase preparation for 15 minutes. Analysis indicated that 0.638 μmole P_i had been released (or about 16% hydrolysis). Chromatography of an incubation mixture aliquot which contained about 0.1 μmole of nucleotide, revealed that about 22% of the total OD_{260} for the nucleotides corresponded to AMP. In duplicate samples, one showed 2.3% of the total OD_{260} as ATP, while the other showed none. Essentially, no significant amount of ATP was produced from ADP.

12. Detection of XDP in XTP Hydrolysis

Early chromatographic examinations of the products from the apyrase reaction at pH 7.8 revealed no accumulation of ADP when ATP was the starting substrate. Only AMP and unreacted ATP were detected. More recent experiments have shown that at pH 5.8 (malonate) ADP is detectable as a product from ATP hydrolysis by the apyrase. At this pH the diphosphates from UTP, ITP, CTP, and GTP have also been detected. Evaluated as a percent of the total nucleotide optical density in the UV the ratio of the diphosphates to monophosphates ranged from 1:2.2 to 1:4.2.

13. Competition Between Nucleotides

Since the apyrase preparation can hydrolyze both ATP and ADP, the possibility is raised that more than one enzymatic entity may be present in the solution. There are three models which may be used to explain the hydrolysis of two substrates:

a. Two (or more) enzymes are present—each specific for the binding and hydrolysis of one substrate.

b. One enzyme is present which has two independent non-interacting binding and hydrolysis sites which are specific for each of the substrates.

c. One enzyme is present, which has one binding and hydrolysis site for both substrates.

Models a and b are not kinetically distinguishable and may be classified as one, a'. One possible way of distinguishing between models a' and c is to compare the rates of hydrolysis of ATP and

ADP when assayed individually and together. Model $\underline{a}'$ would predict that the rate of P_i release from ATP and ADP present simultaneously as substrates should be the sum of the individual rates of hydrolysis of ATP and ADP. Model $\underline{c}$ would predict that the hydrolysis rate of a mixture of ATP and ADP could be anything but an additive sum of the individual rates for ATP and ADP. The results in Table XII indicate that Model $\underline{c}$ is supported, i.e., one enzyme with one activity site appears to hydrolyze both ATP and ADP.

TABLE XII. REACTION OF ATP AND ADP WITH APYRASE

Sample	Substrate input	μmole P_i formed
A	2 μmole ATP	1.58
B	2 " ATP 4 " ADP	3.07
C	4 " ADP	3.11

Assay notes: 0.1 M maleate pH 6.1, 0.01 M $CaCl_2$

With the observation that several kinds of nucleoside triphosphates are hydrolyzed by the apyrase, the question was raised whether one or several triphosphate diphosphohydrolases were present.

A simple (but not definitive) approach to this question was to examine the rates of phosphate released from various nucleoside triphosphates individually and in mixed pairs. Fig. 16 a-d shows the time course patterns of some of the nucleotide pairs.
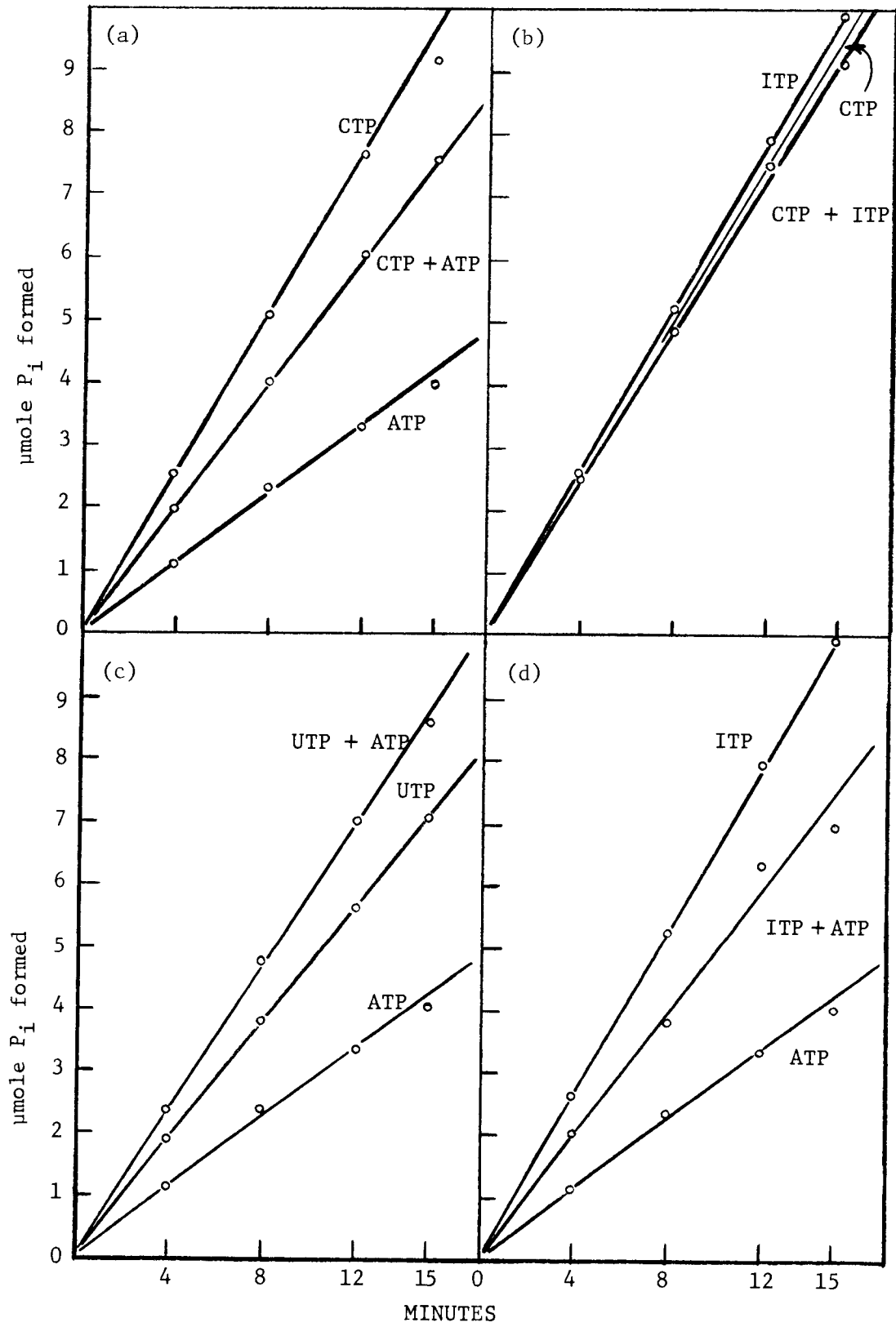
Figure 16. The hydrolysis of various nucleotides by apyrase.

It can be seen generally that the simultaneous presence of two different nucleoside triphosphates does not result in a rate of phosphate release that equals the sum of the rates for the individual nucleotides.

14. Co-purification of ATPase and ADPase

Another suitable criterion for verifying the postulated model $\underline{c}$ in section 13 is to see if the ATP hydrolysis rate and the ADP hydrolysis rate are in constant ratio to each other through different purification procedures. Table XIII shows a fair constancy of the ADPase/ATPase ratio over a 38-fold purification through three steps.

TABLE XIII. COMPARISON OF ADP AND ATP REACTIONS OF APYRASE AT
VARIOUS STAGES OF PURIFICATION

Procedure	ATPase pH 5.5	ADPase pH 7.4	ADPase / ATPase
Acetone powder solution	1.05 $\frac{U}{mg}$	1.54 $\frac{U}{mg}$	1.46
Ammonium sulfate fraction, 2.0-3.6 molal;	1.34 "	2.22 "	1.65
Protamine sulfate	1.33 "	2.34 "	1.75
CMC chromatography (most active fraction)	36.4 "	59.2 "	1.62

Assay notes: 0.01 M $CaCl_2$, 0.002 M ATP or 0.004 M ADP, 0.1 M acetate pH 5.5 or Tris-maleate pH 7.4, and appropriate aliquots of enzyme to keep hydrolysis under 30% of maximum possible.

V. DISCUSSION

These studies of the pea seedling apyrase encompass two broad phases: a) purification of the activity from an acetone powder, and b) characterization of some of its properties. The solution of problems encountered in purification unwittingly contributed to the characterization. Some of the observed properties may be integrated into a proposed mechanism of action of the apyrase.

A. Purification

1. The Polyanion Problem

The turning point in the program to significantly purify the apyrase came about when protamine sulfate was observed to precipitate something out of the AS enzyme preparation and yet leave the activity in solution. Another important observation was the failure of the AS preparation to elute through a mixed bed DEAE and CMC. A hypothesis was suggested that the apyrase and a polyanion form a strong complex at low salt concentrations and that this complex be electrically neutral. The low salt concentration may not need to have been stipulated for the AS was precipitated from ammonium sulfate solutions 2.0 molal and higher. In any event, the retention of the preparations on the mixed ion-exchange bed could be explained as follows. In low ionic strength buffer, the addition of DEAE to CMC resulted in a neutralization reaction which brought the oppositely charged groups into very close proximity to each other. The close positioning of the cationic and anionic groups in the mixed bed

permitted these sites to efficiently compete for the oppositely charged components of the neutral enzyme complex and cause them to become separated. Upon separating, the components were then bound to their respective exchangers.

Since the protamine sulfate, which has a pI = 12, did not precipitate the enzyme, the pI of the apyrase protein could be predicted to be between pH 7 and 11. The fact that the PS preparation did not adsorb very well to CMC at pH 7.8 suggested that the pI of the enzyme must be very near to this value. The improved binding of the apyrase to CMC at pH 6.5 confirmed the nature of the enzyme pI and led to improved conditions for protamine sulfate treatment. With the enzyme solution at pH 6.5, the positive charge on the enzyme protein diminished the possibility of co-precipitation when the protamine was mixed in. The latter being a very strong base could displace the interactions of the enzyme with the polyanion species. The fact that the polyanionic material was shown to be RNA (at least in part) eventually illuminated the problems inherent in ammonium sulfate fraction. Heppel (27, p. 137-138) has pointed out that when large amounts of nucleic acids are present (e.g., in bacterial cell extracts) salt fractionation of enzymes is greatly impeded. For example, a series of fractions may be obtained which differ very little in enzymic specific activity. It can be supposed that in these mixtures the prominent, widespread intermolecular interactions prevent a given enzyme to be salted out discretely. For the pea seedling extracts, a wide ammonium sulfate concentration range (2.4-4.0 molal) brought down the apyrase with only a modest yield. By the time Method b was devised,

the enzyme solution was improved in its preparation (S'_{HS}) by the treatment with protamine such that a slightly narrower salt range (2.7-4.0 molal) permitted high recoveries (see Table VIII).

2. Ion-exchange Chromatography

In spite of the success of being able to chromatograph the apyrase on cation exchangers, one still wonders why the enzyme activity is eluted into two zones: one (A) which emerges at the solvent front or V_O of the column and the other (B) which is somewhat retained on the column and requires either a longer eluting time (in the BR-70 case) or stronger solvent power (e.g., where NaCl gradients are used) to effect its elution from the column. In the efforts to improve the yield of the high purity enzyme it was assumed that the zone A activity corresponded to protein which had not been completely freed from binding with the polyanionic material. But even where large amounts of protamine have been used, the best distribution of A to B has been 1 part to 20 parts respectively. The residual acitivity in zone A may be due to a) a limitation determined by the equilibrium constant of the protamine reaction, or b) a different protein species from that in zone B.

The attempt to chromatograph apyrase (PS) on P-cel was suggested by the notion that stronger basicity of the phosphate ester anion would promote a different and more improved purification of the enzyme. It was supposed that a stronger adsorbant would cause a change in the relative distribution pattern of the various protein components present in the PS preparation when these were eluted from the

adsorbent with a NaCl gradient. This was apparently not realized; hence further refinements were not pursued. Improved results might be obtained by using a shorter column of the same diameter as before and a more rapidly rising NaCl gradient.

In contrast to the hydrophilic nature of the cellulosic cation exchangers, BR-70 offers a hydrophobic matrix while the functional group (-COOH) is the same as in CMC. Since inter-peptide hydrogen bond formation can be facilitated by a medium of low dielectric strength (31) it can be supposed that a carboxylic group situated in a hydrophobic resin matrix will offer stronger interactions with functional groups of proteins due to the relatively low dielectric constant in the immediate molecular environment of that carboxylic group. This had been borne out by the elution techniques used in IRC-50 chromatography. Although the results obtained with BR-70 were less than perfect, this medium should be investigated in any attempts to further purify the apyrase beyond 80-100 fold. The recommendations arise from the qualitative difference between CMC and BR-70 in the protein/ion-exchanger interactions.

3. Gel Filtration

This approach to apyrase purification commanded most attention when the conventional ammonium sulfate fractionations (Method a) were providing only modest recoveries of activity. Because gel filtration amounts to molecular sieving through a polymeric matrix (19, p. 35), the fractionation of a protein mixture results in a separation on the basis of hydrodynamic volume (which corresponds to a certain molecular

weight). The utility of this approach seemed especially promising

when an apparent change in molecular size seemed to take place on G-

200 (exclusion limit about 200,000 MW) with a buffer containing 0.25

M NaCl (Fig. 5). The change in the V_e (elution volume) for the apy-

rase was not apparently due to dissociation of the enzyme-polyanion

complex since the activity obtained from the gel effluents was not

sorbed onto CMC. When the activity purified on G-200 was filtered

through G-75 (exclusion limit about 50,000 MW) more UV absorbing

material was removed resulting in further purification—the activity

emerged at the V_o and the other UV absorbing peak was at about V_e =

2.5 V_o. In addition to the purification attained, the two Sephadex

filtrations permitted a molecular weight estimation of 50,000 to

100,000 for the non-protamine treated enzyme. As already pointed

out, the expenditure of six days for the purification process based

on Sephadex filtration was considered unfavorable, especially since

so many manipulations were involved—e.g., the enzyme solution had

to be concentrated after each gel filtration.

4. Final Purification Scheme

In the slow evolution of purification procedures the efforts have

always been to maximize three factors: a) high degree of purifica-

tion, b) high yield of activity, and c) ease and/or brevity of opera-

tions for achieving a) and b). Not a few tangents were taken from

the path towards a simple, effective purification scheme that is sum-

marized on Table VIII. The effectiveness of conventional or estab-

lished purification methods speak for themselves with respect to

their efficacy. But seemingly minor procedural details such as maintaining constant pH at 7.8 and the inclusion of EDTA in the ammonium sulfate fractionation were required for complete success. The use of protamine sulfate as a polyanion (RNA and/or DNA) precipitant is not new or novel but a systematic study regarding conditions for its most effective use has unfortunately not yet appeared in print. Hence, even in the present work a degree of empiricism was manifested in the small effort to find the best conditions for treatment of apyrase solutions. The efficacy of the purification scheme summarized on Table VIII is attested by the fact that a subsequent preparation using this scheme has yielded a 110-fold purified enzyme with a recovery in excess of 100% of the starting activity.

Further purification of the enzyme requires the accumulation of several milligrams of the CMC purified enzyme, since the handling of sub-milligram amounts of protein quickly becomes impractical in procedures like column chromatography.

B. Characterization

1. Enzyme Stability

In spite of the evidence suggesting that the apyrase is reasonably stable, there has been some concern as to the factors which cause the tremendous variability in enzyme assays. To reduce this problem, obvious efforts were made such as changing the mode of cleaning glassware, etc., but any improvements that were noted seemed to be temporary. Even the addition of ovalbumin as an adjuvant to the purified

enzyme solution (62) did not eradicate the problem. Another observation that may be related to the question regarding enzyme stability is the upward curvilinear relationship of the reaction velocity to the amount of enzyme used to catalyze the reaction. This phenomenon, which has been noted only a few times, could be explained by a) an equilibrium of the enzyme with an activator (15, p. 63) or b) an equilibrium of the enzyme with its structural sub-units—the equilibrium being concentration dependent (50, p. 395). In some cases, dilution of the enzyme seemed to result simply in the denaturation of the enzyme.

2. pH Effects

The variation in initial velocity as a function of pH may be attributed to at least three major factors: a) the inherent effect on the V_m itself, b) the effect on the affinity of substrates to enzyme, and c) the effect on the stability of the enzyme (15, p. 116-118). The observed velocities presented in Figures 10 and 11 probably reflect the result of a combination of the three effects. One can experimentally distinguish the pH effect on V_m by obtaining data which will permit the determination of K_m and V_m at each pH (see Fig. 15). The data obtained at high extrapolated values of the substrate concentration eliminate the effect of pH on the affinity of substrate to enzyme (15, p. 117). A comparison of the v_o vs. pH (Figures 10 and 11) and the V_m (app) vs. pH curves reveals a shift in the pH optima for both ATP and ADP reactions. For the ATP hydrolysis the optima are pH 5.8 and 6.1 respectively from v_o and V_m (app) curves,

while ADP hydrolysis reveals pH 6.8 and 7.1. According to Laidler (36, p. 141) the rise in the pH optimum with increase in (S) could correspond to the formation of an ES complex where the substrate complexes only at an acidic site on the enzyme. Unfortunately, the rest of the activity-pH data do not conform to his hydrolysis model. Taking the data from V_m (app) curves to yield the true optima, the values obtained compare well to those of potato apyrase in the literature (39, 46), that is, they are found in the pH 5.9 to pH 7.7 range.

There appears to be a correlation between the multiplicity of pH optima exhibited by an apyrase preparation and the difference in the rates for ATP and ADP hydrolysis catalyzed by that preparation. For example, one of the apyrase A preparations of Molnar and Lorand (46) shows a pH optimum for ATP at 5.9 and for ADP at 7.7. At pH 6.7 the γ-phosphate from ATP is released about nine times faster than the β-phosphate from ADP. On the other hand, one of their apyrase B preparations which exhibits optimal activity for both ATP and ADP at pH 5.9, liberates the γ-phosphate from ATP at virtually the same rate as the β-phosphate from ADP. Examples from the work of Liebecq, et al., (39) show similar patterns. Since the V_m reflects only the rate of breakdown of the ES complex, the two pH optima observed with the V_m (app) data would suggest that the pea seedling apyrase has a different phosphate release site and/or mechanism for the γ-phosphate of ATP and the β-phosphate of ADP. This notion does not necessarily rule out a single substrate binding site for both nucleotides.

The study of the effect of pH on activity also produced some distinctive patterns wherein the level of activity observed was dependent

on the type of buffer ion supporting the reaction. While a detailed investigation has not been made of the ATP hydrolysis by apyrase in Tris-maleate and imidazole buffers, one may suppose that the lower activity level in these buffers is due to some special binding of the ionic species so that the functional groups at the active site are adversely perturbed (1, p. 145). More importantly we have here a means of distinguishing the activities for ATP and ADP. The difference in buffer inhibition can be used as corraborative evidence to suggest that the γ-phosphate of ATP is hydrolyzed at an active site different from that of the β-phosphate of ADP.

The relationship of the β-phosphate hydrolysis constant (k^β) to the γ-phosphate hydrolysis constant (k^γ) as a function of pH has provoked interest in the pea seedling apyrase from the earliest observations. In contrast to apyrases from potato (12, 46) and insects (22) the enzyme from pea seedlings appears to hydrolyze ADP faster than ATP, hence a biphasic time course pattern in phosphate release has not been observed (12). This presents a problem in the efforts to describe the sequential reaction mechanism whereby ATP is hydrolyzed ultimately to AMP and two P_i. The relationship of the two sequential hydrolytic reactions is much like that of the reactive steps in a two-step reaction mechanism, where no information is kinetically obtainable concerning the course of a reaction after the rate-determining step (23, p. 175).

While k^β is readily obtained from following ADP hydrolysis by P_i assay, the value for k^γ is harder to determine as shown in the following development. When the rate of ATP hydrolysis is measured by

P_i release, the observed rate of product formation must be the sum of two sequential reactions:

$$\text{ATP} \xrightarrow{k_1 (\text{or } k^\gamma)} \text{ADP} + P_i \qquad \text{Eq. 1a}$$

$$\text{ADP} \xrightarrow{k_2 (\text{or } k^\beta)} \text{AMP} + P_i \qquad \text{Eq. 1b}$$
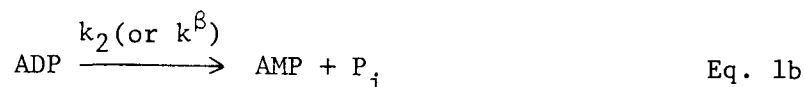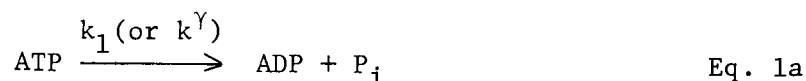
The overall rate of P_i formation is:

$$\frac{dP_i}{dt} = k_1 (\text{ATP}) + k_2 (\text{ADP}) \qquad \text{Eq. 2}$$

To express P_i formation in terms of ATP, we must eliminate the (ADP) term (21, p. 166); first we note that

$$\frac{d(\text{ATP})}{dt} = -k_1 (\text{ATP}) \qquad \text{Eq. 3a}$$

integrates to $\quad (\text{ATP}) = (\text{ATP})_o \exp(-k_1 t) \qquad \text{Eq. 3b}$

which is substituted into, $\quad \dfrac{d(\text{ADP})}{dt} = k_1 (\text{ATP}) - k_2 (\text{ADP}) \qquad \text{Eq. 4}$

to obtain $\quad \dfrac{d(\text{ADP})}{dt} = k_1 (\text{ATP})_o \exp(-k_1 t) - k_2 (\text{ADP}) \qquad \text{Eq. 5}$

Equation 5 is integrated by use of integrating factors (44, p. 434-435), and if $(\text{ADP})_o = 0$, we can get

$$(\text{ADP}) = \frac{(\text{ATP})_o k_1}{k_2 - k_1} \{\exp(-k_1 t) - \exp(-k_2 t)\} \qquad \text{Eq. 6}$$

The expression for (ADP) is then substituted into equation 2 to obtain

$$\frac{dP_i}{dt} = k_1 (\text{ATP}) + k_2 \frac{(\text{ATP})_o k_1}{k_2 - k_1} \{\exp(-k_1 t) - \exp(-k_2 t)\} \qquad \text{Eq. 7}$$

The equation above suggests that it would be very difficult to obtain k^γ from measuring ATP hydrolysis by P_i assay alone. The most practical

way of measuring k^γ would be to follow the release of radioactivity from γ-P^{32} labelled ATP (62).

Chromatographic analysis of reaction products indicated that the k^β/k^γ ratio apparently changes as a function of pH. With the apyrase reaction at pH 7.8, 36% of the nucleotides was found as AMP and none as ADP. At pH 5.8 one found 45% AMP and 20% ADP in the reaction products. The data also indicated that most probably the k^β/k^γratio is always greater than one over the entire pH range wherein the enzyme is active.

3. Kinetic Constants

The apparent Michaelis constants (K_m app) for the pea seedling apyrase reaction with ATP and ADP are somewhat high compared to those reported for potato apyrase preparations by Molnar and Lorand (46). This would indicate that the enzyme in vivo would require a very un-physiologically high level of nucleotides to be fully active.

The apparent maximum velocities (V_m app) are of comparable orders of magnitude to the enzymes from potato. Molnar and Lorand (46) found their apyrase A had a V_m = 46.4 (μmole P_i/min/mg protein) with ATP substrate and V_m = 5.5 with ADP substrate. Their apyrase B showed V_m = 101 with ATP and V_m = 82 with ADP. By comparison the pea seedling apyrase shows V_m (app) = 222 with ATP and V_m (app) = 171 with ADP. The fact that V_m (app) for the ATP reaction is greater than for the ADP reaction does not contradict the notion that k^β is greater than k^γ. As noted in a preceeding section, the determination of V_m (app) by P_i release involves a sum of the k^β and k^γ reaction steps.

Hence, depending on the $k\beta/k\gamma$ ratio it is possible for the V_m (app) for ATP to be greater than the V_m (app) for the ADP.

Close examination of the double-reciprocal plots for ATP and ADP hydrolysis by apyrase reveals some interesting features. The ATP reaction shows an upward deviation of the 1/v at low 1/(s) (Fig. 13). This is usually interpreted as inhibition of the reaction at high substrate concentration (36, p. 71-77).

For a model scheme by Laidler

$$\begin{array}{ccccc}
 & & \xrightarrow{} ES \xrightarrow{k_2} P & & \\
 & K'_t \nearrow & & \searrow K_s & \\
SES & & & & E \\
 & K'_s \searrow & & \nearrow K_t & \\
 & & SE & & \\
\end{array}$$
Eq. 8
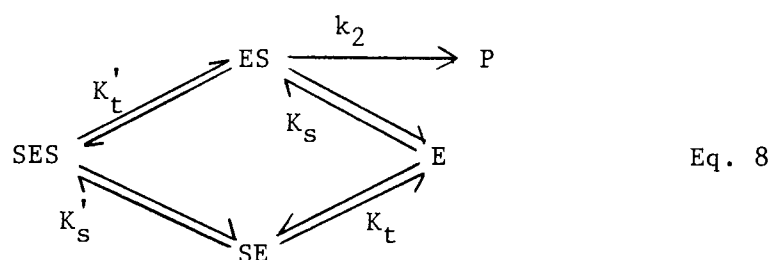
it has been shown that

$$v = k_2(ES) = \frac{k_2 K_s (E)_o (S)}{1 + (K_s + K_t)(S) + K'_s K'_t (S)^2} \qquad \text{Eq. 9}$$

The equation yields zero rate at high (S) and a maximum rate at $(S)^2 = 1/K_s K'_t$.

The double-reciprocal plot analysis can be made by writing the equation 9 in the form:

$$\frac{1}{v} = \frac{1}{k_2 K_s (E)_o (S)} + \frac{K_s + K_t}{k_2 K_s (E)_o} + \frac{K'_s K'_t}{k_2 K_s (E)_o}(S) \qquad \text{Eq. 10}$$

At low (S) or when $1 + (K_s + K_t)(S) \gg K_s K'_t (S)^2$ the plot will be linear. But when (S) is large the 1/v will show an upward deviation. The ordinate intercept will be $(K_s + K_t)/(k_2 K_s (E)_o)$ and not simply $1/k_2(E)_o$ or $1/V_m$. At high (S) a plot of 1/v vs. (S) should be linear

and the slope is equal to $(K_s' K_t')/(k_2 K_s (E)_o)$.

The double-reciprocal plot for the ADPase reaction (Fig. 14) exhibits a concave deviation at high $1/(S)$. This can be interpreted as a reaction which is activated by substrate (15, p. 82). A possible model scheme is

$$
\begin{array}{c}
\text{E + S} \xrightleftharpoons[K_{sa}]{K_s} \quad
\begin{array}{l}
\text{ES} \\[2em]
\text{S} \\
+ \\
\text{ES}_a \xrightleftharpoons{K_s'} \text{ES}_a\text{S} \xrightarrow{k} \text{P}
\end{array}
\end{array}
\qquad \text{Eq. 11}
$$
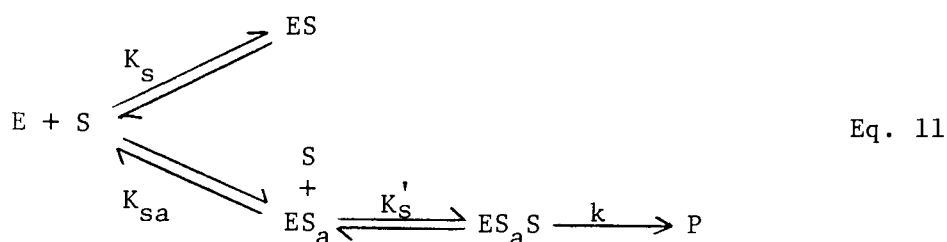
where it can be shown that

$$
v = \frac{k(E)_o}{1 + \dfrac{K_s'}{(S)}\{1 + \dfrac{K_{sa}}{K_s} + \dfrac{K_{sa}}{(S)}\}}
\qquad \text{Eq. 12}
$$

In the reciprocal form we find

$$
\frac{1}{v} = \frac{1}{V_m} + \frac{K_s'}{V_m}\{1 + \frac{K_{sa}}{K_s}\}\frac{1}{(S)} + \frac{K_{sa}K_s'}{V_m}\cdot\frac{1}{(S)^2}
\qquad \text{Eq. 13}
$$

Owing to the $1/(S)^2$ term a curved reciprocal plot will be obtained—this is the basis for interpreting the concentration dependent pattern of the ADP hydrolysis by apyrase. At high (S) the third term (containing $1/(S)^2$) becomes negligible and the plot will approximate a straight line, which gives an apparent Michaelis constant of $K_s'(1 + K_{sa}/K_s)$. The true Michaelis constant is corrected by the quantity in the brackets which corrects for the competitive effect of the E + S $\rightleftharpoons$ ES reaction (see Eq. 11).

4. Effect of Divalent Cations

The requirement for divalent cations is shown by most enzymatic reactions involving hydrolysis or transfer of adensoine nucleotide groups (6, p. 31) and the pea seedling apyrase is no exception. Since ATP and ADP are known to readily form complexes with divalent cations, at least one prominent role is suggested for these metal ions in the apyrase reaction. To arrive at some basis for explaining the particular order of activation by metal ions for the apyrase, a comparison can be made to stability constant data. ATP forms complexes with divalent cations in the order: $Ni^{++}>Zn^{++}>Mn^{++}>Co^{++}>Mg^{++}>Ca^{++}$ (60). ADP-cation complexes show the same order as in ATP except that $Co^{++}>Zn^{++}$ (59). In comparison, the apyrase reactivity shows the order: $Mn^{++}>Ca^{++}>Mg^{++}>Co^{++}>Zn^{++}>Ni^{++}$ for the ATP substrate. It seems evident that the stability of complex formation alone does not explain the specificity in the enzyme reaction. Hammes and Levison (26) have studied the kinetics of metal ion interaction with ATP and ADP as an approach to elucidating the divalent cation specificity of nucleotide hydrolases. The rate constants for formation and dissociation of ATP/metal-ion complexes showed the following increasing order: $Ni^{++}<Mg^{++}<Co^{++}<Mn^{++}<Ca^{++}$. The dissociation rate constant for ATP/Ca^{++} is about 1000 x greater than for the Ni^{++} complex. Although dissociation rates couldn't be the only determinant in the metal-ion preference by the apyrase, the data certainly suggest them to be a significant factor. The non-enzymic hydrolysis of ATP and ADP at pH 5 has revealed distinct patterns of catalytic effectiveness

by divalent cations (61). The ATP reaction exhibits the following order of catalytic specificity: $Zn^{++} \gg Mn^{++} > Ni^{++} \simeq Ca^{++} > Co^{++} > Mg^{++}$. A somewhat different pattern is noted for the ADP reaction: $Zn^{++} \gg Ca^{++} > Co^{++} > Ni^{++} > Mg^{++} > Mn^{++}$. CLearly any analogies drawn between non-enzymic and enzymic reactions will have to be carefully qualified.

Some workers have suggested that ATP and ADP each form different chelates with different divalent cations, thereby making possible the various ATP-cation specificities for the variety of enzymes (64). Even though precise structural assignments have not yet emerged, the nuclear magnetic resonance (10) and the infra-red (7) studies indicate that different types of metal-ATP and -ADP interactions do exist, indeed the ATP-Mn^{++} complex may exist in several forms. This structural approach to determining the cation specificity of enzymes which interact with nucleotides should be the most revealing. In contrast to the other approaches, this one will ultimately have to examine the specificity resulting from contribution of enzyme ligands to the non-phosphate bonded orbitals of these octahedrally co-ordinating metals. The specificity of the apyrase for $Mn^{++} > Ca^{++} > Mg^{++}$ probably results from a balance of mainly two factors: a) the high rate of cation-nucleotide interaction and b) the effective contribution of enzyme ligand groups to the stability of the metal-ion/ nucleotide complex.

5. The True Enzyme Substrate

Cori, et al., report that on the basis of inflection points in pH

vs. K_m or V_m curves, free ATP or ADP are ruled out as substrates for their potato apyrase preparations (12). They suggest that Ca^{++}/ATP and Ca^{++}/ADP could be the more probable substrates. An attempt was made in the present work to see if this could be suggested for the pea seedling apyrase, but apparent V_m values obtained at the various pH have not yielded plots (15, p. 128-145) which would permit meaningful extrapolations.

The Ca^{++}/ATP complex could be designated as the true substrate for the apyrase, if the K_m for the Ca^{++} and the Km for ATP are found to be the same (15, p. 436). It has been found that in malonate buffer at pH 5.5 the apparent Km for ATP = 0.95 mM, while the apparent Km for Ca^{++} = 0.5 mM. While the two values do not coincide, they can be taken to suggest that the true substrate for the pea seedling apyrase is the metal-ion/nucleotide complex.

6. Agents Which Affect Apyrase Activity

The agents which were added to apyrase incubation mixtures were of several types: a) inhibitors of phosphate transfer reactions: NaF (2, 6); b) stimulators of mitochondrial ATPase: oleic acid and DNP (53, p. 56-60): c) potential competitive inhibitors: AMP and adenosine; d) surface acting agent: SDS; and e) plant herbicide: 2,4-D and analog: DCB. Contrary to expectations the effects of the DNP and oleic acid and of the NaF were reversed. Instead of being a stimulant, the oleic acid caused a slight inhibition that suggested surfactant properties analogous to but considerably milder than the SDS, which was very lethal to the activity. The reduction in acitivity by the

presence of AMP and adenosine suggests that the apyrase may be competitively inhibited by its reaction products. The effects of 2,4-D and its analog on purified apyrase appear rather mild.

7. Substrate Specificity

The results indicate the purified apyrase has no phospho-esterase or inorganic pyrophosphatase activity and that the hydrolytic activity seems to be confined to nucleoside di- and tri-phosphates. The limitation of the apyrase to catalyze only the hydrolysis of phosphoric anhydride linkages of nucleotides suggests that this preparation is rather homogeneous functionally. But the facility with which a variety of nucleotides are hydrolyzed may suggest the presence of a heterogeneous nucleotidase population.

That activity towards ADP and ATP is due to one enzyme has been supported mainly by the observed constancy in the ADPase/ATPase ratio over a 38-fold purification procedure. The finding that the ADPase and ATPase activities are not additive also lends support to the notion that one enzyme can hydrolyze both substrates. The observation of different pH optima for ATP and ADP need not imply that two enzymes are present; this will be expanded upon later. The similar requirements for divalent cations in the ATPase and ADPase reactions speak for a single enzyme. A better criteria for deciding the identity of the two enzyme activities would be to compare the apparent molecular weights associated with the activities. If they are the same, this would verify the presence of one enzyme. Gel filtration (3) and sucrose gradient centrifugation (62) would be feasible approaches to

this study. Dixon and Webb (15, p. 202-203) point out that if a constant ratio between the two activities is maintained when the enzyme undergoes inactivation this also would indicate an identity between the two activities. This needs to be done.

Assuming that the diphosphohydrolase and monophosphohydrolase activities reside on one enzyme, there is still the matter of the broad nucleotide specificity which must be settled. Essentially the same sorts of criteria used in judging the identity of the ATPase and ADPase activities are applicable here. At present only the "mixed-substrate" method has been tried (Fig. 16). The non-additivity of activities rules out the presence of independent (and highly specific) enzymes but does not provide conclusive evidence for the presence of only one enzyme, since if several were present each enzyme might be competitively inhibited by a substrate of another (15, p. 203). Hence a more definitive position on the homogeneity of these enzyme preparations requires evidence from other corroborative criteria. For comparisons, it can be noted that Liebecq, et al., (39) observed their potato apyrase preparations to hydrolyze five varieties of nucleoside di- and triphosphates, but ATP was the most active. Raacke (54) has reported the presence of four distinct triphosphatases in pea seedling ribosomal and cytoplasmic fractions, but the criteria for the independency of each activity have not appeared in print.

8. Adenylate Kinase

The kinase activity was examined for two reasons: a) the non-accumulation of ADP as an intermediate in the apyrase catalyzed

hydrolysis of ATP at pH 7.8 could have been due to the presence of a very active adenylate kinase and a slow hydrolase[1], and b) the upward

$$ATP \xrightarrow{\;k^{hyd}\;} ADP \;+\; P_i$$

$$+$$

$$\overset{k^{kin}}{\diagdown} ADP$$

$$AMP \qquad\qquad \text{where } k^{kin} \gg k^{hyd} \qquad\qquad \text{Eq. 14}$$

concave divergency in the double-reciprocal plots for the ADPase reaction (Fig. 14) suggested a $(S)^2$ dependency at low substrate concentrations. The negative results in the ADP equilibrium experiment tend to rule out the presence of the kinase. The fact that P_i release from ATP is slower than from ADP is also in favor of ruling out the enzyme, since a rate determining hydrolysis reaction (k^{hyd}) would predict one common rate of P_i release from both ATP and ADP.

9. Mechanism for Apyrase Action

The preceeding pieces of information and discussion can be brought together in a proposed model for the mechanism of action of apyrases in general (see Fig. 17). The structural details of the enzyme-substrate complex model were derived from suggestions by a) Laidler (36, p. 135-140) on the function of acidic and basic groups at the active site, b) Tetas and Lowenstein (61) on metal-ion/nucleotide interactions for hydrolysis, and c) Kosower (33, p. 254-271) on pyrophosphorolysis and metal-ion catalysis. The model consists of an enzyme with a single active site which contains three functional loci: a nucleosidic binding locus and two pyrophosphorolysis loci. Also

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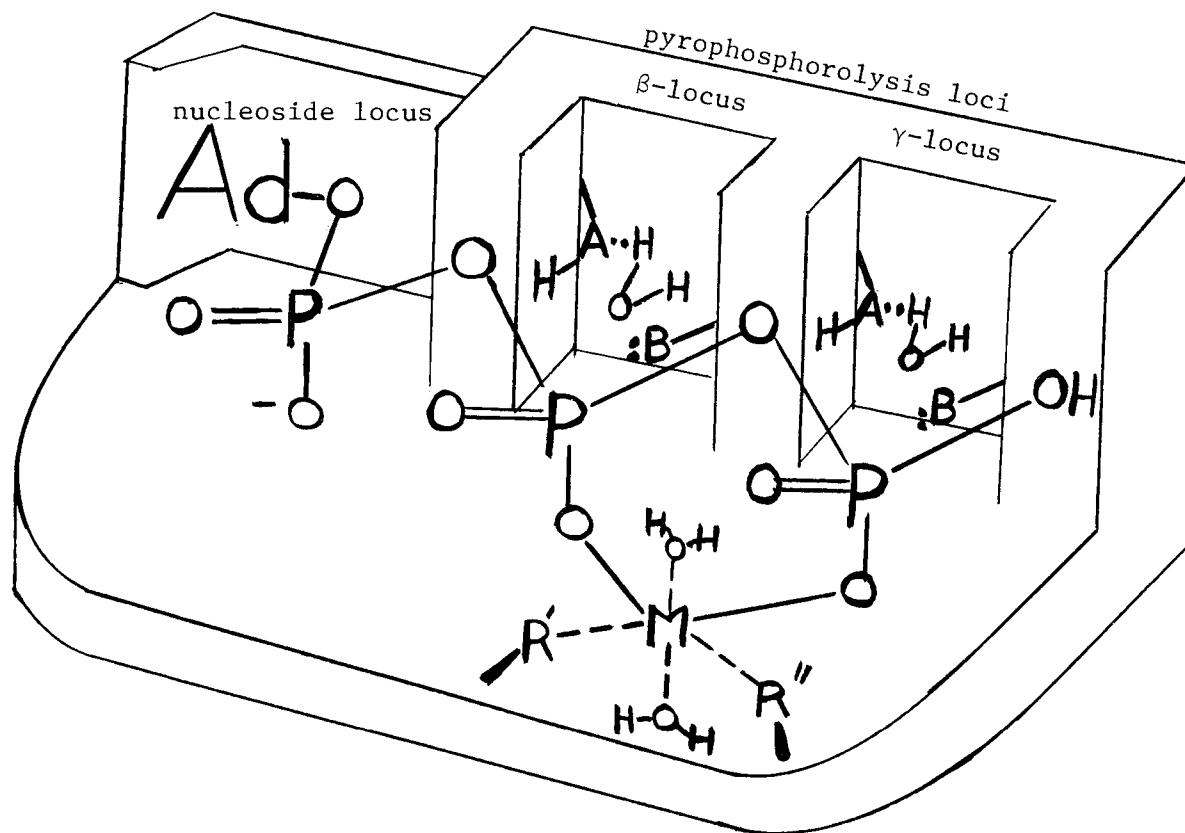[1] Suggested by Dr. 0. Cori in private communication.

Figure 17. A proposed model for the enzyme-substrate complex of apyrase.

present are two minor functional specificities, which are the metal

binding ligands. These groups (R' and R") are postulated to make it

possible for the enzyme to show a divalent cation specificity. The

nucleosidic binding locus will discriminate against non-nucleotide

compounds. But it may be sensitive to competitive inhibition by nuc-

leosides, e.g. adenosine. The pyrophosphorolysis loci may be designa-

ted as β- and γ-loci. The catalytic functional bases contained there-

in may or may not be identical for the two sites. For the pea seedling

it is proposed that the functional base in the β-locus has a pK great-

er than that of the base in the γ-locus. Hence, the rate of the γ-

phosphate liberation does not equal that of the β-phosphate. In the

potato apyrase B of Molnar and Lorand (46) it may be supposed that the

β- and γ- loci contain identical functional bases. Each pyrophospho-

rolysis locus contains an acidic group whose function is to donate

protons at the right time in the reaction and to position a molecule

of water in a suitable geometry to facilitate reaction.

Cohn and Meek (11) were able to show by O^{18} incorporation that

potato apyrase catalyzes a phosphoryl transfer reaction in the hydro-

lysis of ATP. These reactions are typified by the cleavage of the bond

between O and the terminal P. This mode of hydrolysis probably holds

for pea seedling apyrase. Hence this finding is taken into account in

our model. The hydrolytic reaction may be visualized as depicted on

Fig. 18. Cleavage is initiated by the base (B:) attack on the phospho-

rus displacing a pair of electrons to the anhydride oxygen which ab-

stracts a proton from water yielding a hydroxyl ion which in turn dis-

places the base (B:). A phosphoric acid molecule is released when a

rapid shift in the metal-ion coordination is completed.

It is evident that the divalent cation plays two important roles in the mechanism of hydrolysis: a) it orients the pyrophosphoryl linkages into the appropriate geometry for reaction (33, p. 259) and b) it "neutralizes" some negative charges on the terminal phosphate and increases its susceptibility to nucleophilic attack by the base (B:) (33, p. 261).
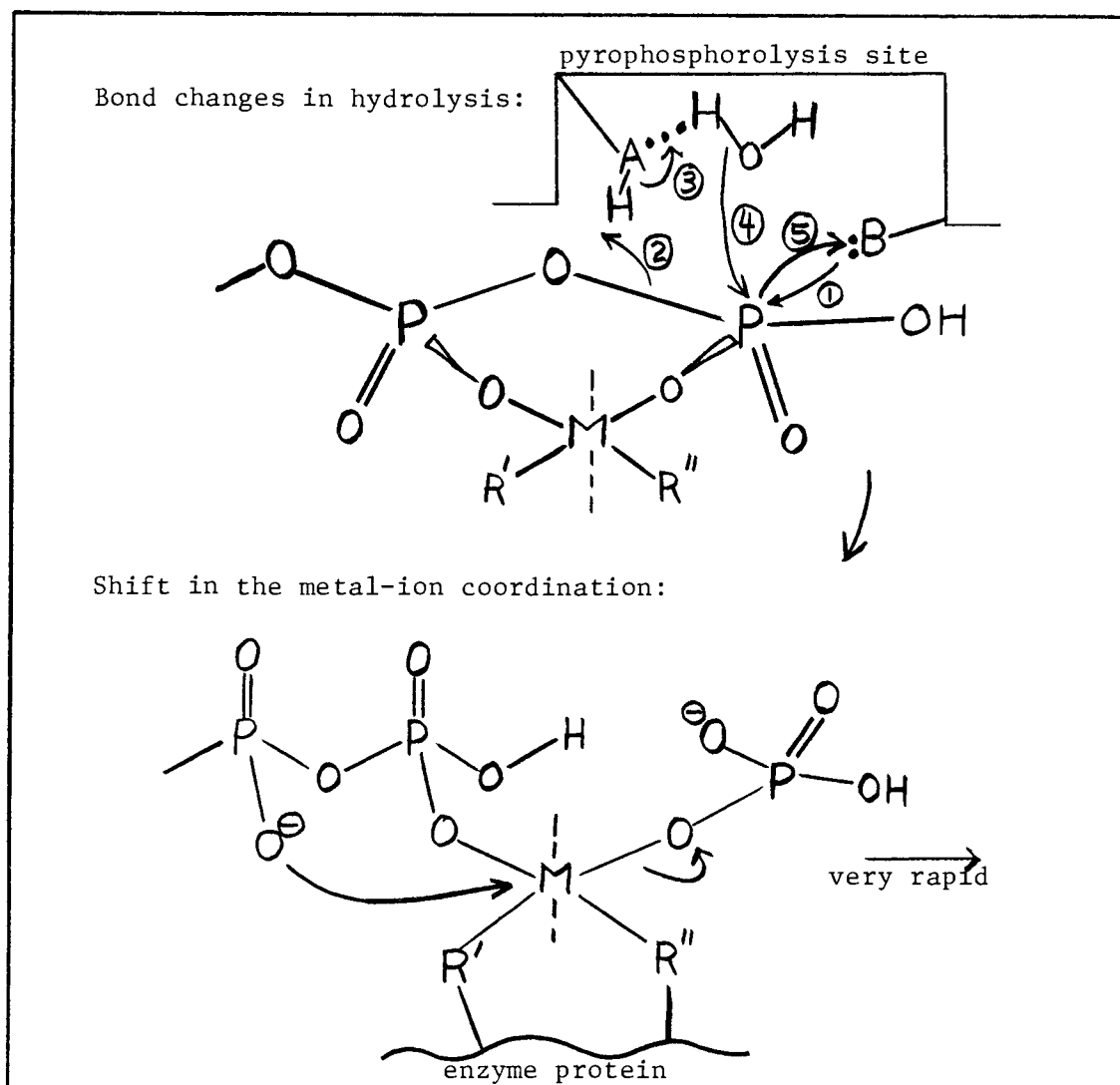

Figure 18. Postulated reaction sequence for ATP hydrolysis.

VI. Summary

Alaska pea seedlings which have been etiolated for 60 hours yield a potent soluble apyrase in the cytoplasmic fraction of the tissue extracts, which were obtained using a hypertonic extracting medium. An ethanol-acetone desiccation procedure was developed to convert the soluble enzymes into a dry powder, which was convenient to accumulate and use for further purification studies.

Ammonium sulfate fractionation (Method a) of acetone powder extracts afforded modest purification (2- to 5-fold) and yield (28 to 64%). Attempts to further purify the ammonium sulfate purified enzyme (AS) on ion-exchange celluloses were frustrated until protamine sulfate was observed to precipitate polyanionic material from the AS preparations and leave the enzyme activity in solution. Chromatography of the protamine treated AS preparations (PS) on carboxymethyl cellulose (CMC) columns with gradient NaCl elution afforded 10- to 14-fold purification and greater than 75% recovery.

The modest results of Method a ammonium sulfate fractionation prompted a study of apyrase purification using high porosity dextran gel bead (Sephadex G-200 and G-75) columns. These mild procedures of molecular sieving coupled with a last step of CMC chromatography afforded an overall yield of 40% and an 88-fold purification. The six days needed for the entire procedure was a deterent to its continued use.

Renewed efforts were made to improve the ammonium sulfate fractionation of the apyrase. The acetone powder solution was directly treated with protamine, then made up to 0.01 M in EDTA. This solution

was constantly monitored at pH 7.8 through three ammonium sulfate steps (Method b). The third (2.7-4.0 molal salt) precipitate yielded the enzyme which was then film dialyzed and chromatographed on CMC. The overall yield (of three steps) ranged 67% to >100% and the degree of purification varied 79- to 100-fold.

The enzyme purified through CMC showed a temperature optimum at 30°C with ATP as substrate. The activity appeared to be rather stable, but wide fluctuations in replicate samples were often noted.

With ATP as substrate the apyrase (ATPase activity) showed a pH optimum at 5.8; the V_m(app) was optimal at pH 6.1. With ADP as substrate the apyrase (ADPase activity) showed a pH optimum at 6.8; the V_m(app) was optimal at pH 7.1. The double-reciprocal plots of the apyrase reaction with ATP showed a deviation at high substrate. On the other hand, with ADP as substrate the plots showed a deviation at low substrate concentration which could correspond to activiation by substrate. The ATPase activity differed from the ADPase activity by showing less activity in Tris-maleate and imidazole buffers.

Divalent cations were required for apyrase activity. The order of effectiveness was the same for both ATPase and ADPase: $Mn^{++} > Ca^{++} > Mg^{++} > Co^{++} > Zn^{++} > Ni^{++}$ except that $Zn^{++} > Co^{++}$ for the ADPase. The purified apyrase showed no phosphoesterase or inorganic pyrophosphatase activity. It was observed that CTP, UTP, ITP and GTP are hydrolyzed faster than ATP. The enzyme was slightly inhibited by DNP, DCB and 2,4-D. NaF was slightly stimulatory. Oleic acid, AMP and adenosine were moderately inhibitory. The apyrase activity was completely abolished by 1 mM SDS.

The ATPase and ADPase activities were co-purified some 38-fold and a "mixed-substrate" reaction shows no additive rates of ATP hydrolysis and ADP hydrolysis. Chromatographic and kinetic data showed that the γ-phospate of ATP was cleaved at a slower rate than the β-phosphate of ADP. This probably holds over the entire pH range where activity is expressed.

In studying the mechanism of apyrase action, the presence of an adenylate kinase type of activity was ruled out since ADP could not be converted to ATP. The multiplicity of nucleoside triphosphate disphos- phohydrolases is not yet conclusively determined. The mixed-substrate experiments only indicated that the rates are not additive when two different nucleotides are present simultaneously.

The action of pea seedling apyrase was summarized by the use of a model. The enzyme contains only one active site for ATP and ADP, but has a different locus and functional group for the hydrolysis of γ- and β-phosphates. The catalytic base in the β-phosphate locus of the enzyme must have a higher pK and greater nucleophilicity than the base in the γ-phosphate locus.

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