A NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE FROM RED BLOOD CELLS OF THE RABBIT

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ABSTRACT

A NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE
FROM RED BLOOD CELLS OF THE RABBIT

By

Ching Jer Chern

A unique nucleoside triphosphate pyrophosphohydrolase catalyzing the hydrolysis of ITP and certain other nucleoside triphosphates with the release of inorganic pyrophosphate and corresponding nucleoside monophosphate as products has been observed in preparations obtained from rabbit reticulocytes. Over 2,000 fold purification of pyrophosphohydrolase from the crude lysates was achieved by a procedure combining ammonium sulfate fractionation, Sephadex G-100 filtration and DEAE cellulose chromatography with a yield of approximately 20%. Later application of preparative disc gel electrophoresis with a 15% acrylamide gel obtained a further 2.8 fold purification of the enzyme from that obtained with DEAE chromatography. Recovery of enzyme activity from the gel electrophoresis step was 40%. Analytical disc gel electrophoresis of this final enzyme preparation indicated one single protein band on polyacrylamide gel at pH 8.7 and two protein components

at pH 5.5. The purified enzyme preparation catalyzed the hydrolytic breakdown of ITP and dITP most actively with the release of PP_i and corresponding nucleoside monophosphate. XTP, UTP, dUTP, GTP and dGTP possessed about 71, 12, 13, 10 and 6%, respectively, of the rate of ITP and dITP hydrolysis. Neither ATP nor dATP could serve as substrates. Marked substrate inhibition was observed. IDP had the most potent inhibitory effect on pyrophosphohydrolase among the nucleotide derivatives tested. Constant ratio of the catalytic rates of ITP/GTP/XTP throughout different stages of the purification, one single pyrophosphohydrolase activity observed in polyacrylamide gels when different substrates by histochemical staining technique and the additive activity assays strongly suggested that pyrophosphohydrolase was a single protein molecule with multiple specificity. The enzyme required magnesium and sulfhydryl compound, and catalyzed the hydrolysis of nucleoside triphosphates at an optimum rate at a pH of 9.75. The apparent Km values for ITP and GTP were estimated at 3.37×10^{-5}M and 4.0×10^{-4}M, respectively. The enzyme has a molecular weight of approximately 37,000 and an isoelectric point of 4.3. Rabbit erythrocytes had levels of activity of pyrophosphohydrolase comparable to that of rabbit reticulocytes, while human erythrocytes possessed one-sixth of the activity of rabbit reticulocytes. No exchange of $^{32}PP_i$ with ITP was detectable with the pyrophosphohydrolase was incubated with ITP

and $^{32}PP_i$. Several hypotheses about the function of pyrophosphohydrolase have been tested.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

IMP, IDP, ITP, inosine 5'-mono, di-, and triphosphates, respectively. GMP, GDP, GTP, guanosine 5'-mono-, di- and triphosphates, respectively. AMP, ATP, adenosine 5'-mono- and triphosphates. UDP, UTP, uridine 5'-di and triphosphates. XTP, Xanthosine triphosphate. CTP, cytidine triphosphate. dITP, dGTP, dCTP, dATP, deoxy-inosine, deoxy-guanosine, deoxy-cytidine, and deoxy-adenosine triphosphates respectively. TTP, thymidine 5'-triphosphate, P_i, PP_i, orthophosphate and pyrophosphate. Tris, tris-(hydroxymethyl)-aminomethane. NAD^+, NADH, nicotinamide adenine dinucleotide and its reduced form. $NADP^+$, NADPH, nicotinamide adenine dinucleotide phosphate and its reduced form. GSH, glutathione. DTT, dithiothreitol. DEAE, diethylaminoethyl. PEI, polyethyleneimine. PPO, 2, 5'-Diphenyloxazole. Dimethyl-POPOP,1,4 bis-(2-(4-methyl-5-phenyloxazolyl))-Benzene. ADP, Adenisine 5'-diphosphate.

INTRODUCTION

Free purines and pyrimidines do not occur in appreciable quantities in blood. They are almost always found in combination with ribose and phosphate as nucleotides. Values for blood nucleotides have been published by several investigators using similar anion-exchange columns (1, 2, 3, 4). Among all the nucleotides, ATP is present in the largest quantity, i.e., around 1,000 µmoles/liter red cells. The concentration of ADP is 10-20% that of ATP and AMP is 1-2%. No IMP is normally found in fresh blood, but is formed promptly at the expense of adenine, guanine or hypoxanthine (5, 6). The only purine other than adenine found in fresh blood is guanine. This is present as GTP although small amounts of GDP and GMP have been reported.

Lowy et al. (7) incubated rabbit erythrocytes with labeled precursors, such as glycine and formate, and were unable to demonstrate incorporation of these precursors into the erythrocyte purines under the conditions that allowed preformed purines to be incorporated into erythrocyte nucleotides. Bishop (5) was similarly unsuccessful in obtaining incorporation of ^{14}C-glycine into

nucleotide, either human blood or chicken blood with its nucleated erythrocytes. However, Lowy and Williams (8) subsequently showed that the mature rabbit erythrocytes could synthesize ATP and GTP from labeled formate in the presence of 5-amino-1-ribosyl-4-imidazolecarboxamide. The in vitro incorporation of labelled formate or glycine into blood purines by the rabbit with a high reticulocyte count was reported (9). The biosynthetic capacity was apparently lost when the reticulocytes matured. Inasmuch as mature anucleate erythrocytes could not completely synthesize purine, and since the red cell nucleotide purines were in a constant state of metabolic turn over, it followed that other tissues must supply these preformed purines to mature red cells.

Lajtha and Vane (84) concluded that in the mammal the liver was the main supplier of purines for bone marrow cells and perhaps for other tissue as well. Henderson and LePage (85) concluded that purines might be transported among mouse tissues by blood cells, adenine being taken up as the blood passes through the liver and later released in other tissues.

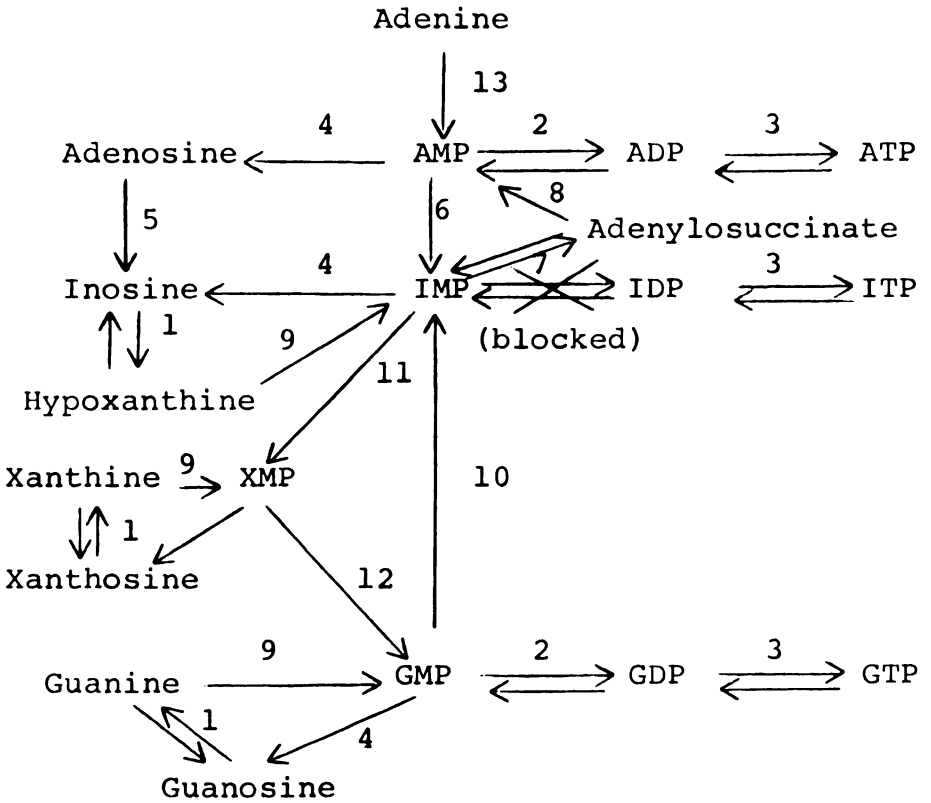
Preformed purines such as adenine, guanine, and hypoxanthine are readily incorporated into red cell nucleotides, implying that these nucleotides are building up and breaking down to purines continuously. Free adenine is rapidly incorporated into adenine nucleotides, free

hypoxanthine, xanthine, or guanine is preferentially incorporated into guanine nucleotides (essentially GTP) (5, 7, 8, 10, 11). Hershko et al. (6) have reported that hypoxanthine appears in the rabbit erythrocyte cells in the form of IMP. The divergent incorporation pattern of hypoxanthine was attributed to the absence of a nucleoside monophosphate kinase capable of catalyzing the phosphorylation of IMP to its diphosphate derivative. The absence of ATP:IMP phosphotransferase activity in the calf liver and pig kidney cortex has also been noted (12, 13).

Hypoxanthine and xanthine proved to be the major purine bases released from red cells, irrespective of the nature of the (8-^{14}C) purine used for labeling the cells. The extracellular hypoxanthine originated from IMP arising within the cell by deamination of AMP and GMP, while xanthine derived from the excess of XMP which failed to convert to GMP. The breakdown of ATP was reported to stop at hypoxanthine (86), because there was no xanthine oxidase present in the blood. These purine bases may be transported by blood cells to other tissues and catabolized.

When nucleosides instead of free bases were used for incorporation studies, the results were essentially the same as with the free bases except in the case of adenosine. This nucleoside was deaminated so quickly that it behaved like hypoxanthine or inosine. The pathways of

purine nucleotide metabolism may be summarized as follows (6):



The numbers represented different enzymes: (1) nucleoside phosphorylase, (2) nucleoside monophosphate kinase, (3) nucleoside diphosphate kinase, (4) 5'-nucleotidase, (5) adenosine deaminase, (6) adenylate deaminase, (7) adenylosuccinate synthetase, (8) adenylosuccinase, (9) nucleoside pyrophosphorylase (IMP or GMP: pyrophosphate phosphoribosyl transferase), (10) GMP reductase, (11) IMP dehydrogenase, (12) XMP aminase, (13) nucleoside pyrophosphorylase (AMP: pyrophosphate phosphoribosyl transferase).

The proper partition of IMP between the competing biosynthetic and catabolic pathways, as well as the coordination of the relative rates of purine assimilation and excretion appeared to be insured by a number of regulatory mechanisms known to be operative along the different enzymatic pathways such as nucleoside pyrophosphorylases (14), adenylosuccinate synthetase (15), IMP dehydrogenase (16), and GMP reductase (16).

Not all of the enzymes in the pathways have been well characterized in red cells. Among other enzymes in relation to purine nucleotide metabolism in blood are nucleoside triphophatase (17, 6), nucleoside diphosphatase (18), adenylate kinase (myokinase) (19), and nucleoside kinase, which phosphorylated inosine to IMP with ATP (18).

During the course of the investigations of nucleotide metabolism by cell free preparation of rabbit reticulocytes in our laboratory, a unique nucleoside triphosphate pyrophosphohydrolase has been detected.

The enzyme was found to catalyze the breakdown of nucleoside triphosphates to pyrophosphate and corresponding nucleoside monophosphates, by the observations of the ability of the reaction product of nucleoside triphosphate hydrolysis to produce NADPH by coupling with UDP-glucose-pyrophosphorylase coupled assay system and the identification of nucleoside monophosphates on paper chromatograms. The observations were further confirmed by the analysis of

reaction products and stoichiometry of the reaction by Dowex-I resin chromatography.

The enzyme was shown to catalyze the following reaction:

$$NTP \xrightarrow{H_2O} NMP + PP_i$$

(N: Purine or pyrimidine nucleoside)

The name of nucleoside triphosphate pyrophosphohydrolase was therefore assigned for this enzyme, which may relate to the metabolism of purine nucleotides in red blood cells.

Pyrophosphohydrolase is highly active in catalyzing the breakdown of ITP and dITP. XTP is hydrolyzed at a lesser rate while GTP, dGTP, UTP, and dUTP have approximately 10% or less of the activity of ITP or dITP. ATP and dATP were not substrates of pyrophosphohydrolase.

The detection and partial purification as well as some properties of pyrophosphohydrolase have been reported in a recent publication (20). Further purifications of pyrophosphohydrolase, studies of certain kinetic parameters, isoelectric point determination, and other studies have been more recently carried out.

The biological function of pyrophosphohydrolase is still not defined at this stage of study. As detailed in the section under "Discussion," it may be related to the recent findings of small amounts of ITP present in

human erythrocytes (76, 77) and of a relatively high concentration of ITP in human erythrocytes of two siblings, which has been attributed to a genetic trait (77, 82).

MATERIALS AND REAGENTS

I. Reagents

Ribonucleoside mono-, di-, and triphosphates and deoxyribonucleoside triphosphates (except deoxyinosine triphosphate which was a gift of Dr. Fred J. Bollum, Department of Cell Biology, University of Kentucky, Lexington, Kentucky) were purchased from P-L Biochemicals, Milwaukee, Wisconsin. Streptomycin sulfate, U.S.P. (0.740 mg/mg), was from General Biochemicals, Chagrin Falls, Ohio. Analytical reagent grade, pyridine-free ammonium sulfate was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri. Dithiothreitol (Cleland's reagent) was purchased from Calbiochem., Los Angeles, California. Yeast inorganic pyrophosphatase (800 units per mg) was acquired from Nutritional Biochemical Corporation, Cleveland, Ohio. $Na_4{}^{32}P_2O_7$ (inorganic pyrophosphate) was purchased from New England Nuclear Corp., Boston, Mass. Guanosine-5'-mono- and triphosphates-8-(^{14}C), inosine-5'-mono- and triphosphates-8-(^{14}C) were obtained from Schwarz Bio-Research, Inc., Orangeburg, New York. Carrier free inorganic phosphate (^{32}P) was from Tracerlab, Waltham, Massachusetts. Ampholine, carrier Ampholytes was from

LKB-Produkter AB, Bromma, Sweden. The acrylamide and compounds for polymerization of the gels were purchased from Canal Industrial Corporation, Rockville, Maryland. 2-Mercaptoethanol was from Eastman Organic Chemicals, Rochester, New York. Dialysis tubing from Visking Company, Chicago, Illinois was prepared for use according to the procedure of Peterson and Chiazze (21). Sephadex gels and Sephadex columns were acquired from Pharmacia Fine Chemical Inc., Piscataway, New Jersey. Cellex-D (DEAE cellulose), Bio gel HTP (hydroxylapatite) and Dowex-I-resin were obtained from Bio-Rad Laboratories, Richmond, California. Nembutal was from Abbott Laboratory, North Chicago, Illinois. Heparin was purchased from Fisher Scientific Company, Chicago, Illinois. Phenylhydrazine hydrochloride and β-alanine were from Distillation Products Industries, Rochester, New York. Liquifluor (PPO + POPOP), PPO (2,5 Diphenyloxazole), Dimethyl-POPOP {1, 4 bis-[2-(4-Methyl- 5-Phenyloxazolyl)]}-Benzene were from New England Nuclear Corp., Boston, Mass. or Packard Instrument Co., Inc., Downers Grove, Ill. Nitrocellulose membranes were purchased from Carol Schleicher and Schuell Co., Keene, New Hampshire. Polyethyleneimine cellulose-coated plastic sheets were acquired from Brinkmann Instruments, Inc., Westurg, N. Y. Norit A was obtained from Fisher Scientific Company, Fair Lawn, New Jersey. Whatman No. 1 chromatography paper was from W & R Balston Ltd., England. UDP-glucose pyrophosphorylase was a gift of Dr. Hansen,

Department of Biochemistry, Michigan State University, East Lansing, Michigan. All other compounds or enzymes were purchased from Sigma Chemical Co., St. Louis, Missouri.

II. Biological Materials

A. Preparation of Crude Enzyme from Reticulocytes (22, 23)

New Zealand White male rabbits were made reticulocytic by 4 daily subcutaneous injections of 2.5% neutralized phenylhydrazine. After 2 days of rest, the animals were injected intravenously with a solution containing 2,000 I.U. of heparin and 100 mg of Nembutal. Blood was collected by heart puncture. The plasma was separated and decanted from red blood cells by centrifugation at 2,000 xg for 20 min. The cells were washed twice by suspension in NKM solution (a solution containing 0.13 M NaCl, 0.005 M KCl and 0.0075 M $MgCl_2$) followed by centrifugation. The packed cells were lysed by adding 4 volumes of 0.0025 M $MgCl_2$ followed by gentle stirring for 10 min. The cell debris were removed by centrifugation at 15,000 xg for 20 min. The supernatant was then subjected to centrifugation at 78,000 xg for 90 min. to spin down reticulocyte ribosomes. The high speed supernatant fraction so obtained, was then used as the starting material from which nucleoside triphosphate pyrophosphohydrolase was purified.

B. Preparation of γ-IT^{32}P

^{32}P labeled ITP was required for the assay in the phosphate buffer of pyrophosphohydrolase activity after hydroxylapatite chromatography and other analysis. However, it is rather expensive when obtained commercially. Efforts were therefore made to work out a method for the synthesis of γ-labeled IT^{32}P.

A study of the chromatographic separation of ATP and ITP was carried out first. These compounds were observed to resolve well one from the other by Dowex-I resin chromatography during elution with 0.1 N HCl. Conditions for the deamination of ATP were studied initially by using unlabeled ATP as substrate. The analysis of the deaminated product was performed, again, by Dowex-I chromatography. The results revealed that ATP was deaminated quantitatively with nitrous acid to ITP. Synthesis of γ-labeled ITP was therefore carried out by labeling of ATP according to the method of Glynn and Chappell (24) and deamination of labeled ATP to yield γ-IT^{32}P.

1. Synthesis of γ-labeled AT^{32}P γ-AT^{32}P was prepared according to the procedure of Glynn and Chappell (24). The labeling of ATP by this procedure is dependent on the isotopic exchange between $^{32}P_i$ and the γ-phosphate of ATP which is catalyzed by the enzymes phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase. The following solutions were pipetted into a 10 ml beaker:

0.50 ml of 1 M Tris-Cl pH 8.0, 0.03 ml of 1 M $MgCl_2$, 0.50 ml of 0.1 M NaOH, 0.80 ml of Na_2ATP (60 µmoles/ml), 0.20 ml of 3-phosphoglycerate (50 µmoles/ml), 0.10 ml of 0.02 M NAD^+, 0.20 ml of 0.05 M cysteine-HCl (freshly prepared), 2.00 ml H_2O and 0.10 ml of carrier-free $^{32}P_i$ (5 mc/ml). After the solutions were mixed, 10 units of 3-phosphoglyceryl kinase and 1 mg glyceraldehyde-3-phosphate dehydrogenase were added and the mixture was incubated at room temperature overnight. $AT^{32}P$ was estimated by Cerenkov Radiation Method (25, 26), after the partition of $^{32}P_i$ into isobutanol-benzene phase as described by Lindberg and Ernster (27).

2. Deamination of γ-$AT^{32}P$ The reaction mixture containing γ-$AT^{32}P$ so obtained, was brought to 2 N in HCOOH and then, 800 mg $NaNO_2$ in 3 ml of aqueous solution was added into dropwise. The reaction mixture was allowed to stand at room temperature for about 4 hrs. and neutralized to pH 7.0 with NaOH. After the addition of 0.6 ml of 25% $BaCl_2$ and one volume of alcohol, the barium salt of ITP which formed was centrifiged and washed with alcohol. Conversion of the barium salt of ITP to the sodium salt was carried out by dissolving of the Ba salt of ITP in least volume of 0.01 N HCl followed by the addition of Na_2SO_4. The $BaSO_4$ which formed was removed by centrifugation. The concentration of NaCl in the supernatant was maintained below 0.01 N by dilution with water. The sample

was then applied to a Dowex-I column, washed and eluted with 0.1 N HCl as described under the section of "purification of commercial ITP and GTP" in "Analytical Method." A recording ultraviolet analyzer and a radioactivity rate-meter were connected to the column to monitor the effluent absorbance at 254 mμ and the radioactivity at the same time.

The results indicated that γ-labeled $AT^{32}P$ was converted completely to γ-$IT^{32}P$ in good yield.

ANALYTICAL METHODS

I. Purification of Commercial ITP and GTP

Chromatographic analysis of control reaction mixtures during the study of ITP hydrolysis by pyrophosphohydrolase (see Figure 21B) revealed the presence of significant amounts of degradation products in the commercial nucleoside triphosphate preparation, notably IDP, PP_i, and P_i. In addition, nucleoside tetraphosphates have been identified and isolated from commercial nucleoside triphosphates by Garden (71), and Vanderheiden (28).

In view of the inhibitory effect of IDP, IMP and possibly, P_i and PP_i, it becomes necessary to rigidly purify the nucleoside triphosphates obtained from the commercial sources for the kinetic study of ITP or GTP hydrolysis.

Purification of the nucleoside triphosphates used as substrates for the kinetic studies requiring rigidly purified materials was performed by chromatography in the same system used for chromatographic analysis of the reaction products (20). Commercial ITP or GTP in aqueous solution was applied to a Dowex-I column (1 x 10 cm bed volumn), 2% cross-linked, 100 to 200 mesh, and eluted with

0.1 N HCl. Chromatography was carried out at 4°C. Fractions containing nucleoside triphosphates were pooled and concentrated with Norit A at 4°C as follows: a 0.1 ml portion of a 20% suspension of acid washed Norit A per μmole of nucleoside triphosphate was added to the pooled fractions and stirred for 5 min. The Norit was collected by centrifugation, washed with cold water and eluted with 0.05 M NH_4OH in 50% ethanol. The combined elutes were filtered through a nitrocellulose membrane filter to remove remaining traces of Norit and lyophilized to dryness. The sample was redissolved in water and filtered again through the nitrocellulose membrane filter before use in order to remove trace amounts of colloidal charcoal.

The purified preparation of ITP or GTP from Dowex-I resin chromatography was examined on PEI (polyethyleneimine) thin layer developed with 1.6 M LiCl for purity by comparison to reference standards. A preparation free of any detectable nucleotide impurities was obtained by the above procedure.

II. Assay of Nucleoside Triphosphate Pyrophosphohydrolase

The assay for pyrophosphohydrolase activity was performed by using a coupled assay system which included added yeast inorganic pyrophosphatase in order to hydrolyze the inorganic pyrophosphate produced in the reaction to inorganic phosphate. The enzyme fractions were routinely incubated in a reaction mixture (1.0 ml) containing 50 mM

β-alanine buffer (pH 9.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 unit of yeast inorganic pyrophosphatase and 0.5 mM ITP. The reaction was initiated with the addition of either the substrate or the pyrophosphohydrolase solution and incubated at 37°C for 20 min. A reaction mixture lacking pyrophosphohydrolase was used as control in order to correct for small amounts of P_i, and PP_i found in commercial preparations of nucleoside derivatives used as substrates and inhibitors throughout this study. Following incubation, 0.1 ml of 50% (w/v) TCA (trichloroacetic acid) was added to stop the reaction at 4°C. The precipitate which formed (if present) was removed by centrifugation at 2,000 xg for 5 min. The supernatant was analyzed for inorganic phosphate by the procedure of Martin and Doty (29) as adapted for animal materials by Ernster, Zettersterom and Lindberg (30). Inorganic pyrophosphate produced in the reaction was estimated from the inorganic phosphate analyzed by comparison to a standard KH_2PO_4 solution.

III. Protein Determination

Protein concentration was determined by the method of Lowry *et al.* (31). Crystallized bovine serum albumin was used as a reference standard.

IV. Rate Measurements (Km and Vm Determination)

The same coupled assay system as used in the enzyme assay was applied for the determination of the Km values

for ITP and GTP. The reaction mixture (1.0 ml) contained 50 mM β-alanine buffer (pH 9.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 unit of yeast inorganic pyrophosphatase, various concentration of the purified ITP or GTP and the pyrophosphohydrolase. The concentration of pyrophosphohydrolase for the system using GTP as substrate was approximately 20 times greater than that for the system using ITP as substrate. In order to obtain a measure of the true initial velocity of the reaction, reactions were started with the addition of pyrophosphohydrolase and incubated at 37°C for 3.5 min. in the ITP system and for 15 min. in the GTP system.

V. Analytical Disc Gel Electrophoresis

Disc gel electrophoresis was performed by the method of Davis (32). The acrylamide and bisacrylamide were recrystalized from chloroform and acetone, respectively, according to the method of Loening (33). The 7% polyacrylamide gel was prepared as follows: one part solution A [1 N HCl, 48 ml; Tris, 36.6 g; TEMED (N, N, N', N'-tetramethylenediamine), 0.23 ml, water to 100 ml] was mixed with 2 parts of solution C [Acrylamide, 28 g, BIS (bisacrylamide), 0.735 g; H_2O to 100 ml] and 1 part H_2O (including dithiothreitol or 2-mercaptoethanol, in order to make a gel containing 10^{-3} M dithiothreitol or 2-mercaptoethanol). The polymerization reaction was initiated with 4 parts of catalyst (ammonium persulfate,

0.2 g; H_2O to 100 ml) at 4°C. The gel solution was then carefully covered with small volume of H_2O. A gel bed of 0.6 x 6 cm of 7% acrylamide containing 10^{-3} M dithiothreitol or 2-mercaptoethanol was therefore prepared. In some cases, a stacking gel (large pore gel) with a height of one-fourth of that of running gel was polymerized on the top of the running gel. One part solution B (1 N HCl, 48 ml; Tris, 5.98 g; TEMED, 0.46 ml; H_2O to 100 ml) was mixed with 2 parts of solution D (Acrylamide, 10 g; BIS, 2.5 g; H_2O to 100 ml), 1 part E (riboflavin, 4 mg; H_2O to 100 ml) and 4 parts solution F (sucrose, 40 g; H_2O to 100 ml). The protein sample in 2.5% of sucrose or glycerol was loaded on the top of the gel. The gels were subjected to a current of 5 ma per gel cylinder for 1 hr. with 0.025 M Tris-glycine (pH 8.7) containing 10^{-3} M 2-mercaptoethanol as electrode buffer. Gel electrophoresis also was carried out according to the method of Gordon and Louis (34) using 0.05 M Borate at pH 9.2 as electrode buffer.

Electrophoresis at pH 5.5 (0.3 M β-alanine-acetate as electrode buffer) carrying out in 7% gel for 1 hr. at 5 ma per gel was a modification of the procedure by Reisfeld *et al.* (35). A 7% polyacrylamide gel containing 10^{-3} M 2-mercaptoethanol was prepared by mixing one part solution A' (17.2 ml glacial acetic acid; TEMED, 4.0 ml and titrated to pH 5.5 with KOH in 100 ml solution) with 2 parts of solution C, 1 part of H_2O and 4 parts of catalyst as described above.

After electrophoresis, gels were stained for proteins either with 1% Amido-Schwarz in 7% acetic acid for 1 hr. and destained by electrophoresis, or with Coomassie brilliant blue in trichloroacetic acid for 1 hr. as described by Chrambach et al. (36).

VI. Localization of Pyrophosphohydrolase in Polyacrylamide Gels

In order to examine the question of the number of nucleoside triphosphate pyrophosphohydrolase enzyme present in the reticulocytes, a histochemical identification of pyrophosphohydrolase activity in polyacrylamide gel was developed using the lead conversion methods by Gomori (37, 38, 39, 40, 41, 42, 43).

After electrophoresis, the pyrophosphohydrolase was first allowed to react with ITP or GTP by incubation of the gel in a solution containing 50 mM β-alanine (pH 9.5), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM ITP or GTP and 15 mM $CaCl_2$ for 1-3 hrs. at 37°C. The white color of Ca-inorganic pyrophosphate precipitate formed in the "substrate gel" by the action of inorganic pyrophosphate released from ITP or GTP hydrolysis intensified after one hour of incubation in the presence of $CaCl_2$. The Ca-inorganic pyrophosphate, in some cases, was then converted to Pb-inorganic pyrophosphate precipitates by rinsing the gel with water and then immersing the substrate gel in a solution of 0.08 M Tris-maleate buffer (pH 7.0) containing 3 mM $Pb(No_3)_2$ at room temperature for 30 min.

The pryophosphohydrolase active zone could also be stained yellow with triethylamine-molybdate reagent (44, 45). The gels were incubated in the reaction mixture of the standard enzyme assay (including yeast inorganic pyrophosphatase for this latter procedure) for a short time period (5-20 min.), rinsed with water, and immediately immersed in triethylemine-molybdate reagent (5 mM triethylamine-HCl, 4 mM ammonium molybdate in 0.1 N perchloric acid). This method, however, is less useful than Gomori's method because of easy diffusion of inorganic phosphate formed in the gel which is surrounded with excess yeast inorganic pyrophosphatase.

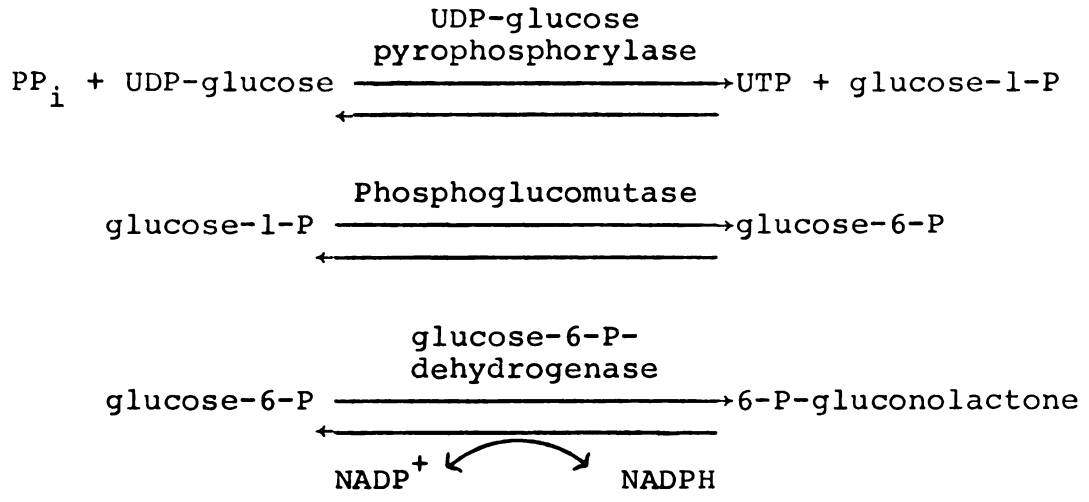
VII. Characterization of Reaction Products of ITP Hydrolysis

A. Identification of IMP by Paper Chromatography and PP_i by UDP-glucose Pyrophosphorylase Coupling Assay

1. Paper chromatography The identity of nucleotides was confirmed by comparison to reference standard on a Whatman No. 1 paper developed with isobutyric acid: $NH_4OH:H_2O$ (57:4:39) for 20 hrs. by descending chromatography. Nucleotides were identified by U.V. absorption under a U.V. lamp.

2. UDP-glucose pyrophosphorylase coupling assay (46) The PP_i released from ITP hydrolysis by nucleoside

triphosphate pyrophosphohydrolase was determined by the following coupled enzyme reactions:



The reaction mixture contained in 0.5 ml:25 μmoles Tris-Cl buffer (pH 9.0), 2 μmoles $MgCl_2$, 0.5 μmoles dithiothreitol, 0.2 μmoles $NADP^+$, 0.5 μmoles UDP-glucose, 0.2 μmoles nucleoside triphosphate, nucleoside triphosphate pyrophosphohydrolase (supernatant of the heated gel purified enzyme, which lacked the heat labile inorganic pyrophosphatase activity), excess phosphoglucomutase and glucose-6-P dehydrogenase and enough UDP-glucose pyrophosphorylase, so that reaction rate was approximately 1 O.D. of A_{340} per 10 min. The reaction was initiated with the addition of pyrophosphohydrolase or $NADP^+$.

B. Identification of Reaction Products by Dowex-I Chromatography

1. Dowex-I chromatography

Chromatographic analysis of the reaction products was carried out by using

a modification of the procedure of Zimmerman and Kornberg (47). A 1 x 10 cm Dowex-I resin (2% cross linkage, 100-200 mesh, chloride form) column was prepared and washed with water. A recording ultra-violet analyzer (Instrument Specialties Company, Inc., Lincoln, Nebraska) was connected to the column, in order to monitor the effluent absorbance at 254 mμ. After the application of sample to the column, the elution was started with 0.02 N HCl. A volume of 7 ml per fraction was collected until P_i and IMP were eluted from the column. The eluant was then changed to 0.1 N HCl and elution continued until PP_i, IDP, and ITP emerged from the column. Nucleotides in the eluate fractions were identified by comparison to reference standards using either paper chromatography, as described above, or PEI cellulose thin layer chromatography.

2. PEI (polyethyleneimine) cellulose thin layer chromatography Ion-Exchange chromatography of nucleotides on PEI cellulose thin layers was performed according to the method of Randerath and Randerath (48). A 20 x 20 cm PEI cellulose precoated plastic sheet (layer: 0.1 mm, cellulose MN 300 polyethyleneimine impregnated) was washed with water previously by ascending chromatography. The samples and reference standards were applied to the prewashed and dried PEI cellulose layer and developed with 1.6 M LiCl for 2 hrs. by ascending chromatography. Nucleotides were visualized with the aid of an ultra-violet lamp.

The identity of the pyrophosphate peak was confirmed by the action of yeast inorganic pyrophosphatase (49) in producing measurable inorganic phosphate.

After localization of each compound in different regions of the eluate fractions, appropriate regions were pooled and the total amount of each material was determined. The absorption at 248.5 mμ in 0.1 N HCl of fractions containing each nucleotide was measured and compared to ITP in 0.1 N HCl as a reference standard.

VIII. Sucrose Density Centrifugation

The sedimentation coefficient of nucleoside triphosphate pyrophosphohydrolase was determined by the method of Martin and Ames (50). Linear sucrose gradients of from 5 to 20% sucrose containing 0.05 M Tris-Cl (pH 7.0), 10^{-3} M $MgCl_2$ and 10^{-3} M glutathione in a volume of 5 ml was prepared in the cellulose nitrate tubes. A sample of 0.1 ml in the same buffer was applied to the top of the gradient and centrifuged at 50,000 r.p.m. for 16 hrs. at 4°C. Gel purified enzyme and the purified enzyme from DEAE cellulose chromatography were analyzed and compared. Rabbit hemoglobin (51) and pancreatic DNAse I (52) were used as reference markers. After centrifugation, contents of the tube were analyzed for their activities by puncturing the bottom of the tube. A volume of 0.19 ml per fraction was collected. Hemoglobin was measured by the absorption at 415 mμ. DNAse I activity was based upon the increase of

U.V. absorption at 260 mμ observed during the course of depolymerization of DNA by DNAse I according to the method of Kunitz (53).

IX. Molecular Weight Determination by Gel Filtration

Molecular weight determination of the purified enzyme was performed by the method of Andrews (54). A 2.5 x 46 cm column of Sephadex G-100 was prepared and equilibrated with 0.05 M Tris-Cl (pH 7.0) containing 10^{-3} M dithiothreitol. Cytochrome C and rabbit hemoglobin, which could be detected spectrally, were used as reference markers. Samples were dissolved in 2 ml of the elution buffer containing 2.5% sucrose and applied to the top of the column by layering under the solution already present. A volume of 3 ml column effluents per fraction was collected with a constant flow rate of 0.3 ml per min. All experiments were carried out in the cold. Hemoglobin and cytochrome C were determined by absorption at 415 mμ and the order of their elution from the column. Pyrophosphohydrolase activity determination followed the procedure of the standard assay.

X. Isoelectric Point Determination

The isoelectric pH of the nucleoside triphosphate pyrophosphohydrolase was determined by the technique of isoelectric focusing which has been previously described by Svensson (55, 56). The determination was made in a

110 ml column containing a sucrose density gradient, which was prepared by layering of 22 to 24 fractions of mixed solution from the aliquots of different proportion of dense solution (diluted 1.9 ml of carrier ampholytes (40% W/V) to 42 ml with distilled water, and dissolved 28 g of sucrose in the solution) and less dense solution (diluted 0.6 ml of carrier ampholytes (40% W/V) to 60 ml with distilled water). Two different pH ranges (pH 3 to 10, and pH 3 to 6) of ampholines, carrier ampholytes, were used in these experiments. Anode solution (0.1 ml of concentrated sulphuric acid diluted with 10 ml H_2O) was loaded on the top of the column. Cathode solution (0.4 ml of ethanolamine diluted with 14 ml of distilled water in which 12 g of sucrose was then dissolved) was layered at the bottom of the column. The enzyme was applied in a concentrated band after the column was approximately one-half filled. The column was cooled to 2°C with a circulating water bath. For a pH range of 3 to 10, the potential of 300 volts was maintained throughout the run for 48 hrs. The potential was raised stepwise to 700 volts for the narrower range of pH 3 to 6. The experiment took about 72 hrs. After the run, 2 ml per fraction was collected by gravity. Aliquots of the fractions were analyzed for pyrophosphohydrolase activity.

XI. Strip Paper Electrophoresis

Whatman No. 1 paper electrophoresis of the most purified enzyme was performed at 200 volts or 400 volts for various time periods, with 0.15 M β-alanine-acetate and maleic-KOH buffers (pH 5.5) containing 10^{-3} M 2-mercaptoethanol or with 0.17 N acetate-KOH buffer (pH 5.5) containing 10^{-3} M 2-mercaptoethanol as electrode buffers. After electrophoresis, the paper was dried and dipped into the ninhydrin solution. The ninhydrin positive spots were developed at 60°C.

XII. Attempted Binding of ITP-8-(^{14}C) to Pyrophosphohydrolase

The purified enzyme from DEAE cellulose chromatography was added to the standard enzyme assay mixture containing 1 μC ITP, but without yeast inorganic pyrophosphatase and incubated at 37°C for different time periods (from 0 to 20 min.). The reaction mixture was then applied to a Sephadex G-25 column (1 x 20 cm). Previously equilibrated with 0.05 M β-alanine buffer (pH 9.5), 10^{-3} M dithiothreitol. The column was analyzed for pyrophosphohydrolase activity and radioactivity.

The technique of nitrocellulose membrane filtration was also employed to measure the binding of enzume with substrate. TCA precipitates of the reaction mixture were filtered through a nitrocellulose membrane filter. The material retained on the nitrocellulose membrane was washed

several times with the same buffer. The membrane was then dried and radioactivity determined in 15 ml of scientillation fluid (0.5% PPO, 0.01% POPOP in toluene) with a Nuclear Chicago Counter.

RESULTS

I. Purification of Nucleoside Triphosphate Pyrophosphohydrolase

A. Streptomycin Sulfate Treatment

Streptomycin sulfate was used to get rid of trace amounts of nucleic acid present in the high speed supernatant.

To the high speed supernatant obtained from the reticulocyte lysates of three rabbits as described under the section of "Biological Material" was added a 100 mg/ml solution of streptomycin sulfate to make a final concentration of 2.8 mg/ml. After 30 min. of gentle mixing, the precipitate which had formed were removed by centrifugation at 10,000 xg for 20 min. The supernatant was then dialyzed against 15 volumes of 50 mM Tris-Cl (pH 7.0), 1 mM $MgCl_2$ and 1 mM GSH for at least 24 hrs. The dialysate was replaced twice during the dialysis. As shown in Table 1, this treatment did not increase the specific activity although removal of some precipitate, presumedly nucleic acid, was observed.

TABLE 1.--Purification of nucleoside triphosphate pyrophosphohydrolase.

Fraction	Total Protein	Total Activity*	Specific Activity	Recovery
	mg	μmoles	μmoles/mg protein	%
Crude Lysate	14,800	3,330	0.224	100
High Speed Supernatant	10,200	2,780	0.273	84
Dialyzed Supernatant	10,100	2,760	0.273	83
40-70% Ammonium Sulfate	300	1,860	5.63	56
Sephadex Column	86.3	1,420	16.4	43
DEAE-Cellulose Column	1.19	630	529	19

*Activity expressed as μmoles of PP_i liberated from ITP per 20 min. under standard assay conditions (see Methods).

B. Ammonium Sulfate Fractionations

In view of the high quantity of hemoglobin present in the high speed supernatant, fractionation on the basis of differential solubilities in ammonium sulfate solutions was employed to achieve an initial fractionation of the enzyme from the hemoglobin present. The dialyzed supernatant was brought to a final concentration of 0.1 M Tris-Cl by the addition of 1 M Tris-Cl (pH 7.5). The solution was then titrated to pH 6.5 with 1 N acetic acid. Powdered ammonium sulfate was added slowly to 40% of saturation over a period of 30 min. The solution was stirred gently for at least 30 min. and the precipitate was removed by centrifugation at 10,000 xg for 20 min. The supernatant was brought to 70% of saturation by the slow addition of powdered ammonium sulfate. After more than 30 min. of stirring, the precipitate containing nucleoside triphosphate pyrophosphohydrolase was harvested by centrifugation as before.

An additional treatment was applied to some preparations. The 40 to 70% ammonium sulfate precipitate as obtained in the above procedure was dissolved in 50 ml of 0.1 M Tris-Cl (pH 7.5), 1 mM $MgCl_2$ and 1 mM GSH and titrated to pH 6.5 with 1 N acetic acid. The solution was brought to 70% of saturation by the addition of powdered ammonium sulfate. After 30 min. of stirring, the washed precipitate was collected by centrifugation and dissolved in

approximately 10 to 20 ml of 50 mM Tris-Cl (pH 7.0) buffer containing 1 mM $MgCl_2$ and 1 mM GSH for gel filtration.

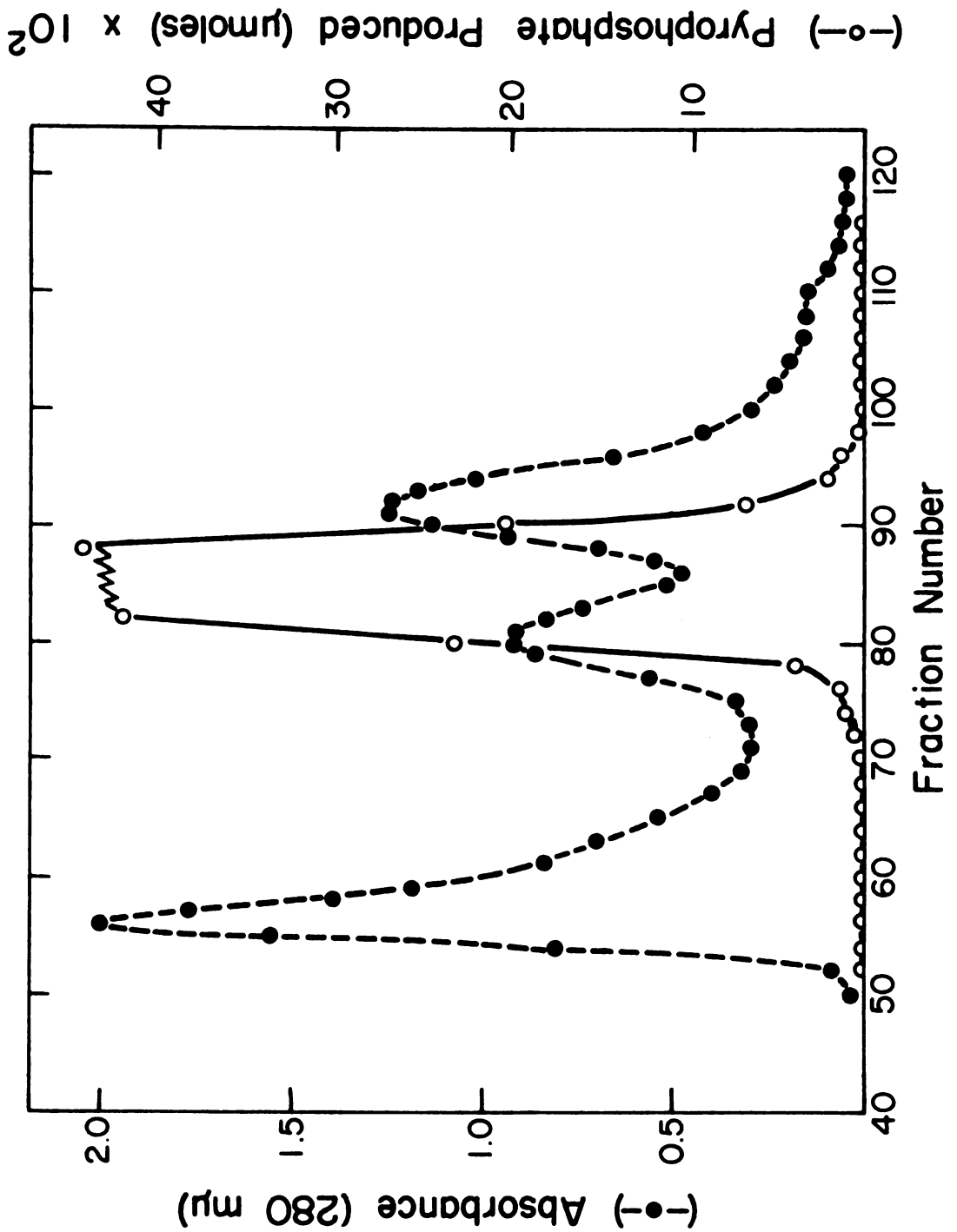
The 40-70% ammonium sulfate fractionation step served to precipitate and to concentrate the pyrophosphohydrolase from solution, leaving most of the hemoglobin in the supernatant fraction. An increase of approximately 20 fold in specific activity was achieved with a recovery of 56%, as shown in Table 1.

C. Sephadex G-100 Gel Filtration

The use of molecular sieves, which fractionate molecules according to size, was adapted to further purification of pyrophosphohydrolase.

The fraction obtained from the ammonium sulfate fractionation step was applied to a 5 x 100 cm column of Septhadex G-100 previously equilibrated with 50 mM Tris-Cl (pH 7.0), 1 mM $MgCl_2$ and 1 mM GSH and eluted with the same buffer solution. The elution was performed ascendingly at a flow rate of 25 ml per hour. A volume of 10 ml per fraction was collected and aliquots of fractions were analyzed for nucleoside triphosphate pyrophosphohydrolase activity. The peak of enzyme was localized at the region of eluate volume from 810 to 950 ml (Figure 1). Fractions of this region were pooled and concentrated either by pressure dialysis against the same elution buffer or by ammonium sulfate addition as mentioned for the wash step described before.

Figure 1. Sephadex G-100 gel filtration of the ammonium sulfate fraction. The sample was eluted with 5 x 10^{-2} M tris containing 1 mM $MgCl_2$ and 1 mM GSH (10.4 ml per fraction). Alternate fractions were analyzed for pyrophosphoshydrolase by incubation at 37° for 20 min. in a reaction mixture containing 50 mM β-alanine (pH 9.5), $MgCl_2$ 10 mM, DTT 1 mM, 1 unit of yeast inorganic pyrophosphatase, column eluate (0.04 ml), 0.5 mM ITP and H_2O to 1.0 ml. Analysis of the inorganic phosphate produced was performed as described in Methods. Control analyses, lacking pyrophosphohydrolase, were carried out using identical conditions in order to correct for small amounts of P_i and PP_i found in commercial preparations of the nucleoside derivatives used as substrates and inhibitors throughout these studies.


(—•—) Absorbance (280 mμ)
2.0
1.5
1.0
0.5
40
50
60
70
80
90
100
110
120
Fraction Number
10
20
30
40
(—○—) Pyrophosphate Produced (μmoles) x 10²

Enzyme preparations obtained using the ammonium sulfate wash step (following the 40 to 70% ammonium sulfate step) and concentration by ammonium sulfate precipitation (following gel filtration) were dialyzed against 50 mM Tris-Cl (pH 7.0), 1 mM $MgCl_2$ and 1 mM dithiothreitol and stored in liquid nitrogen. These preparations are referred to as "gel purified enzyme." However, more recent studies have suggested that both of the latter ammonium sulfate wash steps might be omitted to provide pyrophosphohydrolase preparation with an identical specific activity. In the latter procedure, instead of the wash step, pressure dialysis was used to concentrate the enzyme in the eluates of Sephadex G-100. Yields of the enzyme were markedly improved by this modification.

The Sephadex gel filtration (Table 1) provided a 3 fold increase in specific activity over that of the preparation obtained by the ammonium sulfate fractionation with a total yield to this point of 43%. Small amounts of hemoglobin were still observed in this preparation.

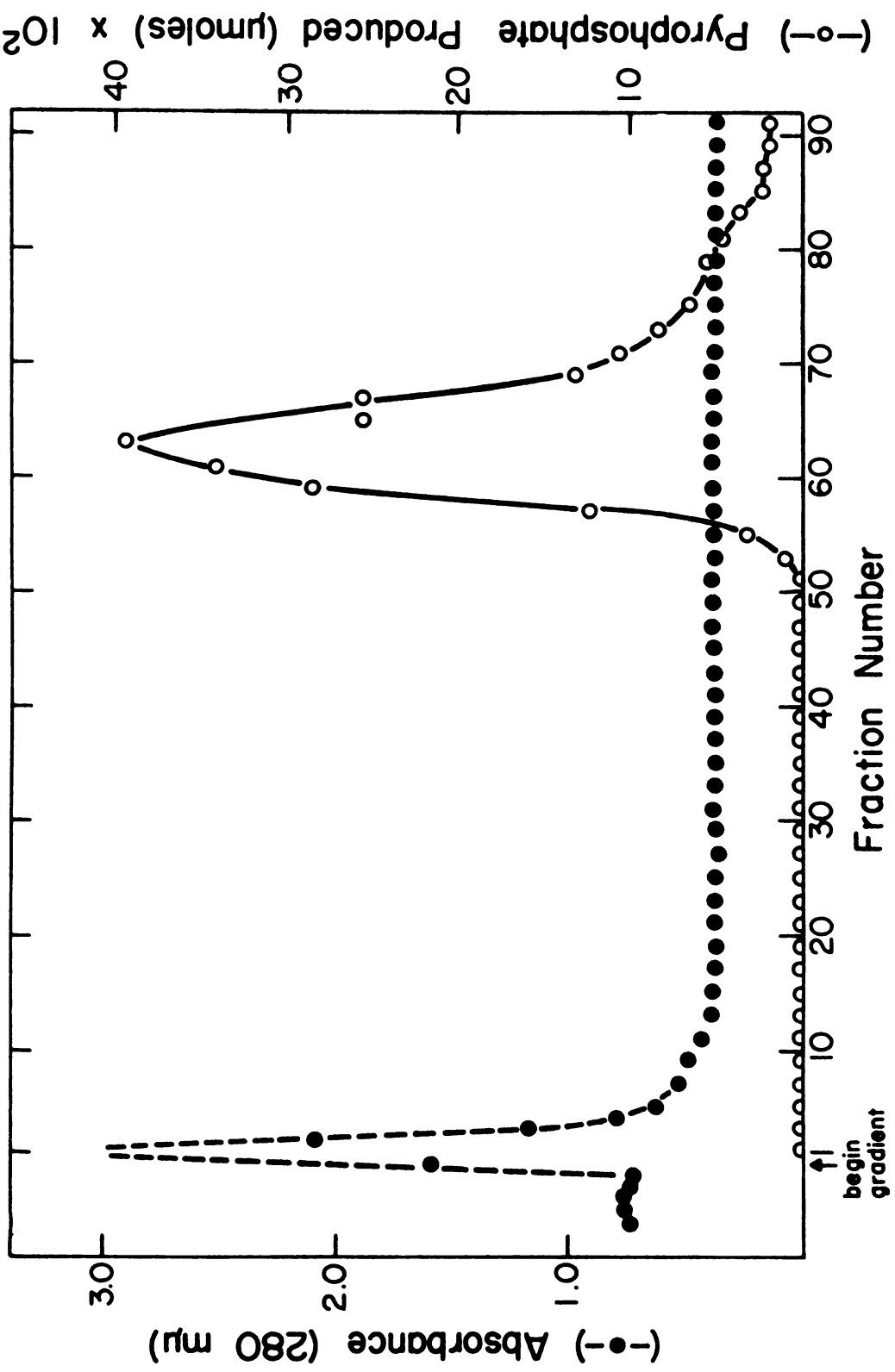
D. DEAE Cellulose Chromatography

In order to remove small amounts of hemoglobin and other impurities, anion exchange chromatography was applied to the purification of pyrophosphohydrolase. The enzyme solution after Sephadex G-100 (obtained by the modified procedure as described) was dialyzed against 50 mM Tris-Cl (pH 8.0), 4 mM $MgCl_2$ and 5 mM dithiothreitol.

Later studies have demonstrated that the eluate fractions from the Sephadex column, containing pyrophosphohydrolase, could be titrated directly to pH 8.0 with a Tris base solution in order to replace the long period of pressure dialysis. This solution was then applied to a DEAE cellulose column directly. It was also found that 5 mM dithiothreitol could be replaced by 1 mM GSH without the loss of any activity. The sample was applied to a DEAE cellulose column (2 x 10 cm, 0.76 meq per g) previously equilibrated with the elution buffer. The column was then washed with the same buffer until the hemoglobin present in the sample began to emerge from the column. Elution of the column was then started using a linear NaCl gradient (Figure 2). It was observed that pyrophosphohydrolase eluted from the DEAE cellulose column contained insufficient protein to be detected directly by U.V. absorption of the eluate fractions at 280 mμ. Fractions containing enzyme activities were identified by their activities of ITP hydrolysis, pooled and concentrated by pressure dialysis against 0.05 M Tris-Cl, 10^{-3} M $MgCl_2$ and 10^{-3} M GSH. These preparations of enzyme were stored in liquid nitrogen. The enzyme activity was stable for long periods by this method of storage.

The resolving power of DEAE cellulose column chromatography in this preparation proved to be especially useful. As shown in Table 1, an increase of 32 fold

Figure 2. Chromatography of the gel purified enzyme on DEAE cellulose. The sample was eluted using a linear NaCl gradient of from 0 to 0.1 M in 50 mM tris Cl (pH 8.0), 4 mM $MgCl_2$ and 5 mM DTT. (200 ml each of the two respective elution media). Eluate fractions (4.0 ml each) were analyzed for pyrophosphohydrolase as described in Figure 1.


(—•—) Absorbance (280 mμ)
3.0
2.0
1.0
(—o—) Pyrophosphate Produced (μmoles) x 10²
40
30
20
10
↑1
begin
gradient
10
20
30
40
50
60
70
80
90
Fraction Number

purification was obtained in this step with total recovery to this point in the purification of 19%. In addition, the preparation was observed to be free of hemoglobin and inorganic pyrophosphatase.

A summary of the results of individual purification steps is presented in Table 1. Overall purification exceeds 2,000 fold in approximately 20% yield. Gel electrophoresis at pH 9.2 of the enzyme preparation from DEAE cellulose chromatography (see Methods) revealed three protein bands in the polyacrylamide gel (Figure 5).

E. Further Purification of Nucleoside Triphosphate Pyrophosphohydrolase

For the purpose of obtaining a homogeneous pyrophosphohydrolase, several approaches have been tried after the step of DEAE cellulose chromatography.

1. Hydroxylapatite chromatography On the basis of the selective absorption of proteins, hydroxylapatite was employed for further purification of pyrophosphohydrolase.

The enzyme preparation from DEAE cellulose chromatography was applied to a 0.9 x 4 cm hydroxylapatite (HTP) column previously equilibrated with 10^{-3} M phosphate buffer (pH 6.8) containing 10^{-3} M DTT. The column was eluted stepwise with successively increasing concentrations of phosphate buffer until the pyrophosphohydrolase activity was eluted. Because of the phosphate buffer used, the

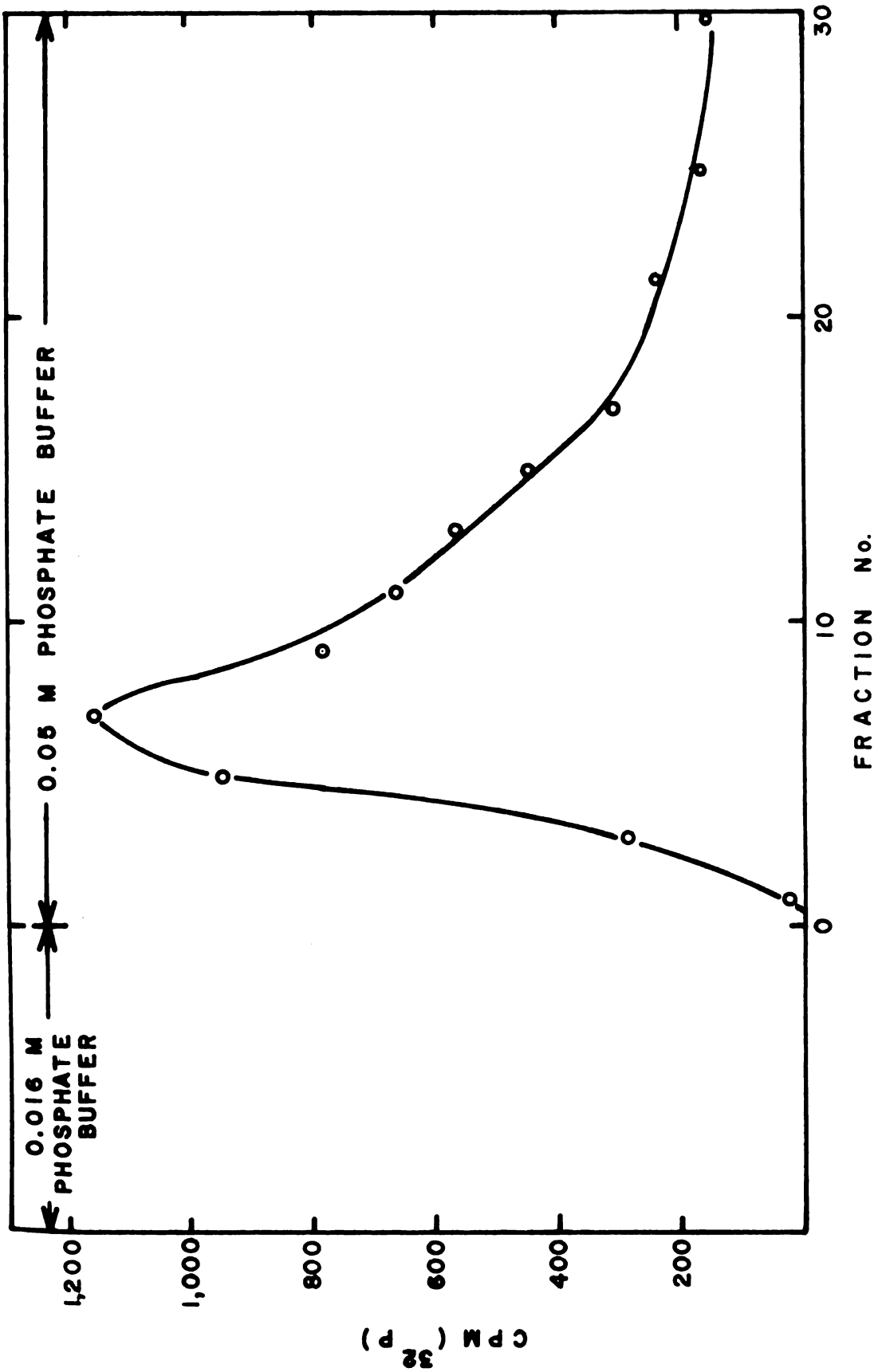
standard enzyme assay system with γ-labeled $IT^{32}P$ as substrate was employed in order to measure the pyrophosphohydrolase activity. Radioactivity of P_i released from $IT^{32}P$ was analyzed by extraction as the phosphomolybdate complex into the organic phase by isobutanol benzene extraction. Aliquots were then counted with a Geiger Muller counter.

A modification of the radioactive assay was also conducted. IMP released in the standard assay mixture lacking yeast inorganic pyrophosphotase was removed by charcoal adsorption. The radioactivity of $^{32}PP_i$ in the supernatant was then counted.

The elution diagram of pyrophosphohydrolase is given in Figure 3. Some "tailing" of the pyrophosphohydrolase activity was observed. A yield of about 50% was obtained from this purification step. Purity of this preparation was analyzed by disc gel electrophoresis (Figure 6) by the method of Davis. Three protein bands were observed.

2. Preparative disc gel electrophoresis The combined mechanisms of the physical sieving and differential electrophoretic mobilities of proteins in polyacrylamide at a certain pH made preparative disc gel electrophoresis applicable as a technique in the purification of pyrophosphohydrolase.

Figure 3. Chromatography of pyrophosphohydrolase from DEAE cellulose preparation on a Hydroxylapatite column. Pyrophosphohydrolase was eluted with 0.05 M phosphate buffer (pH 6.8) containing 10^{-3} M DTT. Enzyme activity was measured by the radioactivity of $^{32}P_i$ released from IT^{32}P hydrolysis. Detailed experimental procedure was described in the text.

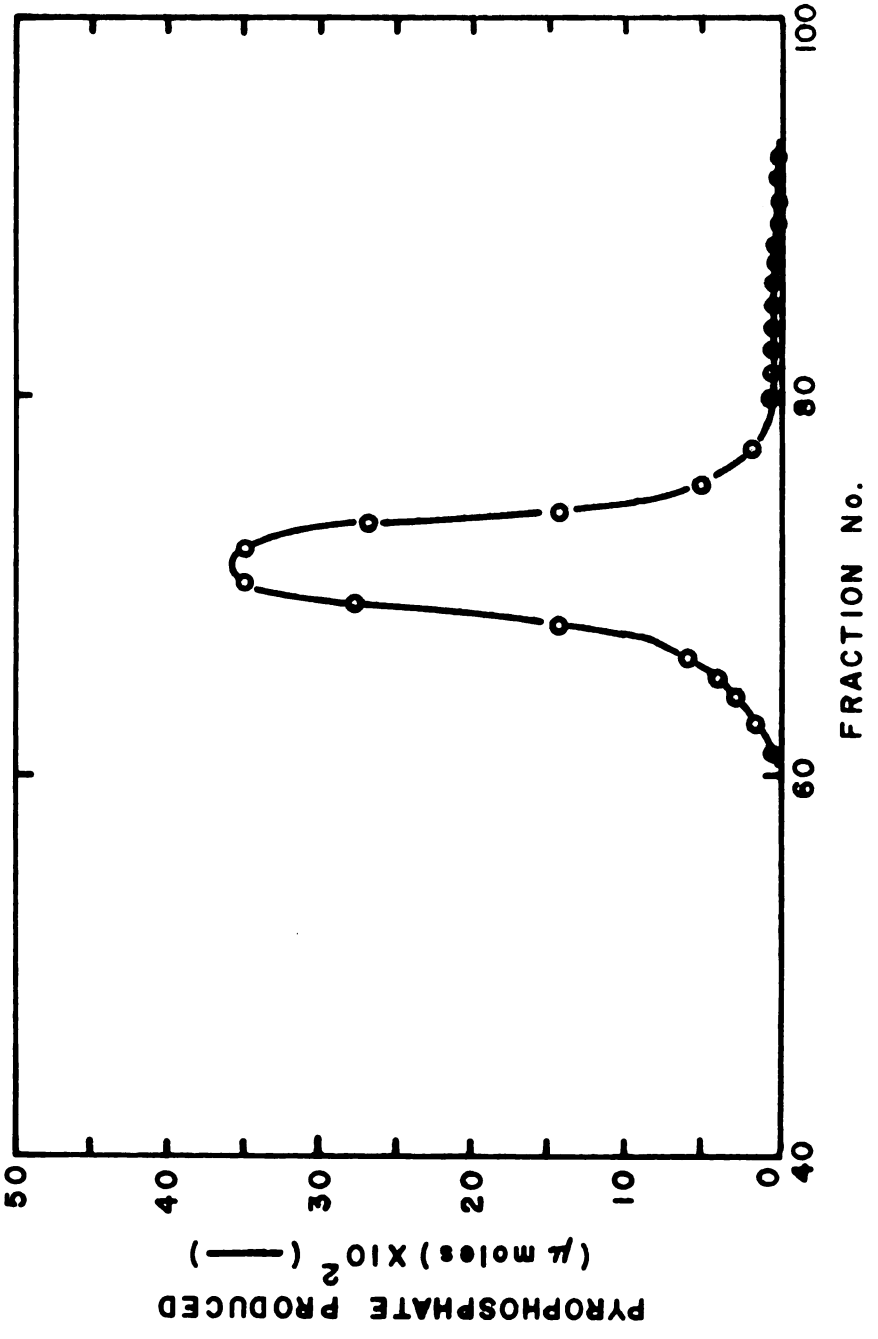

0.016 M PHOSPHATE BUFFER
0.05 M PHOSPHATE BUFFER
1,200
1,000
800
600
400
200
CPM (^{32}P)
0
10
20
30
FRACTION No.

A Canal Co. instrument fitted with a PD 2/150 upper column was used. A separating (or running) gel of 15% acrylamide containing 10^{-3} M DTT (dithiothreitol), pH 9.1 was prepared to a height of 6 cm in the column and polymerized as described in the section of "Analytical Disc Gel Electrophoresis" under "Analytical Method." A 1.5 cm height of stacking gel (2.5% acrylamide containing 10^{-3} M DTT, pH 6.8-7.0) was polymerized on top of the separating gel. The sample was prepared by mixing 3 ml of the enzyme solution from DEAE cellulose preparation in 2.5% sucrose solution, and then carefully layered on top of the stacking gel either before or after the buffer in the upper electrode chamber was in place.

Electrophoresis was started at 4 ma until the sample entered into the stacking gel. The current was then increased to 16 ma for 18 to 20 hrs. The electrode buffer consisted of 0.025 M Tris-glycine buffer (pH 8.7) containing 10^{-3} M DTT. The elution buffer was 0.375 M Tris-Cl (pH 8.0) containing 0.23% N, N, N', N'-tetramethylenediamine (TEMED), 10^{-3} M DTT. A flow rate of 0.3 ml per min. of elution buffer was maintained, and fractions of 3.75 ml were collected. All steps were carried out at 4°C. A profile of pyrophosphohydrolase activity versus fraction no. is shown in Figure 4.

Approximately 39% of the pyrophosphohydrolase from the DEAE cellulose preparation was recovered in this

Figure 4. Elution pattern of pyrophosphohydrolase from preparative disc gel electrophoresis. A volume of 3.75 ml per fraction was collected. Enzymatic activity was determined by the standard enzyme assay (see Methods). The details of electrophoretic procedures are described in the text.


PYROPHOSPHATE PRODUCED
(μ moles) X10^2 (——)
0
10
20
30
40
50
40
60
80
100
FRACTION No.

procedure with approximately 2.8 fold of purification. Homogeneity of the enzyme from this preparation, again, was analyzed by disc gel electrophoresis technique. One single protein was observed in the polyacrylamide gel at pH 8.7 (Figure 6). One more criterion for homogeneity was applied by disc gel electrophoresis of the enzyme at a different pH. Two protein bands were revealed at pH 5.5 (Figure 6). Only one of the two components has enzyme activity. Detailed studies of disc gel electrophoresis and histochemical identification of pyrophosphohydrolase in the polyacrylamide gels are presented in the following section.

3. Fractionation by isoelectric focusing technique Isoelectric focusing was applied to separate the proteins according to their isoelectric points. Detailed experimental procedure was given under "Analytical Method." Nucleoside triphosphate pyrophohydrolase was observed in the pH gradient at the region around pH 4.5 (Figure 27). Low recovery of pyrophosphohydrolase activity was obtained. The occurrence of precipitates in the column and interference of carrier ampholytes with inorganic phosphate assay (unpublished observations) may account for the low enzyme activity recovery observed. Analysis of the pyrophosphohydrolase preparation obtained following the isoelectric focusing technique by the analytical

polyacrylamide disc gel technique revealed the presence of four protein bands (Figure 6).

F. Analytical Disc Gel Electrophoresis and Histochemical Identification of Nucleoside Triphosphate Pyrophosphohydrolase in Polyacrylamide Gels

Homogeneity of the pyrophosphohydrolase preparation was studied by disc gel electrophoresis with histochemical identification of enzyme activity in the polyacrylamide gels. Figure 5 and Figure 6 show the profiles of protein bands and pyrophosphohydrolase activity in the polyacrylamide gels with the enzyme preparations from different purification steps. There is only one protein band in the 7% polyacrylamide gel at pH 8.7 with the enzyme preparation obtained from preparative disc gel electrophoresis. Disc gel electrophoresis was also carried out at pH 5.5. No protein was found in the gel. When the electrodes were reversed, two protein components with slow mobilities were observed. Only the more slowly moving one possessed pyrophosphohydrolase activity as revealed by the histochemical staining method. The pyrophosphohydrolase component constitutes approximately 30% of the total protein in this preparation, as determined by densitometric tracing of entire gel at 600 mμ with a Gilford recorder. A graph of densitometric tracing was shown in Figure 7.

A discrepancy between the observations of the mobility of pyrophosphohydrolase in the polyacrylamide gel

Figure 5. Disc gel electrophoresis of an enzyme preparation from the Sephadex G-100 filtration step (A), and DEAE cellulose chromatography (B) at pH 9.2 according to the method of Gordon and Louis. The protein bands were visualized with Coomassie brilliant blue (see Methods). Nucleoside Triphosphate Pyrophohydrolase activity was identified by the lead conversion method of Gomori as white precipitation band, i.e., Ca-pyrophohphate (C) (see details in the text).

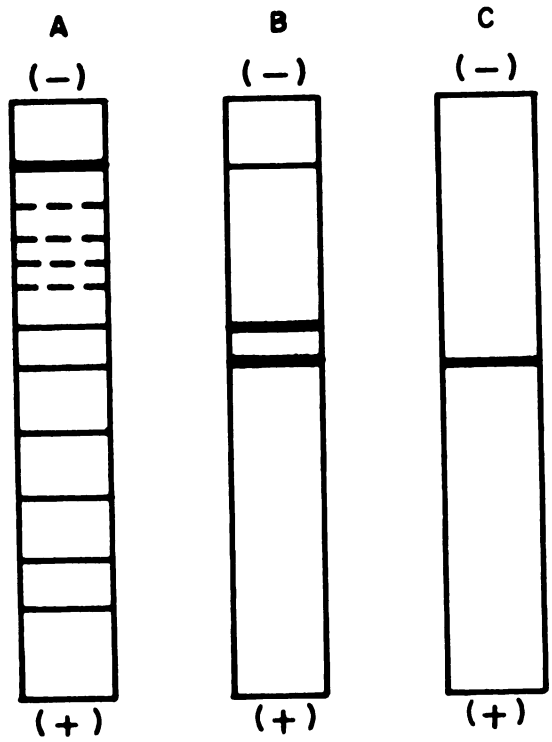

A
(−)
(+)
B
(−)
(+)
C
(−)
(+)

Figure 6. Disc gel electrophoresis of pyrophosphohydrolase preparations from Hydroxylapatite Chromatography (A), Isoelectric Focusing Fractionation (B) and preparative Disc Gel Electrophoresis (C, D) in 7% acrylamide gels. A constant current of 5 ma per gel was conducted from cathode to anode at pH 8.7 for gels (A), (B), (C), and at pH 5.5 for gel (D) (note the polarity of the current applied). After electrophoresis, the gels (A) and (B) were stained with 1% Amido-Schwarz for proteins. Halves of gels (C) and (D) were stained for proteins with Coomassie brilliant blue and halves of each gel (C' and D') were stained for enzyme activity by histochemistry as described in the text. Identical results were observed with duplicate whole gels of (C) and (D), staining one of the duplicate pair for protein with Amido-Schwarz and the other gel for enzyme activity by the histochemical technique.

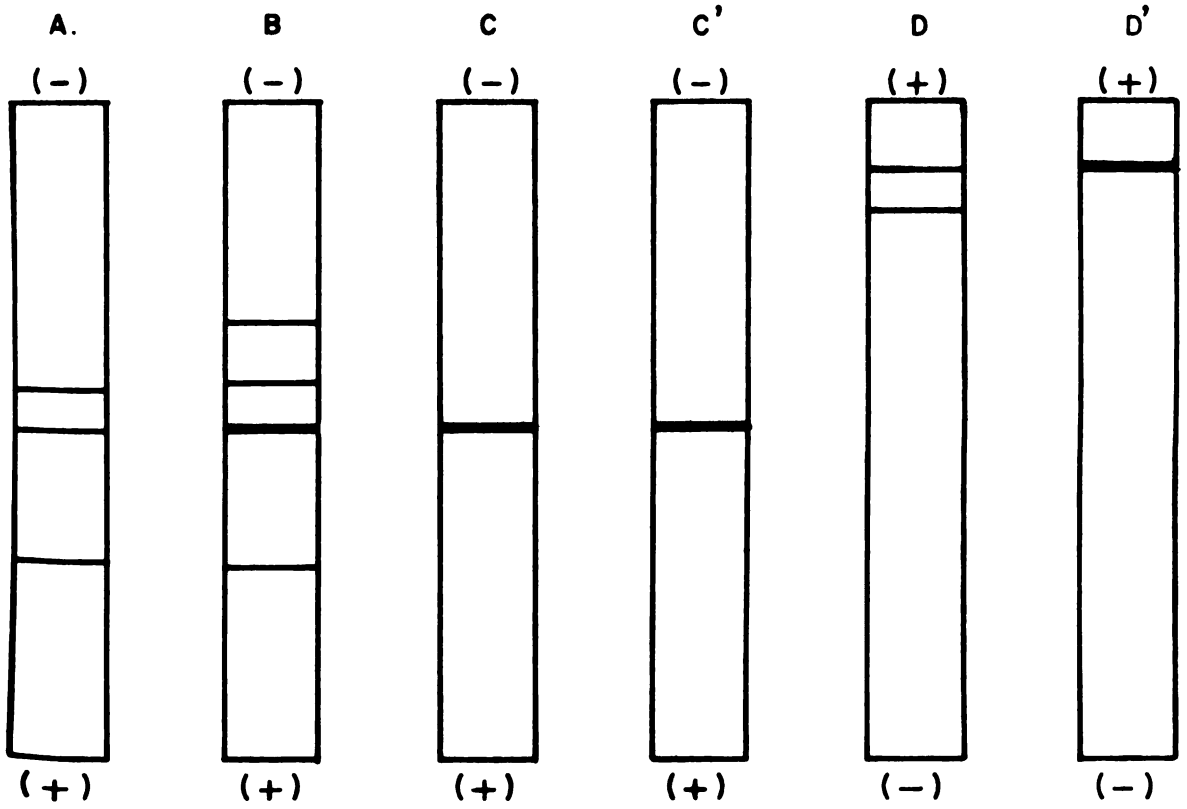
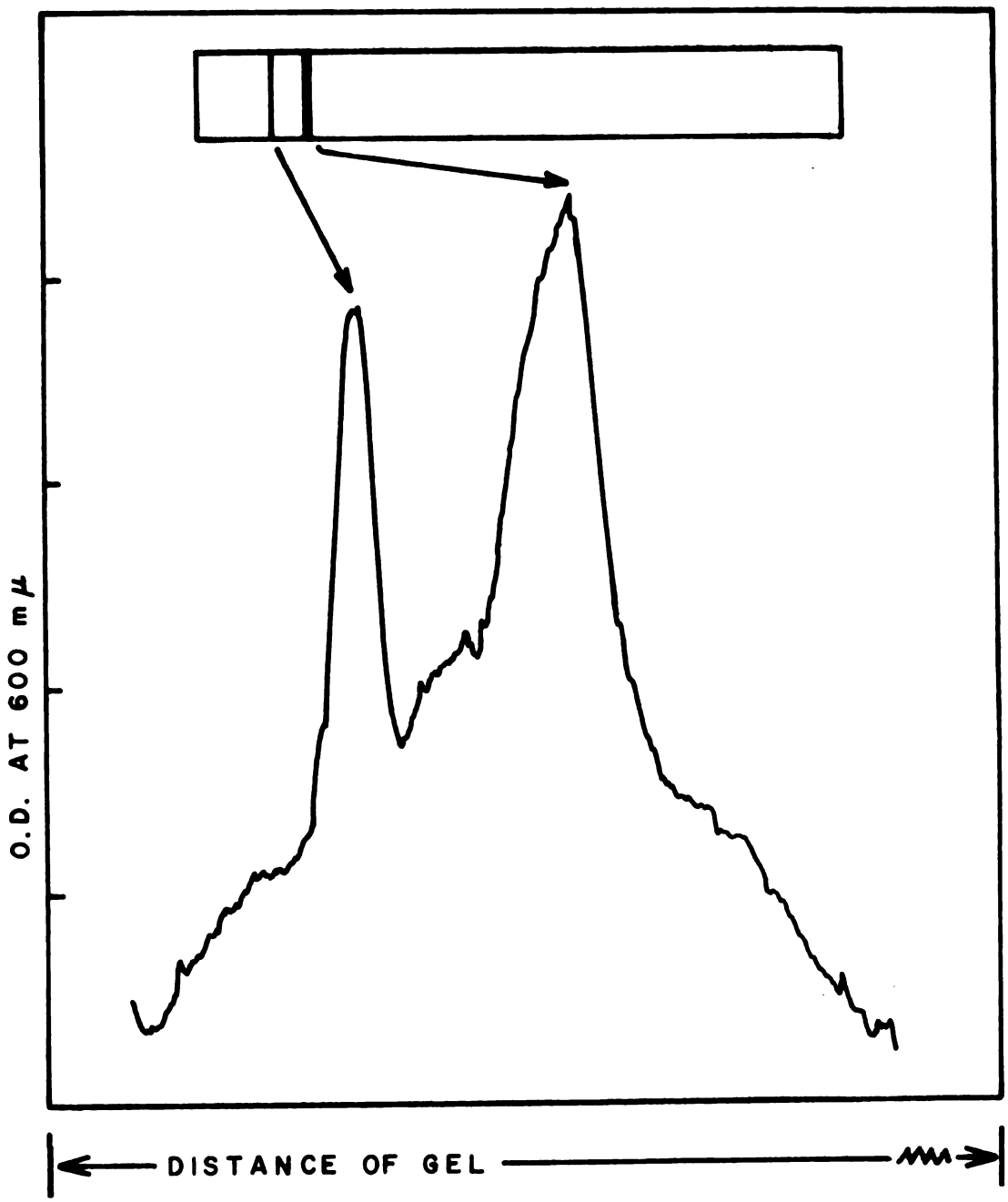

A.
(−)
(+)
B
(−)
(+)
C
(−)
(+)
C'
(−)
(+)
D
(+)
(−)
D'
(+)
(−)

Figure 7. Densitometric tracing of polyacrylamide gel (D) in Figure 6 at 600 mμ.


O.D. AT 600 mμ
DISTANCE OF GEL

(from anode to cathode) and of the pI value of 4.3 for the enzyme was noted. This dilemma, however, was resolved using the paper electrophoresis technique.

G. Paper Electrophoresis

Figure 8 presents the results of strip paper electrophoresis of the enzyme from preparative electrophoresis at pH 5.5 with different solutions as the electrode buffer. The sample was applied at the central line of the paper. The proteins moved toward the cathode β-alanine-acetate was used as the electrode buffer. However, with maleic-KOH or acetate-KOH as electrode buffer, the proteins moved to the reverse direction, i.e., toward the anode. These results suggest that the possible interaction between β-alanine and the protein sample have resulted in a change of direction of electrophoretic mobility of the proteins at pH 5.5. The pH of 4.3 is consequently believed to be the correct value for the pI of the pyrophosphohydrolase. This explanation of the above dilemma was further strengthened by the observed mobilities of the protein bands and pyrophosphohydrolase activity in the polyacrylamide gel electrophoresis at pH 5.5 with acetate-KOH as the electrode buffer (Figure 9).

Figure 8. Strip paper electrophoresis of the pyrophosphohydrolase obtained from preparative disc gel electrophoresis at pH 5.5 with (A) β-alanine acetate, (B) maleic-KOH, (C) acetate-KOH as electrode buffers. Proteins were stained with ninhydrin.

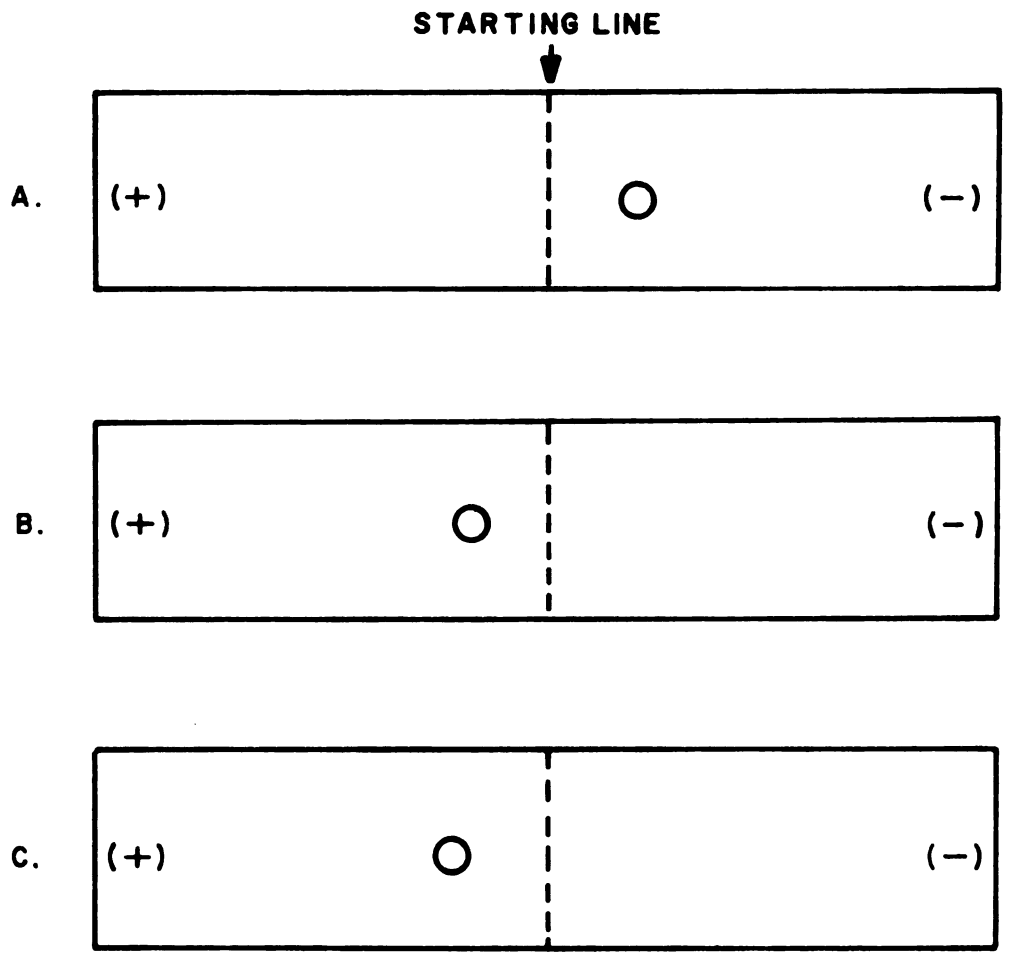
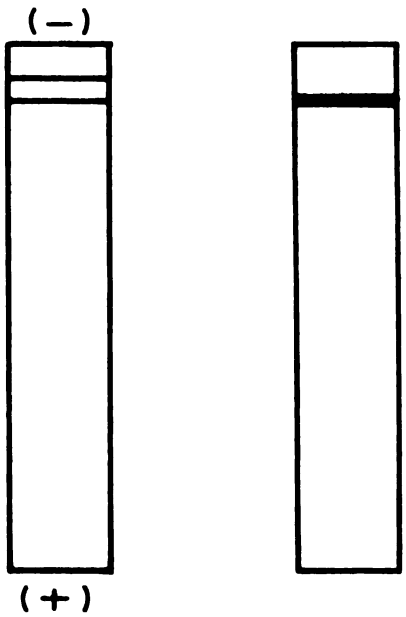

STARTING LINE
A.
(+)
(−)
B.
(+)
(−)
C.
(+)
(−)

Figure 9. Disc gel electrophoresis at pH 5.5 using acetate-KOH buffer (0.17 M) containing 2-mercaptoethanol (10^{-3}M) of pyrophosphohydrolase prepared by preparative gel electrophoresis. A current of 5 ma/gel was conducted from cathode to anode. Protein bands were developed with Amido-Schwarz. Pyrophosphohydrolase activity was stained with histochemical technique (B).


(−)
(+)
(A)
(B)

II. Properties of Pyrophosphohydrolase

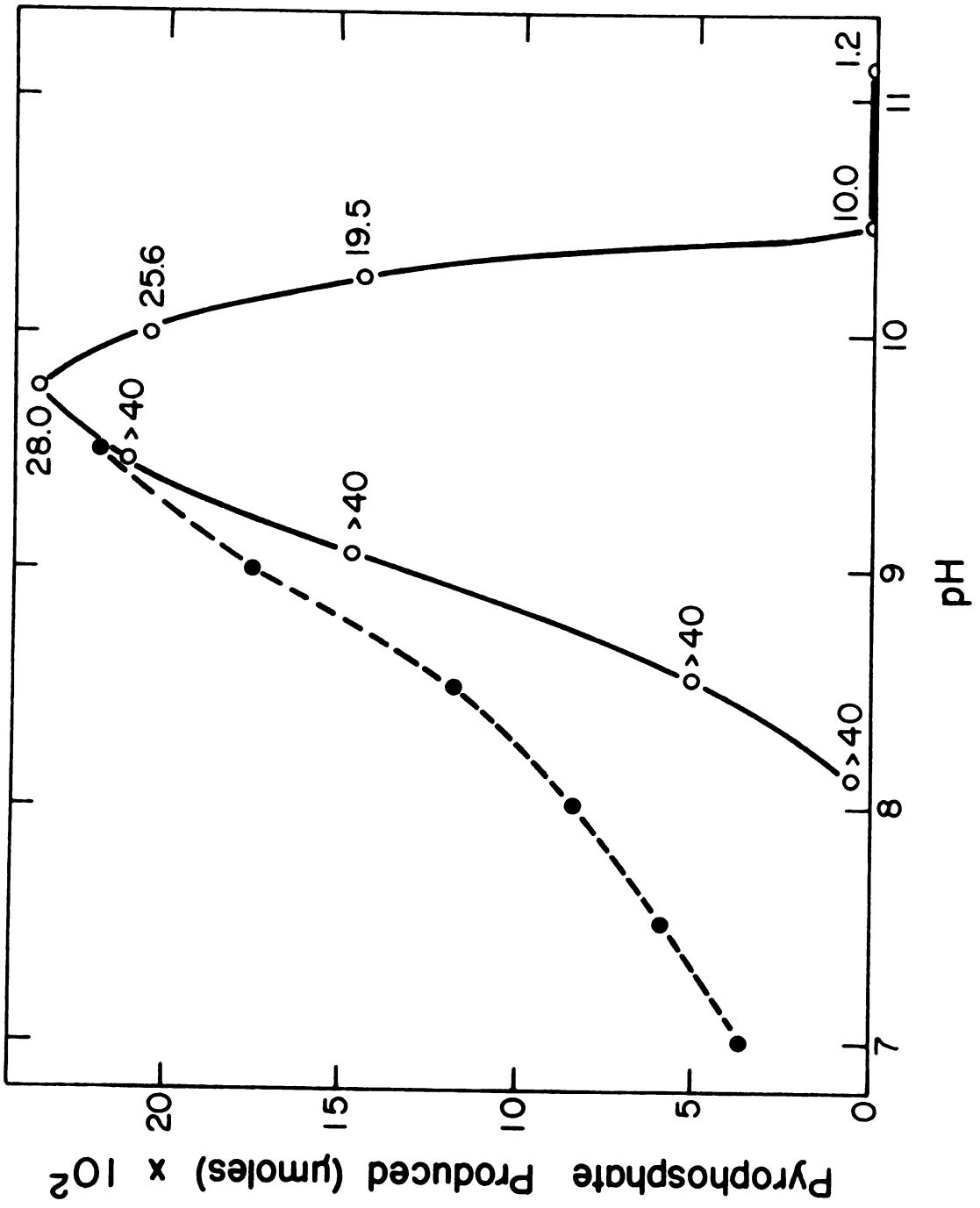
A. pH Optimum

The optimum pH range for the hydrolysis of ITP by pyrophosphohydrolase was studied using two different buffer systems. Figure 9 shows the effect of pH on enzyme activity. A rather sharp pH optimum of 9.75 was obtained with β-alanine buffer. Using this buffer, no observable activity was found either below pH 8.0 or above pH 10.5. However, a considerable activity was observed between pH 7 and pH 8, when Tris-Cl was employed as buffer.

In view of the presence of yeast inorganic pyrophosphatase in the coupled assay system, it was important to check the effect of high pH on pyrophosphatase activity to assure that it was not a limiting factor in the assay system. Yeast inorganic pyrophosphatase (1 unit) was added routinely to each reaction to supplement the endogenous inorganic pyrophosphatase present in the gel purified enzyme. A profile of the activity of pyrophosphatase as a function of pH (using sodium pyrophosphate as substrate) is also presented in Figure 10.

A drastic decrease of the activity of inorganic pyrophosphatase above pH 9.5 was observed, but the activity remaining exceeded that of the total pyrophosphohydrolase activity observed in the coupled assay system. What is more, higher concentrations of yeast inorganic pyrophosphatase in the reaction mixture did not change the

Figure 10. The effect of pH upon pyrophosphohydrolase and pyrophosphatase activity. Pyrophosphohydrolase activity was determined using 50 mM tris Cl (- - -●- - -) or 50 mM β-alanine (- - -o- - -) buffer, gel purified enzyme and other components as described in Figure 1. The effect of pH upon pyrophosphatase was determined using β-alanine buffer, 0.5 mM Na PP_i as substrate and other components as described above. The numerical values obtained for pyrophosphatase action at each pH, i.e., PP_i cleaved x 10^2 (μmoles) per 20 minute incubation at 37°, are shown directly adjacent to the corresponding point for pyrophosphohydrolase action for purposes of comparison.


Pyrophosphate Produced (μmoles) x 10²
pH
0
5
10
15
20
7
8
9
10
11
28.0
25.6
19.5
10.0
1.2
>40
>40
>40
>40

observed results. Therefore, these data are considered to provide reliable information for the pH optimum of the pyrophosphohydrolase. Incubation at pH 9.5 with β-alanine buffer in the coupled assay system was adapted to all the further analyses conducted throughout this study.

B. Enzyme Concentration Curve

Effect of the concentration of ITP hydrolysis was studied with standard coupled assay. The result (Figure 11) revealed a linear relationship between the concentration of pyrophosphohydrolase and ITP hydrolysis over a range of enzyme concentrations which produced from 0 to more than 0.3 μmole of inorganic pyrophosphate.

C. Time Course of ITP Hydrolysis

The effect of time of incubation at 37°C upon ITP hydrolysis was studied by incubating gel purified enzyme in the standard coupled assay at 37°C for various time periods and the analysis of inorganic pyrophosphate released. The rate of the hydrolysis of ITP was linear for at least 45 min. (Figure 12). A period of 20 min. was adapted as the convenient interval of incubation in the coupled assay system in order to measure the reaction in a linear portion of the curve.

Figure 11. Effect of pyrophosphohydrolase concentration upon ITP hydrolysis.

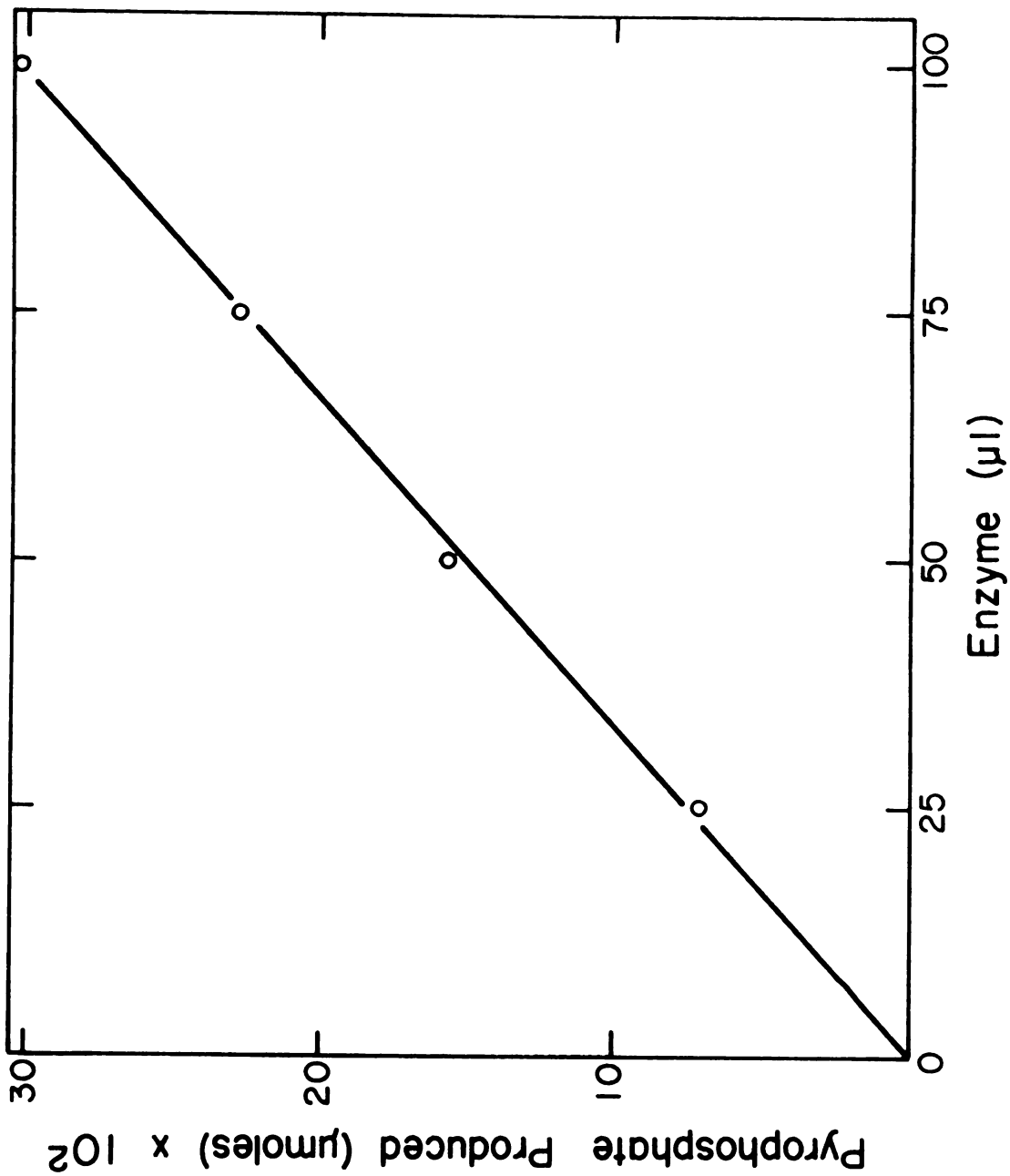
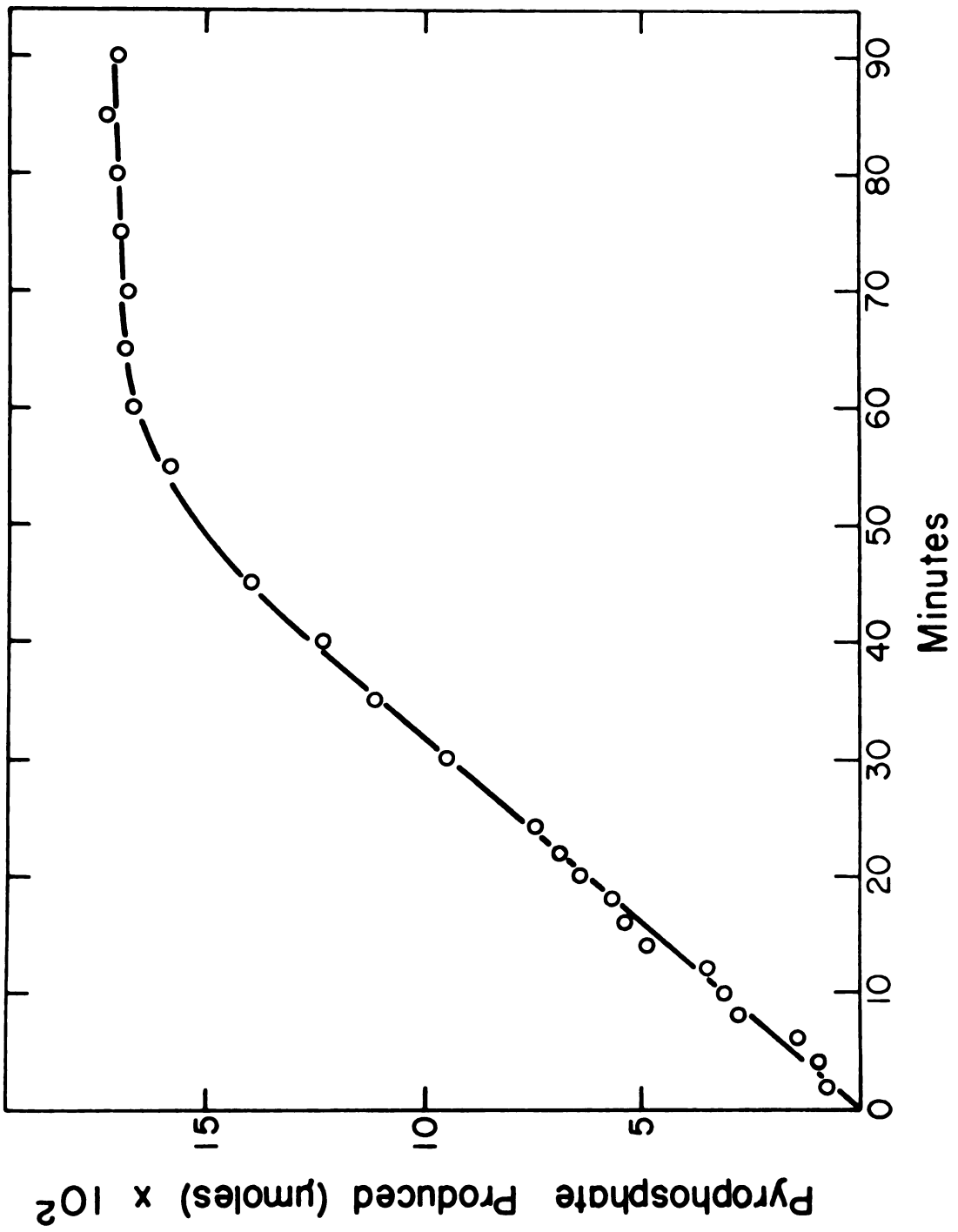

Pyrophosphate Produced (µmoles) x 10²
30
20
10
0
0
25
50
75
100
Enzyme (µl)

Figure 12. Time course of ITP hydrolysis by pyrophosphohydrolase. See Figure 1 for details of the assay.


Pyrophosphate Produced (μmoles) x 10²
15
10
5
0
10
20
30
40
50
60
70
80
90
Minutes

D. Substrate Concentration Curve

A substrate optimum concentration at 5 x 10^{-4} M ITP was obtained from the study of the influence of substrate concentration on the activity of pyrophosphohydrolase (Figure 13). An inhibitory effect was observed at higher levels of substrate concentration. An identical phenomenon was revealed by using GTP as substrate although the rate of hydrolysis was considerably less (see Table 2).

Substrate inhibitions were confirmed by using highly purified ITP and GTP as substrates, which is reported under "Kinetic Studies" (Figure 22 A & B). The difference of levels of inhibition between commercial preparation of ITP or GTP and purified ITP or GTP suggested that some of the substrate inhibition, was due to impurities. The use of purified ITP or GTP eliminated the possibly inhibitory effects of impurities, such as IDP, IMP, PP_i or P_i, presented in commercial ITP or GTP preparations.

E. Requirement of the Sulfhydryl Compound

Figure 14 shows the effect of dithiothreitol (DTT) on the observed activity of pyrophosphohydrolase. These data demonstrated that the activity of enzyme was dependent on the presence of a sulfhydryl compound in the assay. A sulfhydryl compound, either DTT or Glutathione (GSH) was included in the buffer at all stages of purification and analyses throughout this study.

Figure 13. Effect of ITP concentration upon the observed pyrophosphohydrolase activity. Analyses were conducted as described in Figure 1 using ITP as indicated.

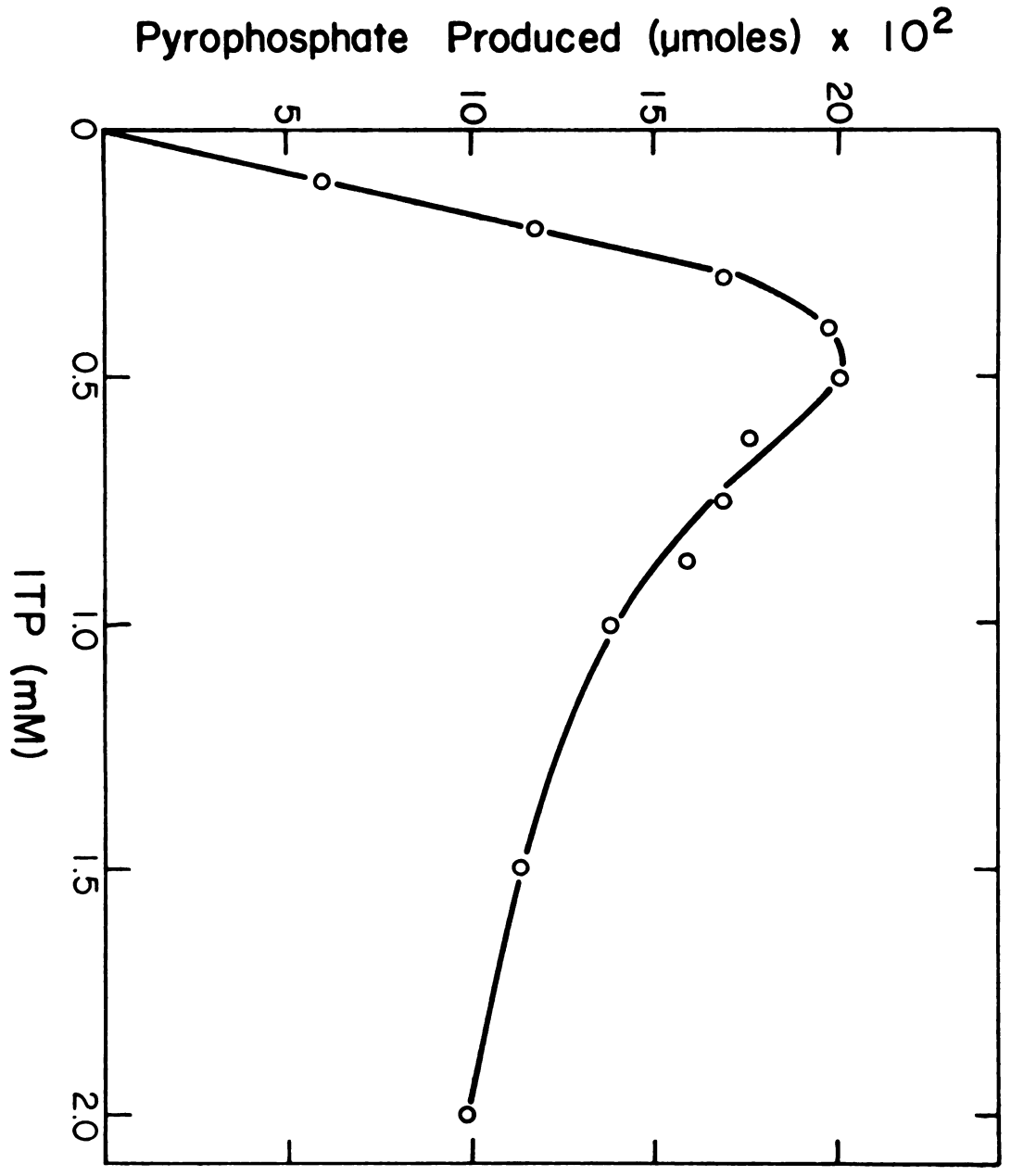
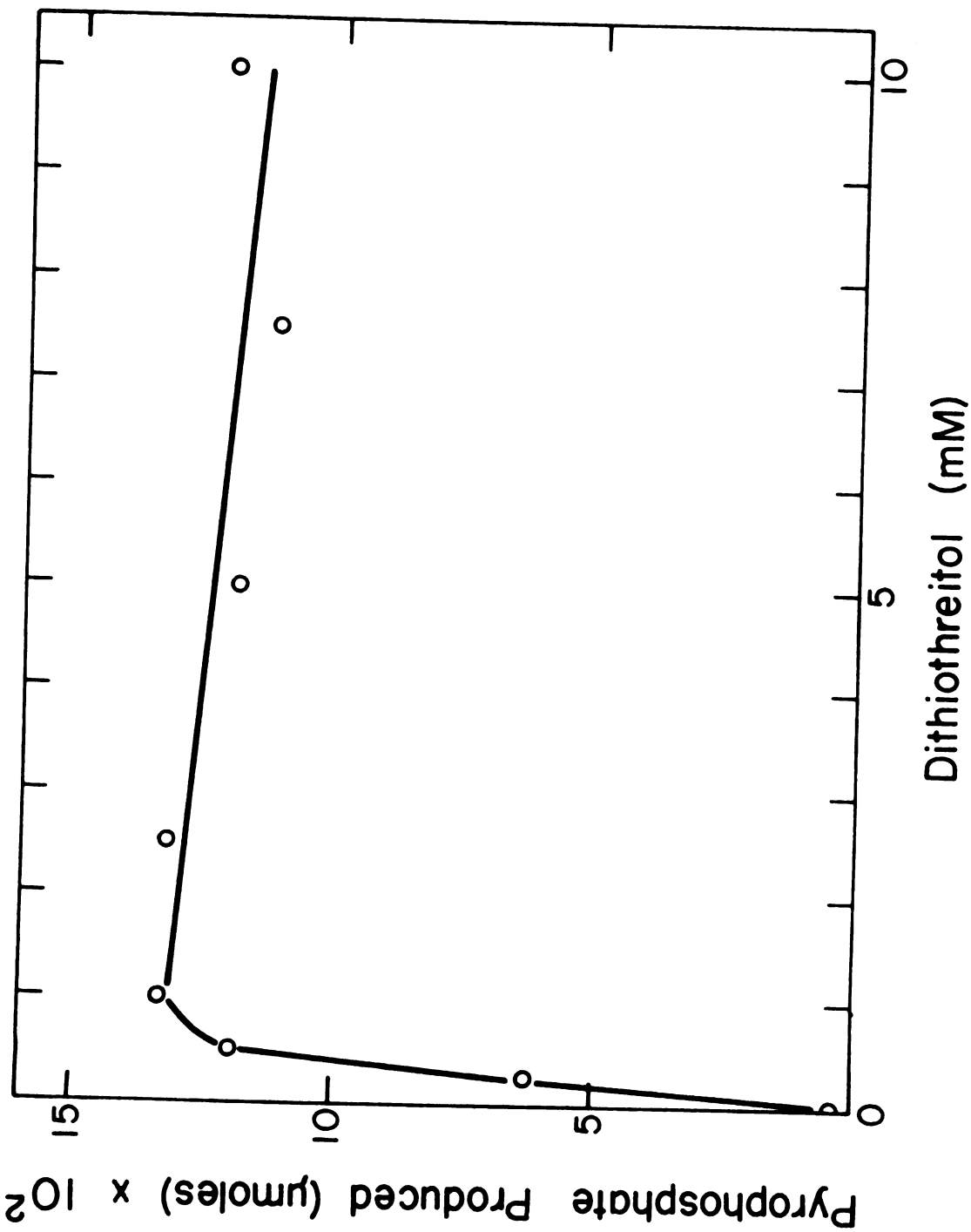

Pyrophosphate Produced (μmoles) x 10^2
5
10
15
20
0
0.5
1.0
1.5
2.0
ITP (mM)

Figure 14. The sulfhydryl requirement of pyrophosphohydrolase.

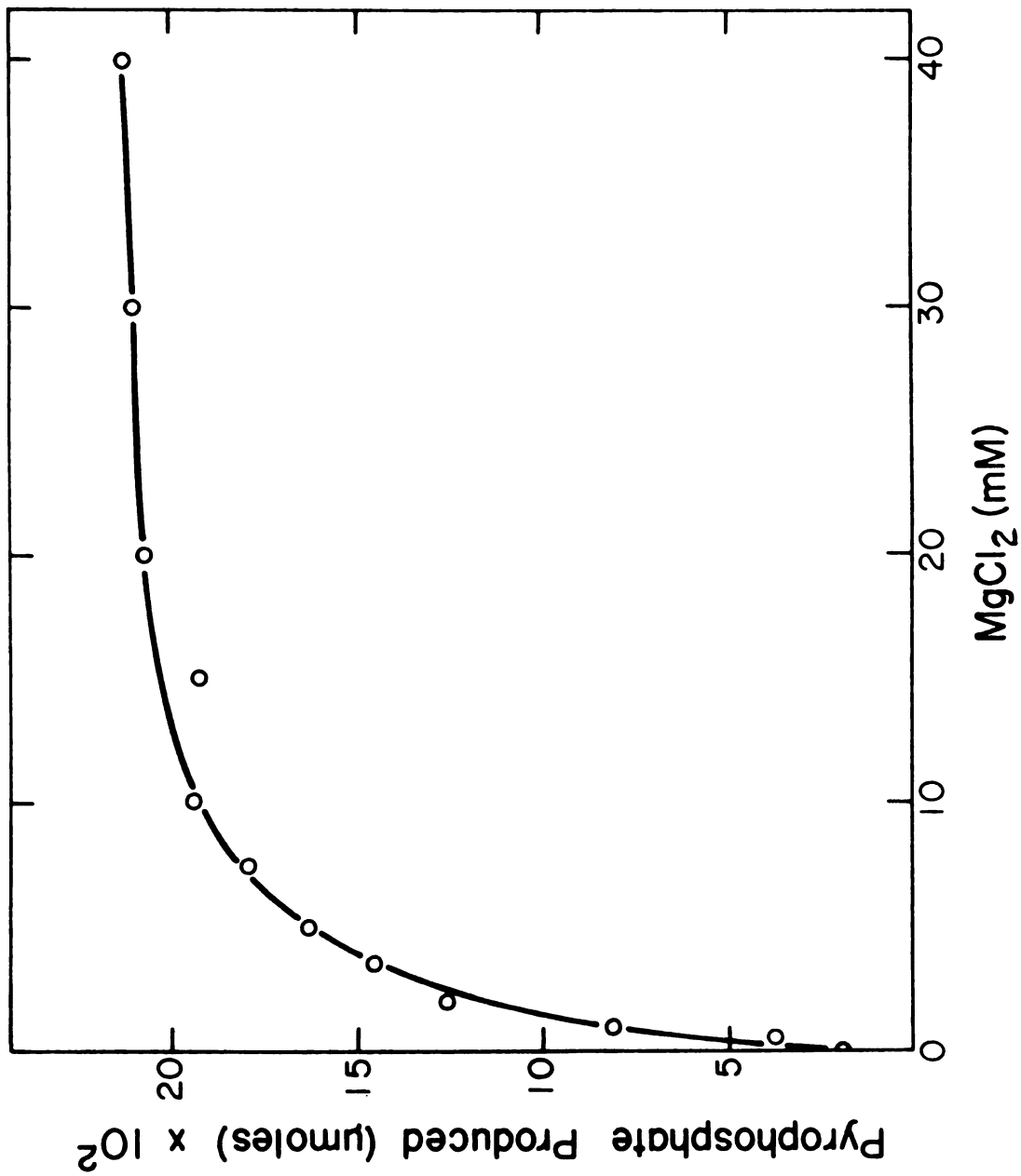

Pyrophosphate Produced (μmoles) x 10^2
15
10
5
0
0
5
10
Dithiothreitol (mM)

F. The Effects of Divalent Metal Ions on Pyrophosphohydrolase Activity

The coupled assay system (in the presence of inorganic pyrophosphatase) was employed in order to study the divalent cation effects on pyrophosphohydrolase activity. There was a measurable amount of ITP hydrolysis observed only in the presence of Mg^{++}. The reaction was shown to reach saturation at 10 mM of Mg^{++} and to remain relatively unchanged with higher concentration of Mg^{++} (Figure 15).

Since Mg^{++} is known to be required for inorganic pyrophosphatase activity (57), the specific divalent ion requirements of pyrophosphohydrolase were studied by the following procedure: Gel purified enzyme was incubated in a reaction mixture containing 50 mM histidine buffer (pH 9.5), mM DTT, 0.5 mM ITP and a divalent ion at 37°C for 20 min. The pyrophosphohydrolase activity was then destroyed by heating the reaction mixture to 85°C for 5 min. After cooling, 1 unit of yeast inorganic pyrophosphatase was added and the reaction mixture was adjusted to 10 mM Mg^{++}. Essentially the same effect of Mg^{++} on pyrophosphohydrolase activity was observed, either by this method or previous procedure. A lesser activity of pyrophosphohydrolase in the presence of Mn^{++} was revealed by the latter assay system. None of the other divalent cations tested ($CaCl_2$, $FeCl_2$, $ZnCl_2$ or $CuCl_2$) resulted in significant hydrolysis of ITP. For the

Figure 15. The magnesium ion requirement of the pyrophosphohydrolase reaction mixture.


Pyrophosphate Produced (μmoles) x 10²
0
5
10
15
20
0
10
20
30
40
MgCl₂ (mM)

comparison of divalent cation effects, it was necessary to use histidine buffer in the assay to prevent the precipitation of Mn^{++} at pH 9.5 observed in the presence of β-alanine. The effects of Mg^{++} on enzyme activity were identical in either buffer.

G. The Effect of Monovalent Cations on the Activity of Pyrophosphohydrolase

The coupled assay system and the pyrophosphohydrolase alone as described above were used to examine the effects of monovalent ions. All monovalent cations were found, without exception, to have significant inhibition of ITP hydrolysis. For example, 70 mM KCl, NaCl and NH_4Cl resulted in 25, 41 and 46% inhibition, respectively.

H. Substrate Specificity

The results of different nucleoside triphosphate compounds tested as substrates of pyrophosphohydrolase (Table 2) revealed that ITP and dITP were the most effective substrates tested, while XTP was degraded to a lesser extent. GTP, dGTP, UTP and dUTP were hydrolyzed at approximately 10% of the rate of ITP and dITP. TTP was hydrolyzed in only trace amounts while ATP, dATP, CTP and dCTP did not serve as substrates. In addition, nucleoside mono- or diphosphates were not hydrolyzed by the pyrophosphohydrolase.

TABLE 2.--Substrate specificity. [All substrates analyzed were 0.5 mM in the assay mixture (see Figure 1). The pyrophosphohydrolase solution added was either the gel-purified enzyme (A) or the enzyme obtained from DEAE-cellulose chromatography (B) (see text).]

Nucleotide Tested	Relative Activity	
	A	B
ITP	100	100
dITP	103	
XTP	71	74
dUTP	13	
UTP	12	
GTP	10	8
dGTP	6	
TTP	3	
CTP	0.5	
dCTP	0.5	
dATP	0.2	
ATP	0	
IDP	0	
IMP	0	

The data suggest that no distinction is made between the ribose and deoxyribose moiety in the specificity of pyrophosphohydrolase during the hydrolysis of either purine or pyrimidine nucleoside triphosphates. The 6-keto-group of purines and pyrimidines is important for the recognition of pyrophosphohydrolase, since replacement of the 6-keto-group by an amino group markedly reduces the ability of the substance to act as a substrate.

No inorganic phosphate was released by pyrophosphohydrolase using either glucose-6-phosphate, ribose-5-phosphate, glycerol-3-phosphate, 2, 3-diphosphoglycerate or ρ-nitrophenylphosphate as substrate, excluded the possibility of a contamination by phosphatase activity in the preparation of pyrophosphohydrolase.

I. Criteria for a Single Enzyme Molecule

Broad specificities of pyrophosphohydrolase toward nucleoside triphosphates raised the question of the number of pyrophosphohydrolase in the reticulocyte cell. This question was examined by the following three experiments:

The ratio of activities of different nucleoside triphosphates as substrates following purification was found to be constant by using gel-purified enzyme and highly purified enzyme from DEAE cellulose chromatography as the sources of enzyme (Table 2). These data provided evidence that the hydrolysis of different nucleoside triphosphates was

the result of a single enzyme molecule with broad specificity. This concept was further strengthened by the results (Table 3) of additive activity assay (additive activity will appear in the presence of two different substrates if the enzyme preparation contains two different enzymes, one specific for GTP hydrolysis and the other specific for ITP hydrolysis).

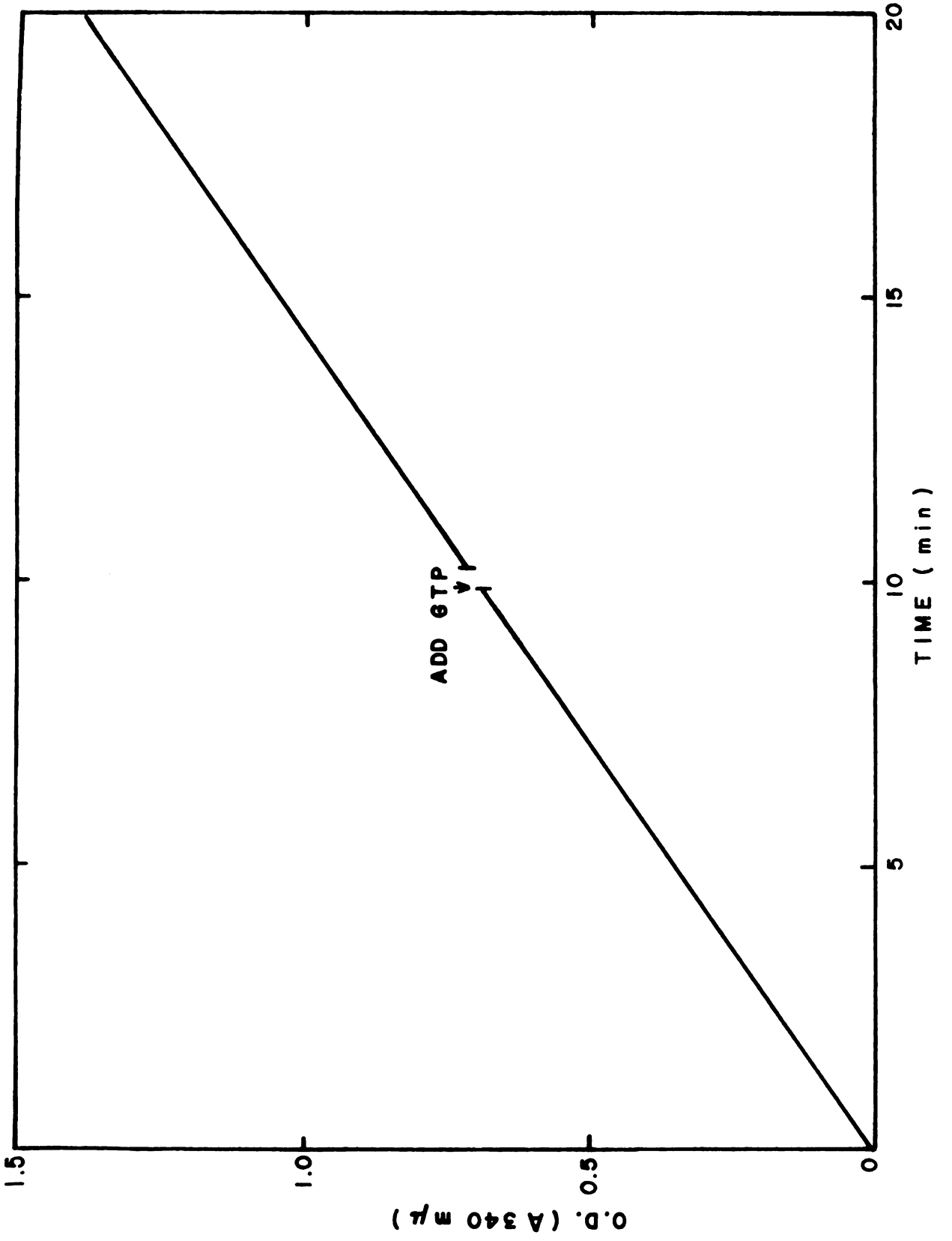
TABLE 3.--Additive activity of GTP and ITP as substrates.

Enzyme(s)	Substrate(s) (μmoles)	A_{680}- Control
G + PPase	0.4 GTP	0.195
G + PPase	0.4 ITP	1.173
G + PPase	0.4 GTP + 0.4 ITP	1.208

G: Enzyme preparation from Sephadex G-100 filtration.

The UDP-glucose pyrophosphorylase coupling assay was also applied to examine the additive activity assay by using the supernatant of heated gel-purified enzyme (heating at 65°C for 15 min. to remove endogenous inorganic pyrophosphatase activity). GTP was added to the reaction mixture after the reaction had already been preincubated for 10 min. in the presence of ITP as substrate. There was no increase of activity (NADPH production) with the addition of GTP (Figure 16).

Figure 16. Additive activity assay by UDP-glucose-pyrophosphorylase coupled system. Tracing of the rate of NADPH production at 340 mμ with a Gilford recorder was recorded. GTP was added to the reaction mixture after the reaction already preincubated for 10 min. in the presence of ITP as substrate (see "Criteria for a Single Enzyme Molecule" in the text).


1.5
1.0
0.5
0
O.D. (A 340 mμ)
ADD GTP
5
10
15
20
TIME (min)

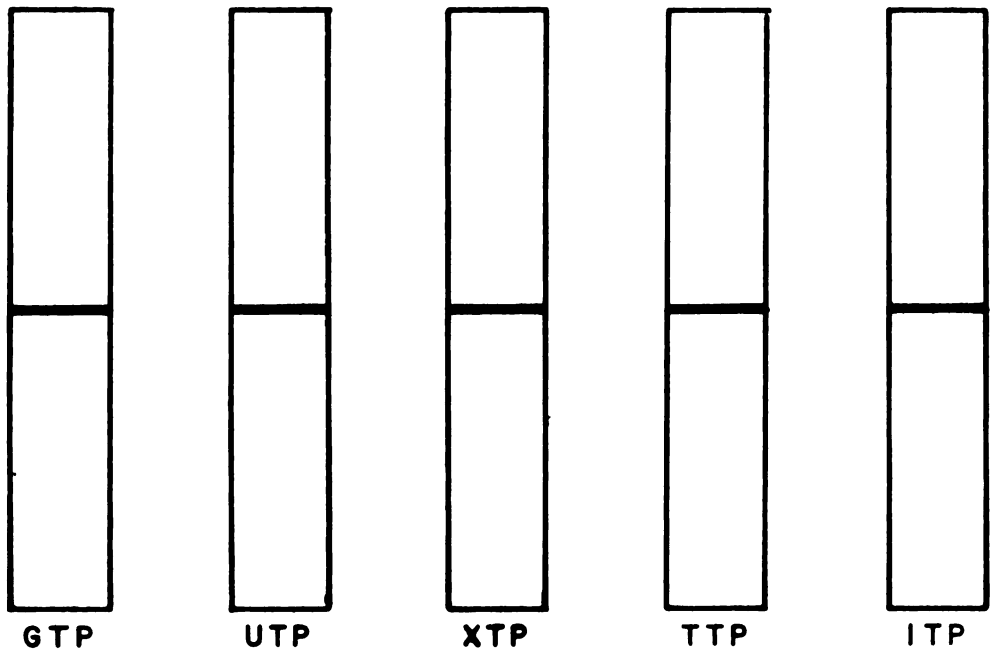
Histochemical identification of pyrophosphohydrolase activity in the polyacrylamide gel was also developed to examine the number of pyrophosphohydrolase in the preparation. The results of the staining of pyrophosphohydrolase activity in polyacrylamide gel (as described under "Analytical Method") using different nucleoside triphosphates as substrates is shown in Figure 17. Gel purified enzyme was used for this study. Presence of the single and same white precipitate band observed in each gel further suggested that there is a single enzyme with multiple specificity.

J. Preliminary Characterization of the Reaction of Nucleoside Triphosphate Hydrolysis by Pyrophosphohydrolase

The pyrophosphohydrolase reported here was initially detected by its ability to release P_i from GTP (58).

Time course and substrate concentration curve of the red cell inorganic pyrophosphatase have been studied by using the high speed supernatant fraction as the enzyme source. The rate of inorganic pyrophosphate hydrolysis was linear for at least 30 min. under the condition tested. A typical substrate concentration curve was observed with a saturation above 0.1 mM PP_i (unpublished data). Hereforth assays for pyrophosphatase were conducted at excess PP_i concentration. Inorganic pyrophosphatases have also been observed in the different stages of the purification

Figure 17. Histochemical identification of pyrophosphohydrolase activity in polyacrylamide gel with gel-purified enzyme using GTP, UTP, XTP, TTP and ITP as substrates (see detailed experimental procedure in the text).


GTP
UTP
XTP
TTP
ITP

of pyrophosphohydrolase, such as, 40 to 70% ammonium sulfate fractionation, streptomycin sulfate treatment, and Sephadex G-100 filtration steps. Preliminary studies indicated that inorganic pyrophosphatase eluted with almost the same eluate volume from the G-100 column as nucleoside triphosphate pyrophosphohydrolase. However, inorganic pyrophosphatase activities have been separated from pyrophosphohydrolase by DEAE cellulose chromatography (Figure 18). Pyrophosphohydrolase free of inorganic pyrophosphatase activities could be obtained by rechromatography (Figure 19) or by a modification of elution (i.e., prewashing the column with the buffer until the hemoglobin present in the sample began to emerge from the column and starting the elution with a linear NaCl gradient of from 0 to 0.1 M (200 ml each of the two respective media) as described before.

Critical understanding of the pyrophosphohydrolase reaction made it necessary to characterize the reaction products of ITP or GTP hydrolysis. Previous studies indicated that the supernatant fraction of gel purified enzyme heating at 65°C for 15 min. was devoid of pyrophosphatase activity (unpublished data). This preparation was used to identify nucleoside monophosphate and PP_i as reaction products of nucleoside triphosphate hydrolysis. Nucleoside monophosphate was identified by paper chromatography through comparison to reference standards (Figure 20).

Figure 18. Resolution of inorganic pyrophosphatase from pyrophosphohydrolase on DEAE cellulose. The sample was eluted using a linear NaCl gradient of from 0 to 0.2 M in 50 mM Tris-Cl (pH 8.0), 4 mM $MgCl_2$ and 5 mM DTT (200 ml each of the respective elution media). A 1.95 ml per fraction was collected. Fractions were analyzed for pyrophosphohydrolase and inorganic pyrophosphatase activities as described in the text.

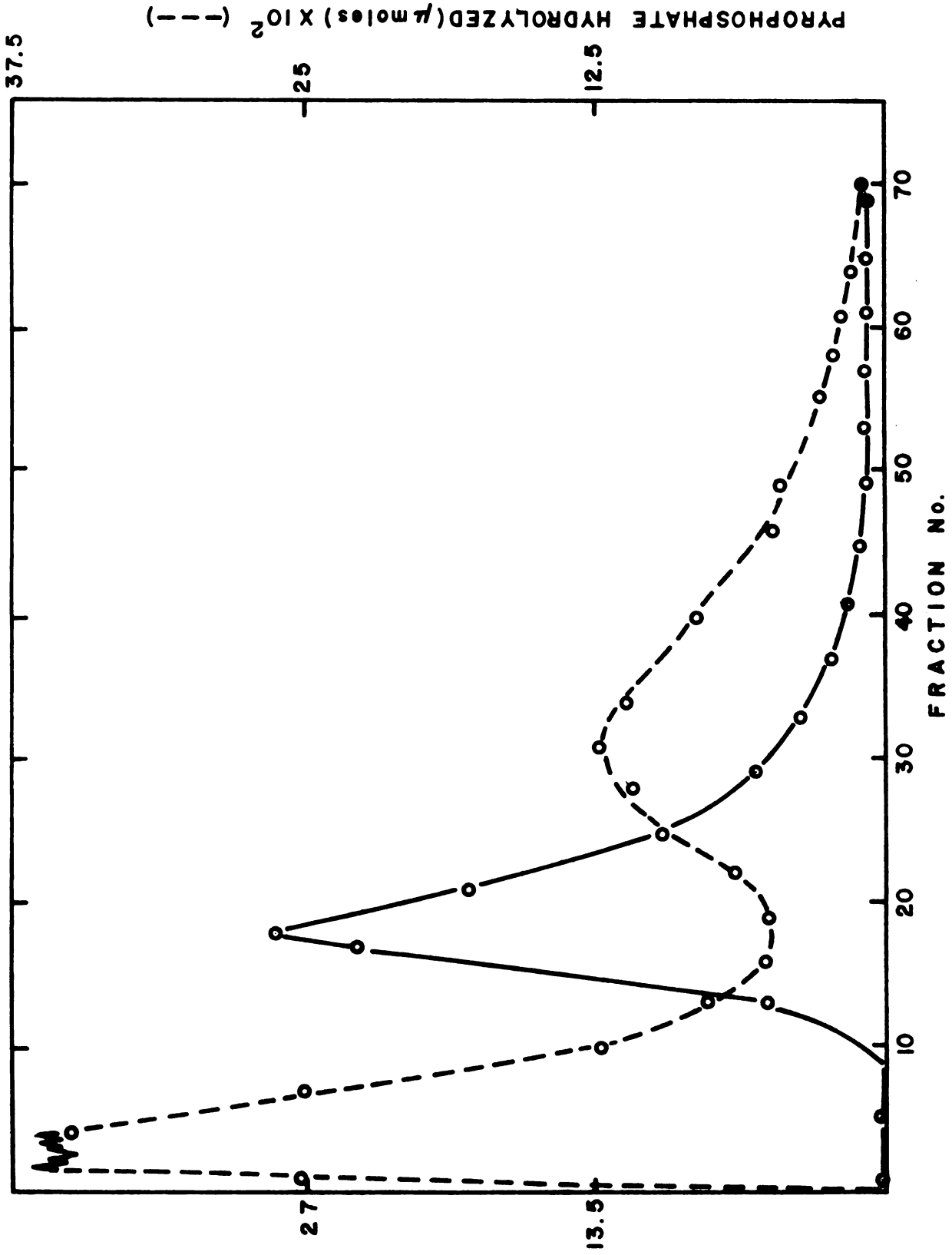

PYROPHOSPHATE HYDROLYZED (μ moles) X 10² (---)
37.5
25
12.5
70
60
50
40
30
20
10
FRACTION No.
PYROPHOSPHATE PRODUCED (μ moles) X 10² (——)
27
13.5

Figure 19. Rechromatography of the pooled and concentrated fractions containing pyrophosphohydrolase on DEAE cellulose. The condition of elution was identical to that as described in Figure 18. A volume of 50 ml, instead of 200 ml, of the respective elution media was used.

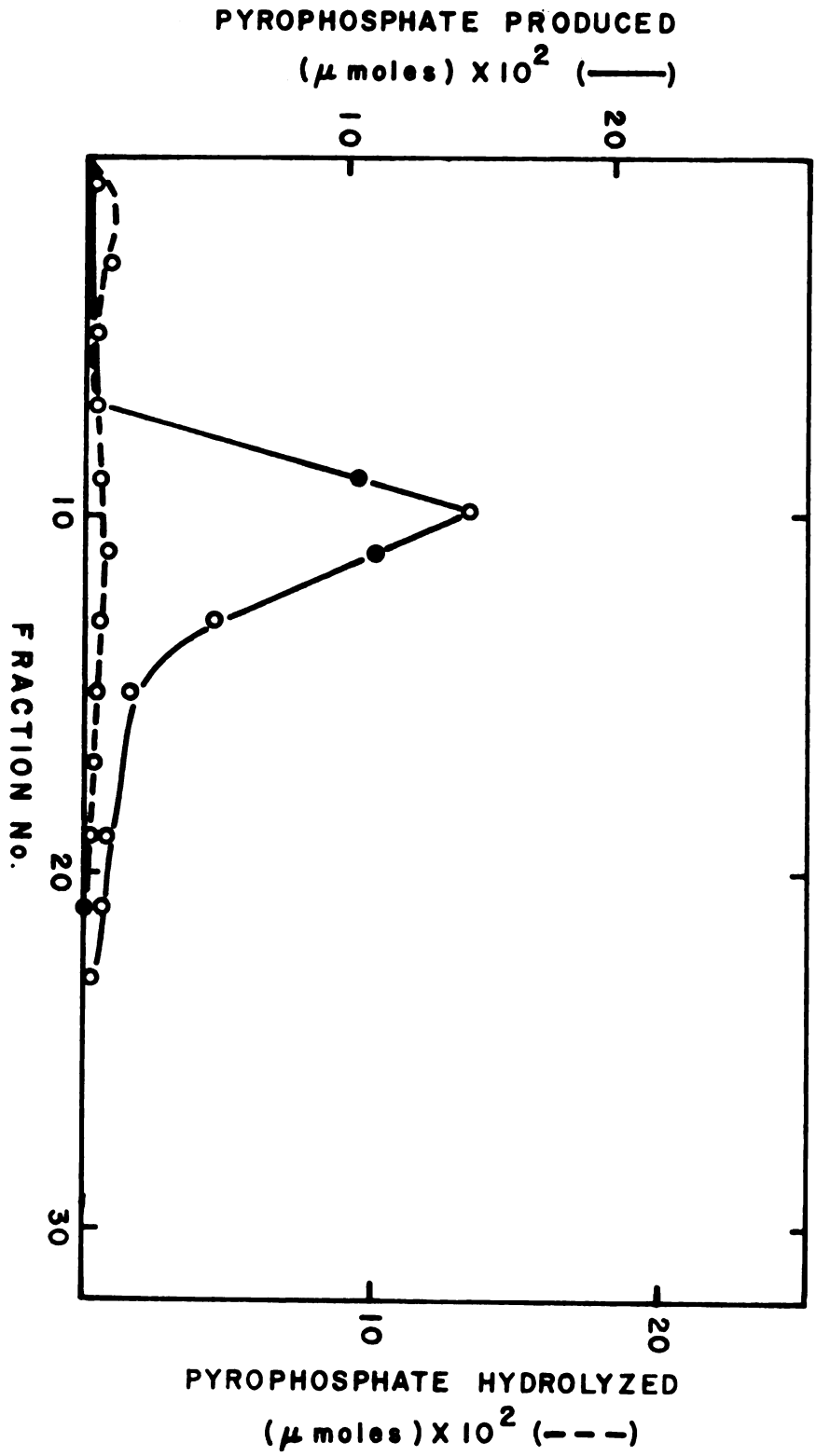
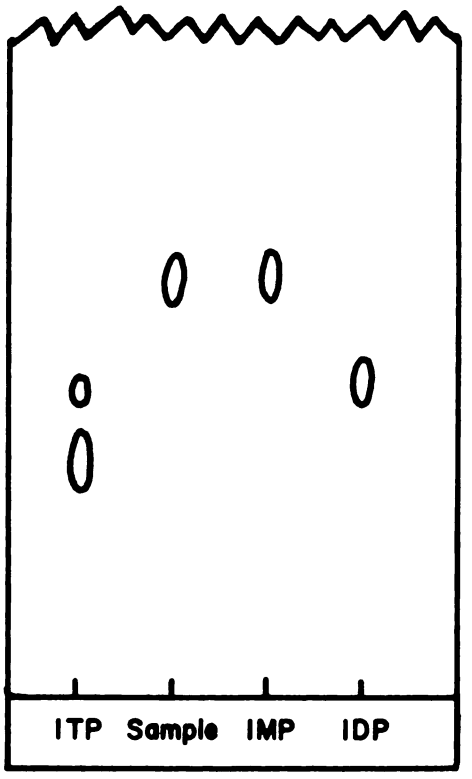

PYROPHOSPHATE PRODUCED
(μ moles) X 10² (——)
10
20
FRACTION No.
10
20
30
10
20
PYROPHOSPHATE HYDROLYZED
(μ moles) X 10² (– – –)

Figure 20. Comparison of the reaction product of ITP hydrolysis by nucleoside triphosphate pyrophosphohydrolase to the reference standards on Whatman No. 1 paper by descending chromatography with isobutyric acid: NH_4OH:H_2O (57:4:39) for 20 hrs. Nucleotides were identified under an U.V. lamp.


ITP
Sample
IMP
IDP

Inorganic pyrophosphate production was determined by NADPH production using UDPG-pyrophosphorylase coupling assay as described under "Analytical Method." It was observed that the heated gel-purified enzyme contained a highly active nucleoside triphosphate pyrophosphohydrolase activity which brought about the release of PP_i from ITP or GTP to produce NADPH in the coupled UDP-glucose pyrophosphorylase assay system. Interestingly, ATP could not be used as substrate of pyrophosphohydrolase to release inorganic pyrophosphate.

The reaction of pyrophosphohydrolase was proposed as follows:

$$NTP \xrightarrow{H_2O} NMP + PP_i \xrightarrow[\text{if any}]{\text{endogenous pyrophosphatase}} 2\ P_i$$

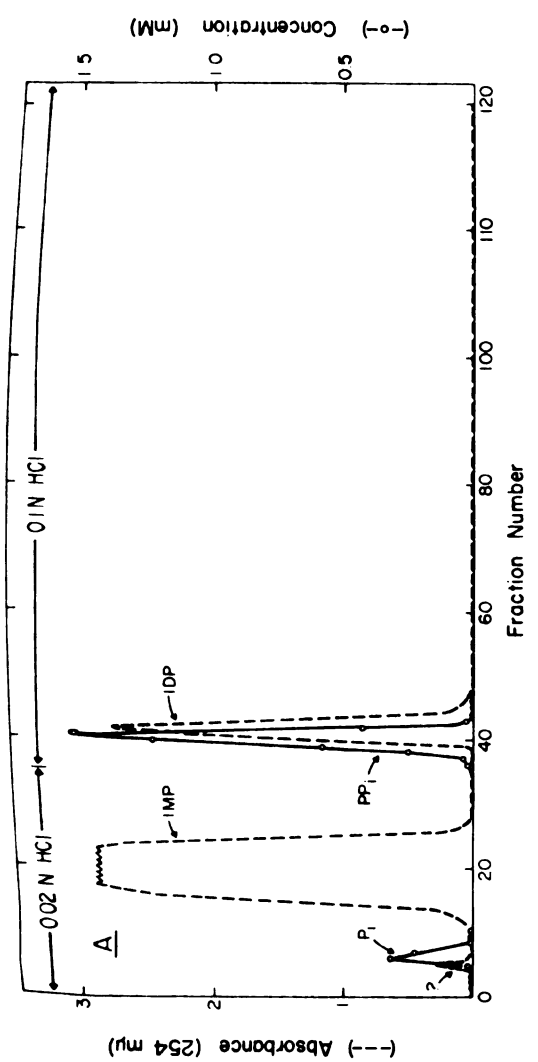
This proposal was further confirmed by the studies of stoichiometry and identification of the products of ITP hydrolysis using the technique of Dowex-I chromatography and the highly purified enzyme obtained from DEAE cellulose chromatography.

K. Stoichiometry and Reaction Products of ITP Hydrolysis

Highly purified enzyme obtained from DEAE cellulose chromatography, free from any detectable inorganic pyrophosphatase activity, was used for this study. A large scale reaction mixture (82 ml), lacking yeast

inorganic pyrophosphatase, was incubated at 37°C for 20 min. and the reaction was stopped by heating to 80°C for 3 min. The sample was then applied to a Dowex-I column. Chromatography and characterization of reaction products was performed as described under "Analytical Method." The result of the procedure is shown in Figure 21A. Inorganic phosphate and pyrophosphate are well resolved from one another, as are IMP, and IDP, ITP. ITP, which was expected near fraction 80, was absent because of its complete hydrolysis by pyrophosphohydrolase. A control reaction mixture without pyrophosphohydrolase was subjected to the same procedure of analysis (Figure 21B) in order to correct for impurities in the commercial ITP preparation and any possible degradation products of ITP caused by the treatment. The difference between these two analyses, which represented the stoichiometry of the pyrophosphohydrolase reaction with ITP as substrate, is summarized in Table 4. The reaction products of ITP hydrolysis by pyrophosphohydrolase were IMP and PP_i. For each μmole of ITP degraded, 1 μmole of IMP and 1 μmole of PP_i were produced. The reaction resulted in complete hydrolysis of ITP in this study.

Figure 21. Chromatographic analysis of the reaction products following ITP hydrolysis by pyrophosphohydrolase. Figure 21A illustrates the elution profile of the reaction products following incubation for 20 min. in the presence of pyrophosphohydrolase. Figure 21B illustrates the elution profile of a control incubation (no enzyme). See Methods for details of the column procedure.


A
0·02 N HCl
0·1 N HCl
IDP
IMP
PPi
Pi
(---) Absorbance (254 mμ)
(-o-) Concentration (mM)
Fraction Number

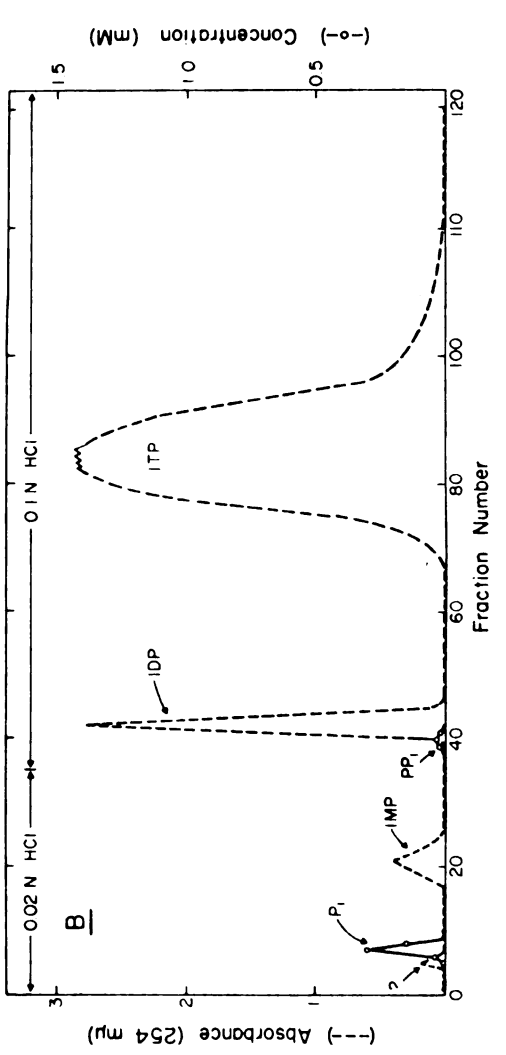

B
0·02 N HCl
0·1 N HCl
ITP
IDP
IMP
PPi
Pi
(---) Absorbance (254 mμ)
(-o-) Concentration (mM)
Fraction Number

TABLE 4.--Products and stoichiometry of ITP cleavage by nucleoside triphosphate pyrophosphohydrolase.

Compound	Plus Enzyme*	Minus Enzyme**	Differences
	µmoles	µmoles	µmoles
IMP	32.0	0.81	+ 31.2
IDP	4.37	4.40	- 0.03
ITP	0.00	31.9	- 31.9
PP_i	29.1	0.66	+ 28.4
P_i	4.21	3.61	+ 0.60
Unknown	0.21	0.15	+ 0.06

*See Figure 21A
**See Figure 21B

L. Kinetic Study of ITP and GTP Hydrolysis

In view of the possibly inhibitory effects of degradation products present in commercial preparation of ITP or GTP and the substrate inhibition observed previously, purified ITP or GTP from Dowex-I column was specially used for this study (Commercial preparation of ITP or GTP was routinely used throughout other analysis in this paper).

Initial rate data for the hydrolysis of purified ITP and GTP are shown in double reciprocal plots of Figure 22A and Figure 22B, respectively. Apparent Michaelis constants (Km) were as follows: 3.37×10^{-5}M for ITP and 4.0×10^{-4}M for GTP. The concentration of purified enzyme

Figure 22. Dependence of the rate of ITP (Figure 22A) or GTP (Figure 22B) hydrolysis on substrate concentration (mM). Assay conditions were described in the text (Analytical Methods). Velocity (v):μmoles pyrophosphate produced per 3.5 min. in ITP system, 15 min. in GTP system.

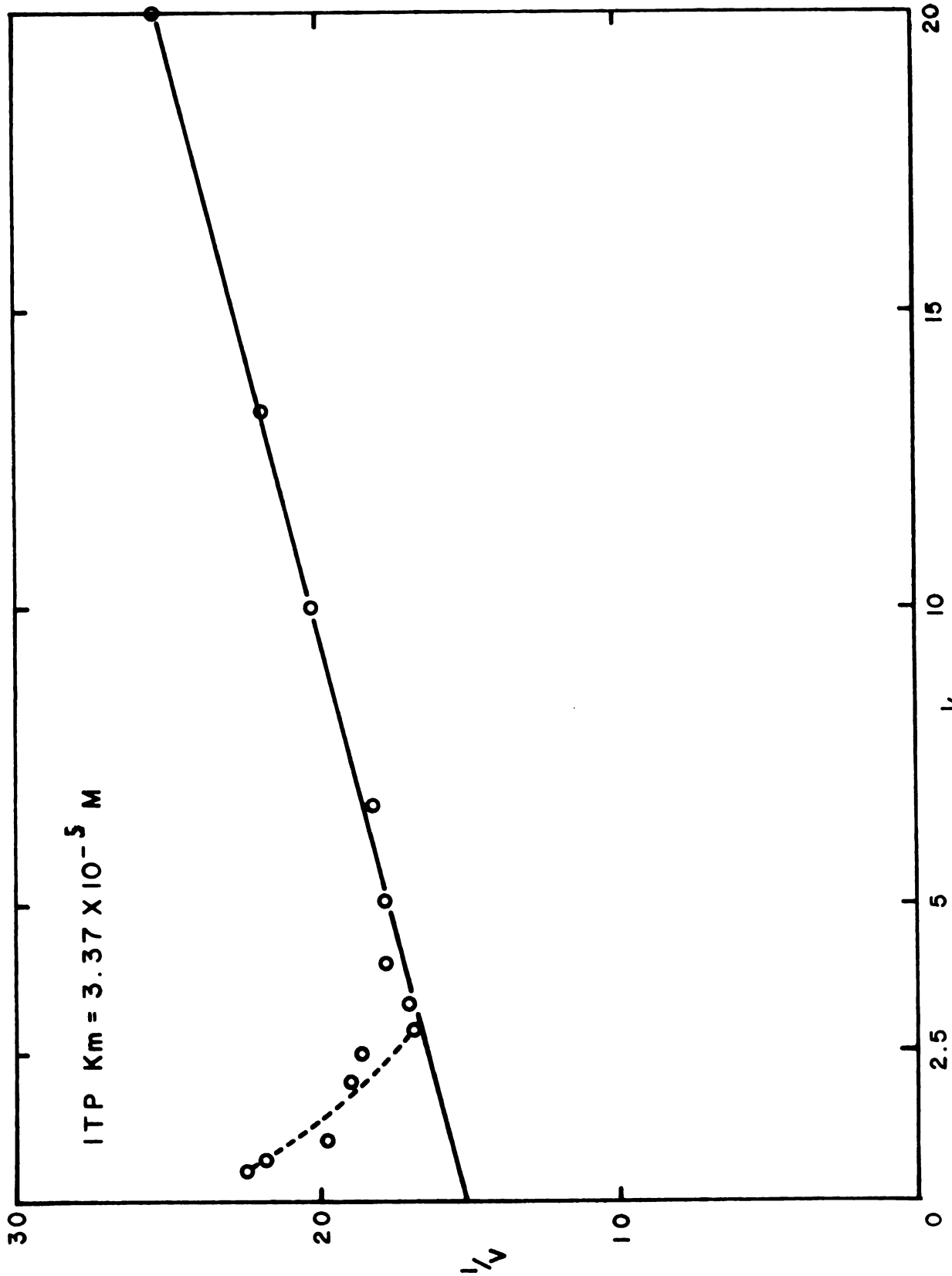


Figure 22A

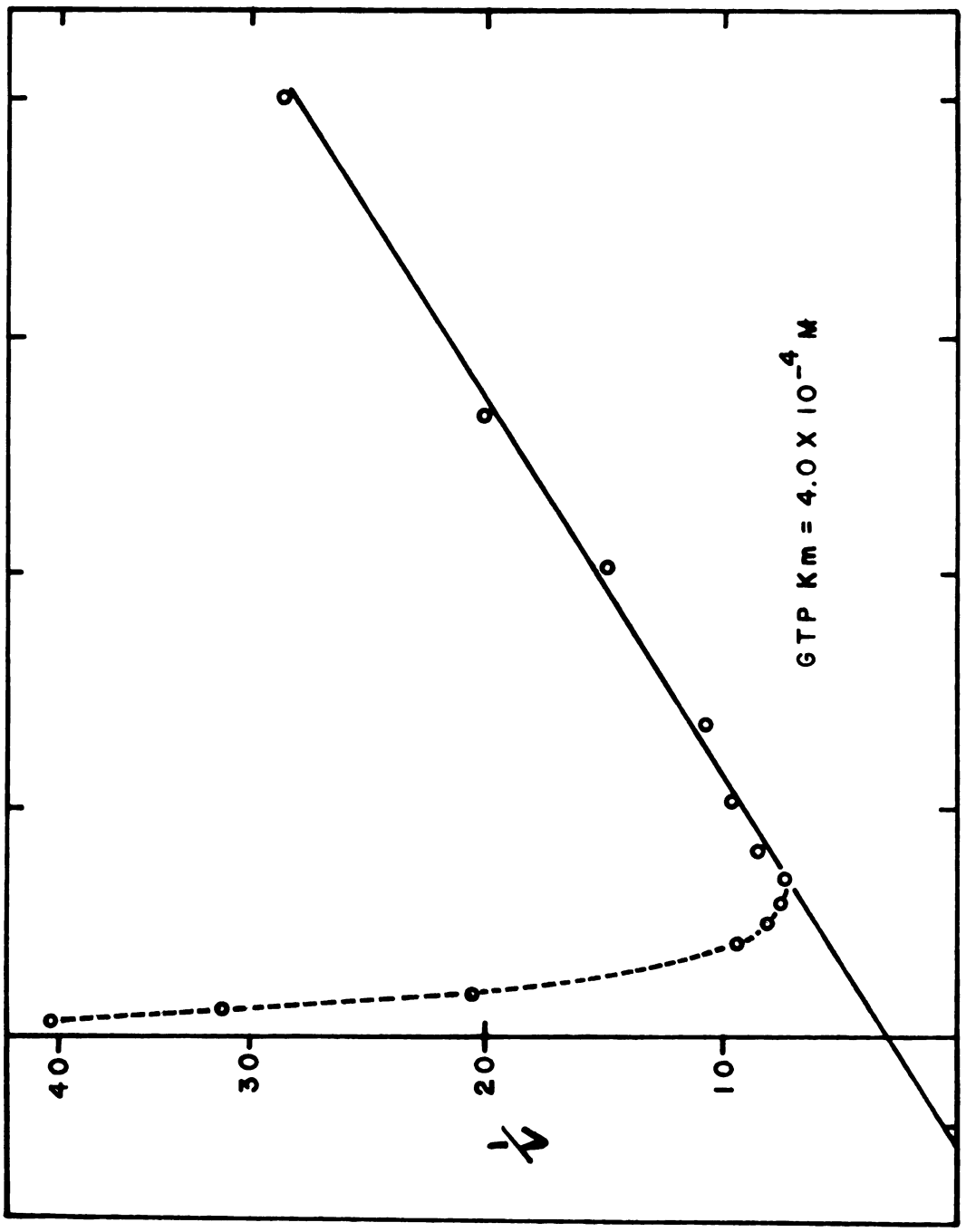


Figure 22B

from DEAE cellulose chromatography and the time period of incubation at 37°C have been adjusted to assure the measurement of true initial velocity. Vm of ITP and GTP was calculated at 0.0152 and 0.0009 μmoles PP_i hydrolyzed per min. per μℓ of pyrophosphohydrolase respectively. The detailed analytical procedure was described under "Analytical Method." The Lineweaver-Burk plots in Figure 22 indicate substrate inhibition at the higher levels of ITP or GTP which further confirm the previous observation of substrate inhibition.

M. Inhibition Studies

1. Nucleotide derivatives Among the different nucleotide derivatives tested, only IDP was observed to have potent inhibitory effect on pyrophosphohydrolase reaction (Table 5). Other nucleotide derivatives, including ADP or ATP, exhibited little effect on the activity of the enzyme.

2. Inorganic phosphate Different concentrations of KH_2PO_4 were added to the standard assay mixture in the presence of γ-labeled $IT^{32}P$ as substrates. After incubating at 37°C for 20 min. 0.1 ml of 1 N HCl was added to stop the reaction and the reaction mixture was then placed in an ice bath. Each reaction solution was treated with 0.1 ml of 20% suspension of acid washed Norit A to remove the nucleotides from the solution.

TABLE 5.--Inhibition of ITP hydrolysis by nucleotide derivatives. [Nucleotide derivatives were added to the assay mixture (see Methods) as shown. The activity observed using ITP alone was taken as 0% inhibition.]

Compound	Molarity	Inhibition (%)
ATP	10^{-3}	25
ATP	10^{-4}	8
ADP	10^{-3}	19
ADP	10^{-4}	6
AMP	10^{-3}	16
AMP	10^{-4}	8
Adenosine	10^{-3}	8
Adenosine	10^{-4}	1
IDP	10^{-3}	57
IDP	10^{-4}	40
IMP	10^{-3}	23
IMP	10^{-4}	11
Inosine	10^{-3}	17
Inosine	10^{-4}	7

A 0.5 ml aliquot of the supernatant was transferred to a planchet, oven dried, and counted with a Geiger Muller counter. Approximately 8% of inhibition per μmole of inorganic phosphate was observed (Figure 23).

N. Sucrose Density Centrifugation

The procedure for the estimation of the sedimentation coefficient of pyrophosphohydrolase was described under "Material and Analytical Method." The sedimentation profiles of the activity of pyrophosphohydrolase and reference markers are shown in Figures 24 and 25. Both gel purified enzyme and the enzyme from DEAE cellulose chromatography had the same sedimentation coefficient of 3.0 s. An estimated molecular weight of 37,000 was obtained for pyrophosphohydrolase. This molecular weight value was based on the assumption that all three protein molecules possess similar molecular shapes in the solutions used for centrifugation.

O. Molecular Weight Study by Sephadex G-100 Filtration

The molecular weight of 37,000 of pyrophosphohydrolase obtained from sucrose density gradient centrifugation was found to conflict with a molecular weight deduced from the elution profiles of the pyrophosphohydrolase from Sephadex G-100. Specifically, it was observed that G-100 gel filtration of the ammonium sulfate precipitated enzyme fraction or refiltration of

Figure 23. Effect of inorganic phosphate concentration on the activity of pyrophosphohydrolase. (See "Analytical Method" for details.)

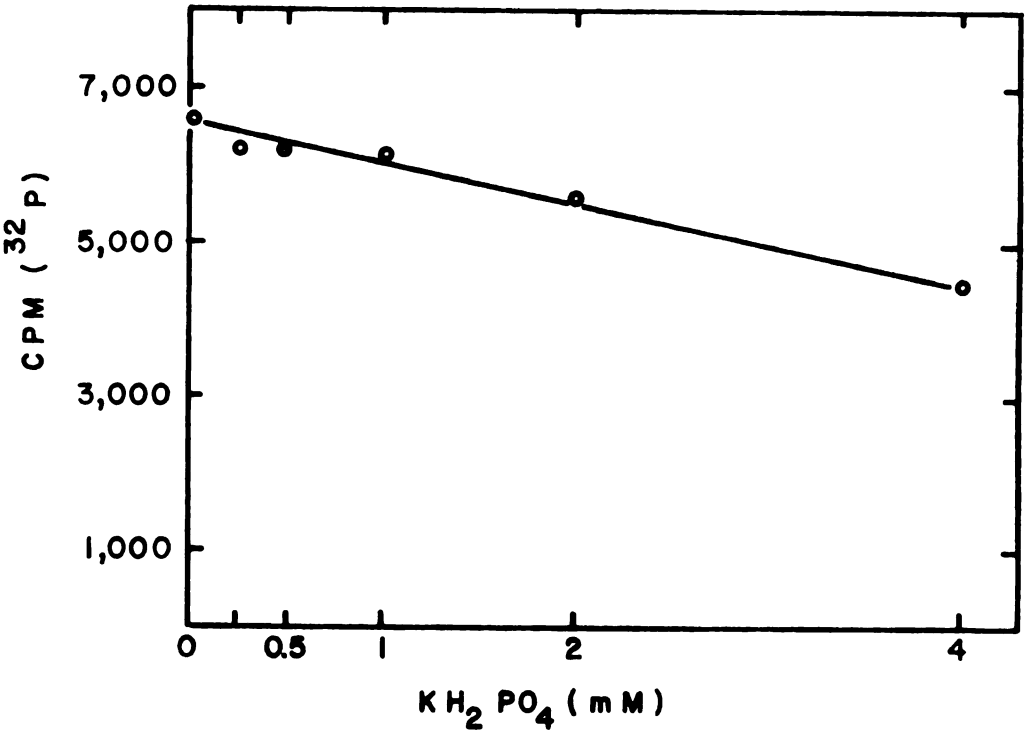
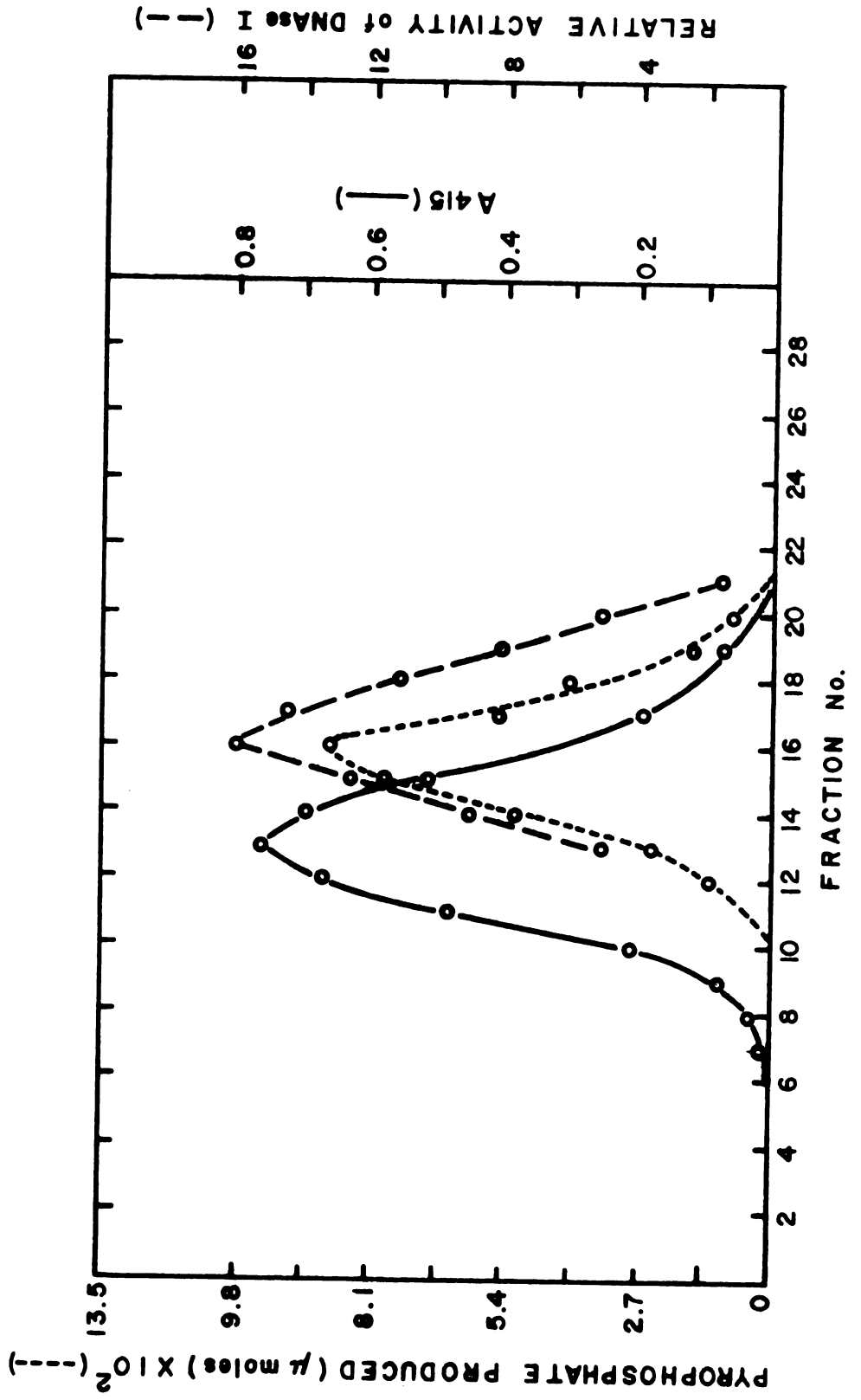

7,000
5,000
3,000
1,000
CPM (^{32}P)
0
0.5
1
2
4
KH_2PO_4 (mM)

Figure 24. Sucrose density centrifugation of the pyrophosphohydrolase obtained from Sephadex G-100 filtration. Centrifugation was carried out for 16 hrs. at 50,000 r.p.m. with a 5 to 20% of sucrose gradient (see "Analytical Method" for details).

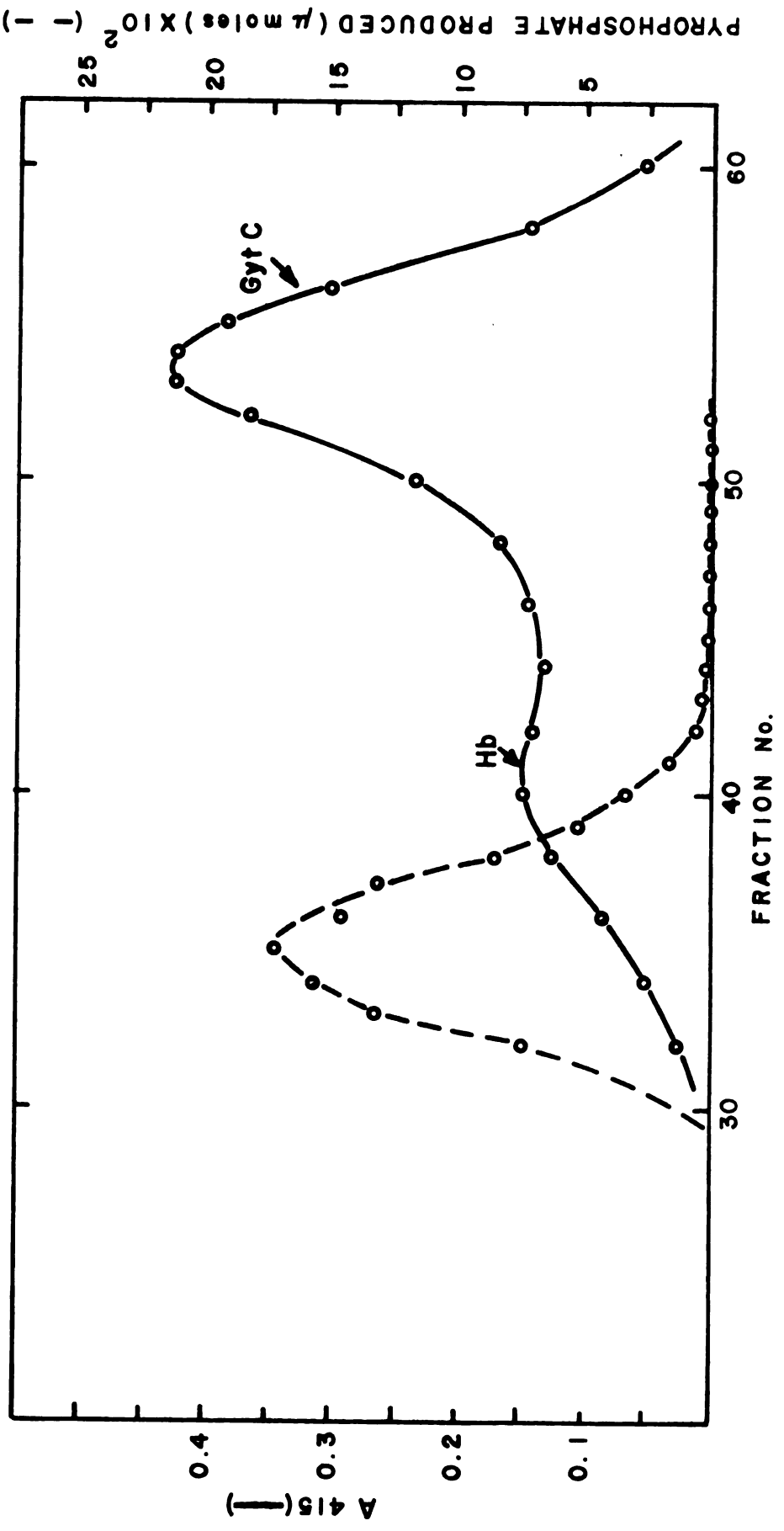
RELATIVE ACTIVITY of DNAse I (— —)
20
10
A415 (——)
10
0.5
2 4 6 8 10 12 14 16 18 20 22 26 28 30
FRACTION No.
27
13.5
PYROPHOSPHATE PRODUCED (μ moles) X10² (---)

Figure 25. Sucrose density gradient of the pyrophosphohydrolase obtained from DEAE cellulose chromatography (see legend of Figure 24 for details).


RELATIVE ACTIVITY of DNAse I (— —)
16
12
8
4
A415 (——)
0.8
0.6
0.4
0.2
PYROPHOSPHATE PRODUCED (μ moles) X 10² (----)
13.5
9.8
8.1
5.4
2.7
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
FRACTION No.

the gel-purified enzyme suggested a molecular weight bigger than that of hemoglobin (M.W. 65,000) for the pyrophosphohydrolase. Sephadex G-100 filtration of the highly purified enzyme obtained from preparative disc gel electrophoresis was carried out according to the method of Andrews (54). The result of this study is given in Figure 26. A molecular weight of approximately 80,000 may be calculated on the basis of this experiment. This result ruled out the possibility of any impurity bound tightly to the pyrophosphohydrolase during gel filtration of the crude enzyme preparation since the relative elution volumes of the crude enzyme and highly purified enzyme are identical. The discrepancy of the molecular weights observed between sucrose density centrifugation and Sephadex G-100 filtration may be attributed to the molecular shape of the protein. However, Andrews (59) has already shown that the apparent molecular weight of hemoglobin on Sephadex G-200 decreased with increasing dilution and approached an ultimate value that was one-half of that generally accepted for hemoglobin. It could be demonstrated by gel filtration on Sephadex 75 (60) and G-100 (61, 62), that the dissociation of oxyhemoglobin was promoted by high ionic strength and that ferrihemoglobin was sensitive to both ionic and pH changes. It may be necessary to use other independent reference standard instead of hemoglobin on molecular weight determination with gel filtration.

Figure 26. Elution diagram for separation of proteins on Sephadex G-100 column (25 x 46 cm) equilibrated with 0.05 M Tris-Cl buffer, pH 7.0 containing 10^{-3} M GSH. Experimental details are given in the text.


PYROPHOSPHATE PRODUCED (μ moles) X 10²
25
20
15
10
5
Cyt C
Hb
A 415 (——)
0.4
0.3
0.2
0.1
30
40
50
60
FRACTION No.

P. Isoelectric Point Determination

Figure 27 shows the pyrophosphohydrolase activity profile from an isoelectric focusing column (pH 3-10), (see details under "Analytical Method"). An isoelectric pH of 4.5 was obtained for pyrophosphohydrolase. A pI of 4.3 was determined with the narrow pH range (pH 3-6) carrier ampholytes (Figure 28).

Q. Occurrence of Pyrophosphohydrolase

The high speed supernatant fraction from rabbit reticulocytes had a specific activity (micromoles PP_i produced per mg of protein per 20 min.) of 0.266 $\pm$ 0.099 of the pyrophosphohydrolase present. High speed supernatant fractions from rabbit erythrocytes had levels of pyrophosphohydrolase comparable to that of rabbit reticulocytes. Human erythrocytes possessed approximately one-sixth of the activity of rabbit reticulocytes.

R. Attempted Binding of ITP-8-(^{14}C) or $^{32}PP_i$ to Pyrophosphohydrolase

Gel filtration technique was used in an attempt to detect the complexes of pyrophosphohydrolase with various substrates. These studies could be of value in investigations of the mechanism of action of the enzyme and in the identification of its active center.

Under the condition (see "Analytical Method") tested, no binding of either ITP-8-(^{14}C) or $^{32}PP_i$ to pyrophosphohydrolase was observed (Figures 29, 30).

Figure 27. Nucleoside triphosphate pyrophosphohydrolase activity and pH profile from an isoelectric focusing column. Details are given under "Analytical Methods." A pI value of 4.5 was obtained with a broad pH range (pH 3-10) of ampholine. A volume of 2 ml per fraction was collected.

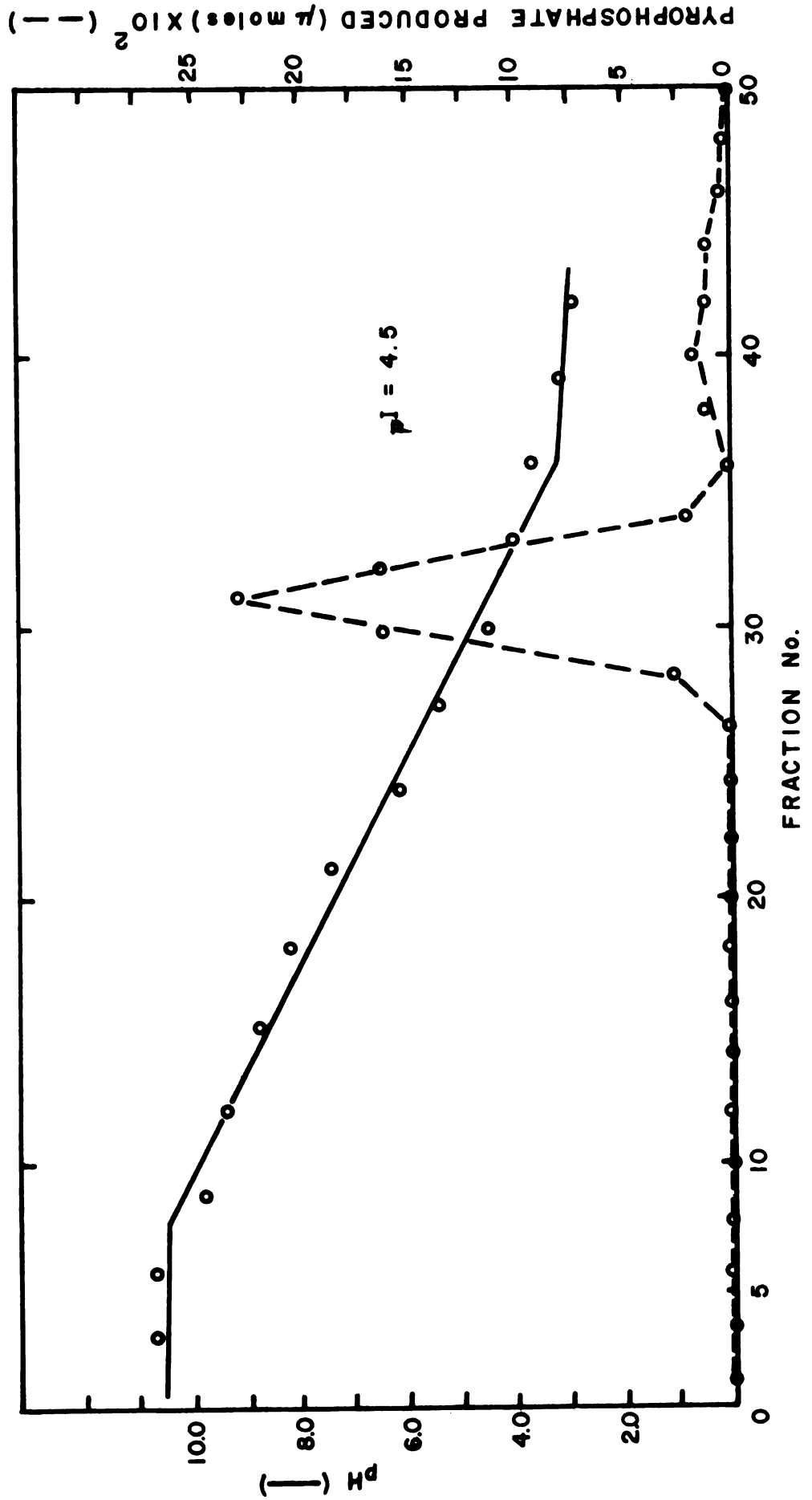

PYROPHOSPHATE PRODUCED (μ moles) X 10² (— —)
pH (——)
FRACTION No.
pI = 4.5
0
5
10
15
20
25
2.0
4.0
6.0
8.0
10.0
0
10
20
30
40
50

Figure 28. Nucleoside triphosphate pyrophosphohydrolase activity and pH profile from an isoelectric focusing column with a narrow pH range (pH 3 to 6) of ampholine.

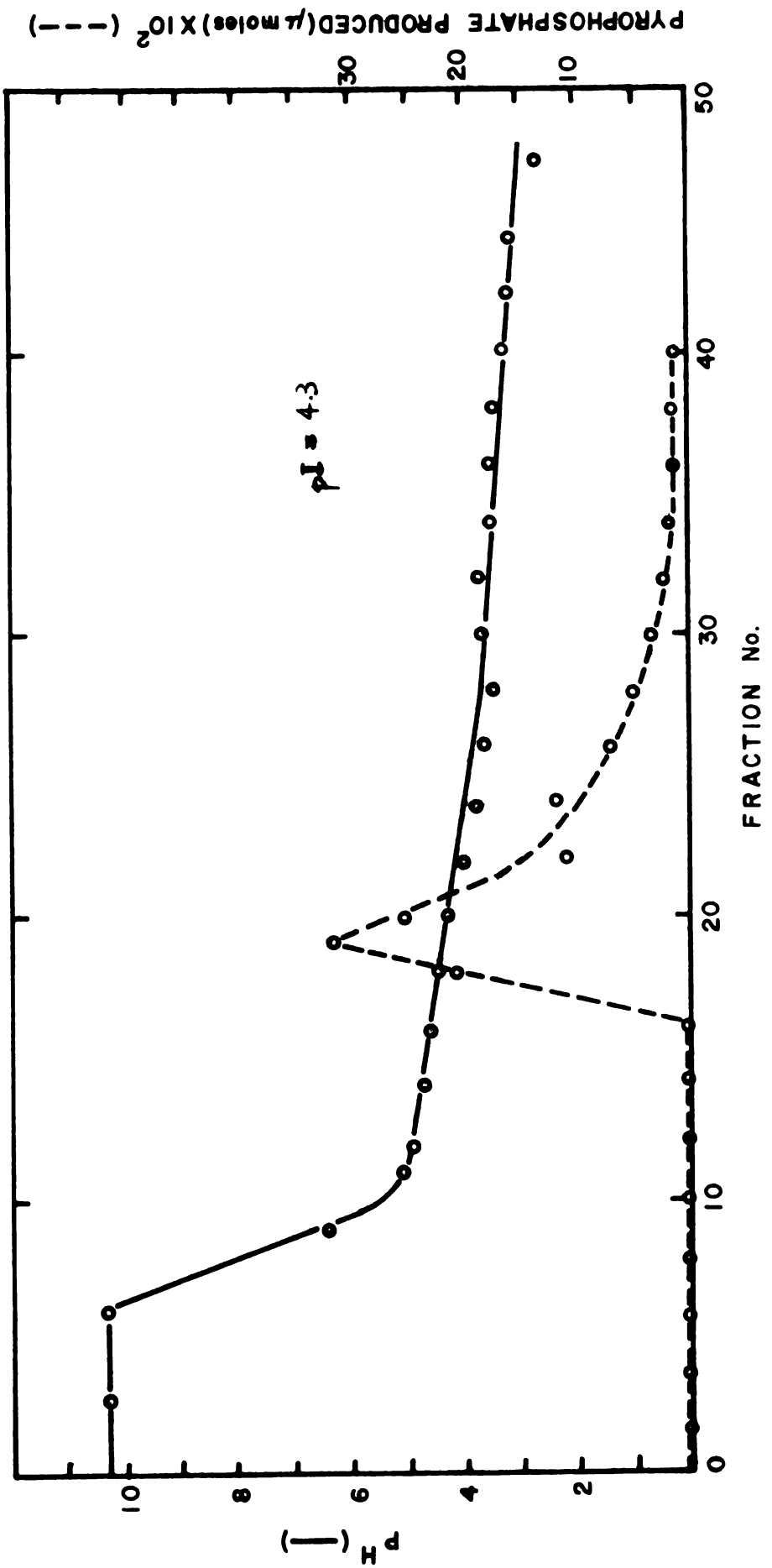

PYROPHOSPHATE PRODUCED(μ moles) X 10^2 (---)
30
20
10
50
40
30
20
10
0
pI = 4·3
FRACTION No.
p^H (—)
10
8
6
4
2

Figure 29. Profile of radioactivity (^{14}C) and relative activity of pyrophosphohydrolase after Sephadex G-25 column (see details in the text).

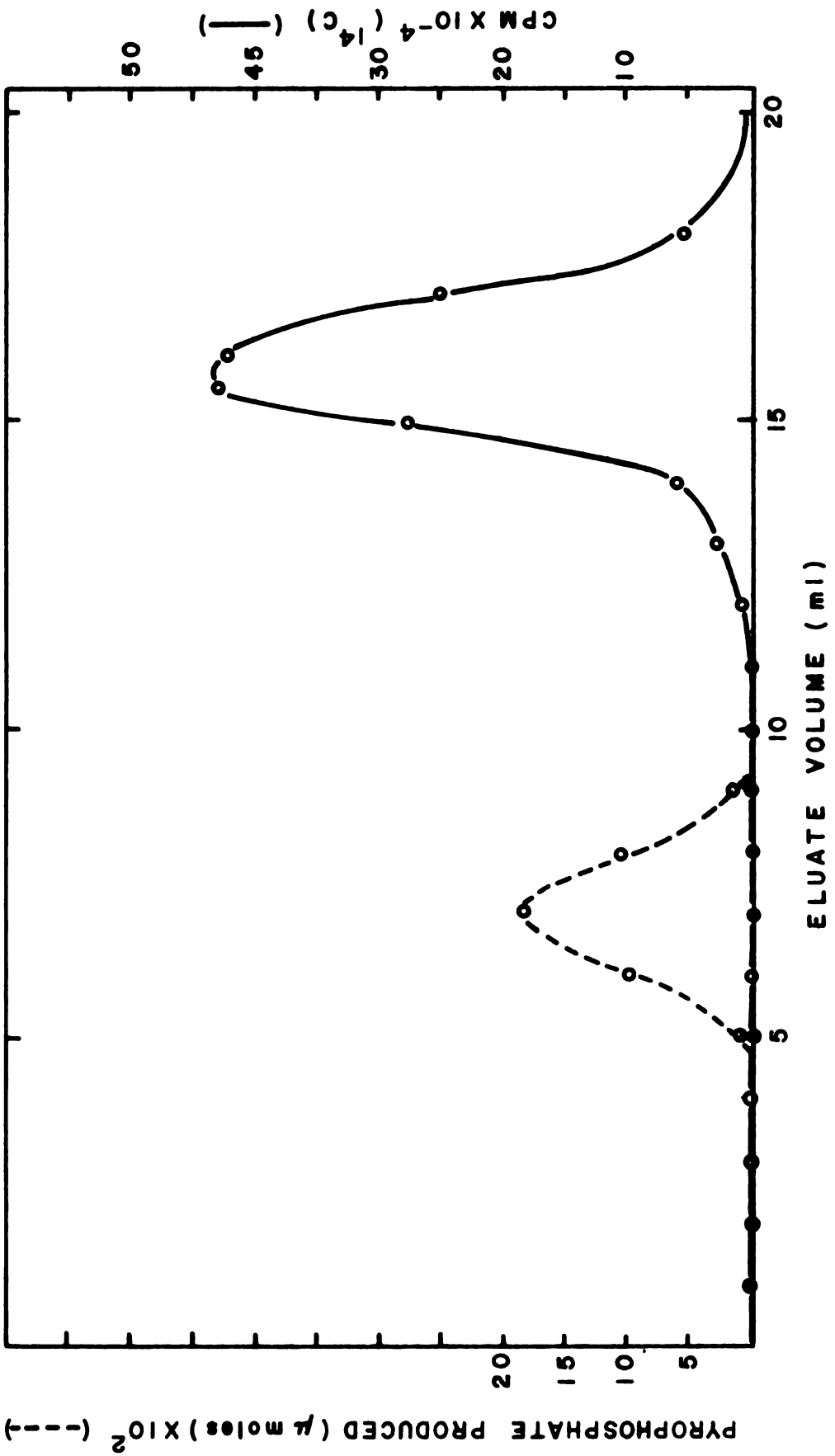
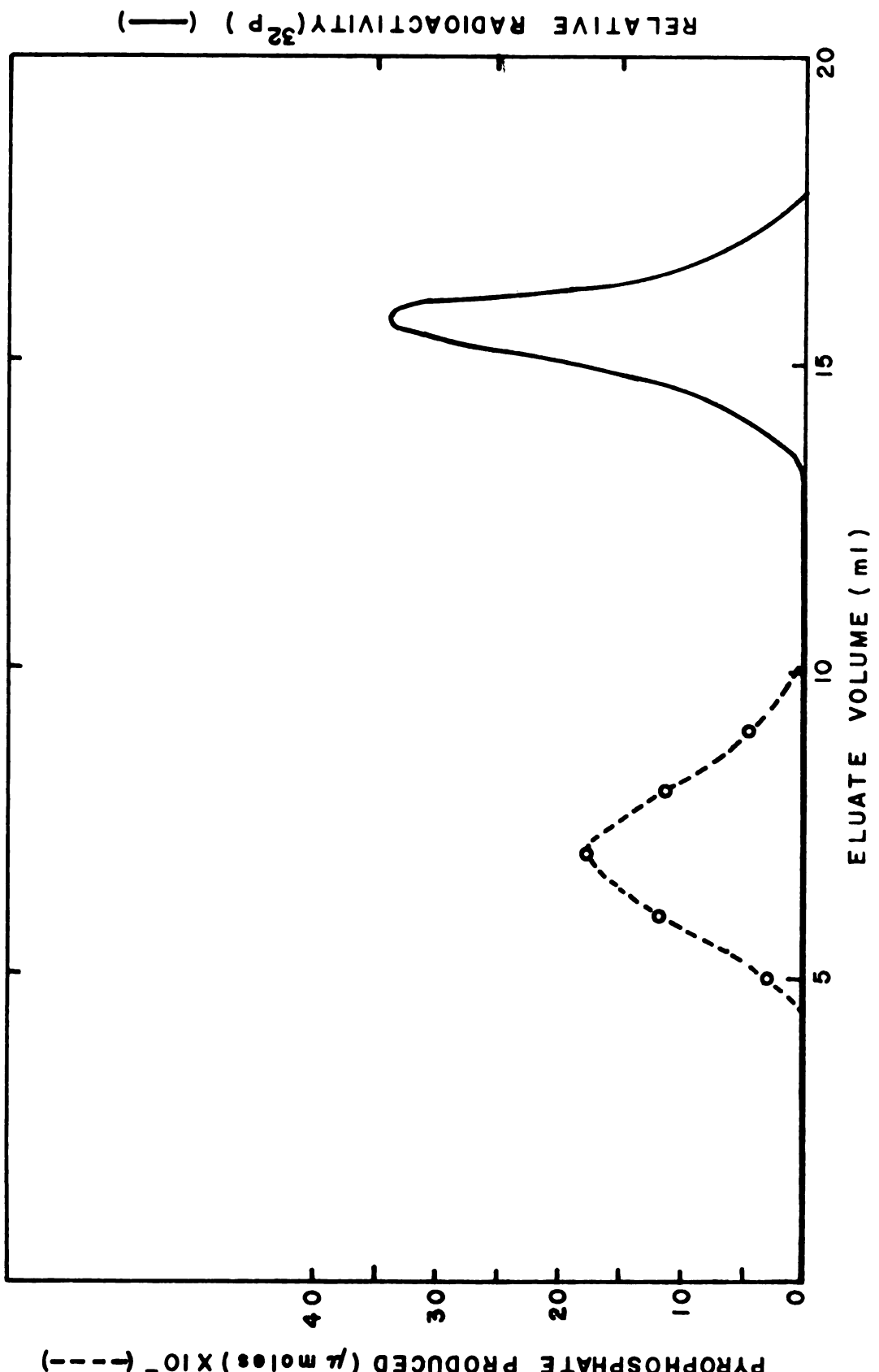

CPM X 10⁻⁴ (¹⁴C) (——)
50
45
30
20
10
20
15
10
5
ELUATE VOLUME (ml)
PYROPHOSPHATE PRODUCED (μ moles) X 10² (----)
20
15
10
5

Figure 30. Profile of radioactivity (^{32}P) versus pyrophosphohydrolase activity in Sephadex G-25 column. $^{32}PP_i$ and equal concentration of ITP were incubated with pyrophosphohydrolase for 20 min. and applied to the column. Radioactivity was recorded with a radioactivity ratemeter connected to a Gilford recorder.


RELATIVE RADIOACTIVITY (^{32}P) (——)
PYROPHOSPHATE PRODUCED (μ moles) X 10^2 (- - -)
0
10
20
30
40
5
10
15
20
ELUATE VOLUME (ml)

S. Studies of the Possible Functions of Pyrophosphohydrolase

1. Polymerization of nucleoside triphosphates

Possible involvement of pyrophosphohydrolase in the polymerization of nucleoside triphosphates and releasing PP_i as by-product was studied by incubating GTP-8-(^{14}C) with gel purified enzyme for different time periods followed by analysis of the trichloracetic acid precipitable material from the reaction mixture (Table 6). No polymerization was detected. The lack of pyrophosphohydrolase participation as an RNA polymerase was confirmed by the more recent study of the stoichiometry and reaction products of ITP hydrolysis as described before (Figure 21A & B).

TABLE 6.--Polymerization study. [Pyrophosphohydrolase was incubated with GTP-8-(^{14}C) at 37°C for various time periods. TCA precipitable materials were analyzed for radioactivity.]

Incubation Time (min.)	*CPM
0	718
5	468
20	173
blank	47

*Average of analysis done in triplicate.

2. PP_i transfer reactions

The idea behind this study was to search for a possible function of pyrophosphohydrolase in carrying out a pyrophosphate transfer during

ITP biosynthesis. Such a reaction would be similar to the phosphate transfer reaction of nucleoside diphosphokinase (63). Since pyrophosphohydrolase was highly active in hydrolysis of ITP at pH 9.5 but not at pH 7.0, assay for the pyrophosphate transfer reaction was carried out at pH 7.0. The possible existence of the following two reactions was examined:

1. IMP + ATP $\rightleftharpoons$ ITP + AMP

2. IMP + GTP $\rightleftharpoons$ ITP + GMP

Each was studied from left to right by incubating of 2 μmoles of nucleoside monophosphates (IMP and GMP were labeled in carbon 8) with 2 μmoles of corresponding nucleoside triphosphates in a reaction mixture containing 50 mM Tris-Cl (pH 7.0), 1 mM $MgCl_2$ and DTT and the enzyme from preparative disc gel electrophoresis. The reaction mixture was incubated at 37°C for 20 or 30 min. The reaction mixture was then directly layered onto a Dowex-I column (1 x 10 cm). The column was washed with H_2O and eluted with 0.1 N HCl. Fractions (4 ml each) were collected and the eluents were measured at 254 mμ with a recording ultra-violet analyzer connected to the column for the formation of ITP.

Radioactivity was counted in the Brays solution (60 g Naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml MeOH,

20 ml ethylene glycol, add dioxane to 1 liter). The results so far obtained revealed no involvement of pyrophosphohydrolase in the reaction of PP_i transfer at pH 7.0.

DISCUSSION

The nucleoside triphosphate pyrophosphohydrolase reported here was initially detected by its ability to release P_i from GTP (58). "GTPase" activity was identified from ribosomal and high speed supernatant fractions. Further studies, however, have revealed that the high speed supernatant fraction of rabbit reticulocyte lysates contained a highly active nucleoside triphosphate pyrophosphohydrolase as well as large amounts of inorganic pyrophosphatase activity. The release of P_i from nucleoside triphosphates was shown to be the result of the combined action of pyrophosphohydrolase and the inorganic pyrophosphatase. The nucleoside triphosphate pyrophosphohydrolase of this study is undoubtedly different from the ribosome-dependent GTPase involved in protein biosynthesis (64, 65). A detailed investigation of the substrate specificity of pyrophosphohydrolase revealed that several nucleoside triphosphate compounds are more rapidly hydrolyzed than GTP.

The presence of "ITPase" activity in human erythrocytes (17) and rabbit erythrocytes (6) has been noted.

However, the reaction products of the "ITPase" reactions were not identified in either report. On the basis of studies described in this thesis, these "ITPase" activities are now thought to be the result of the combined activity of the nucleoside triphosphate pyrophosphohydrolase and the red cell inorganic pyrophosphatase since a rather active pyrophosphatase has been reported in erythrocytes (66, 57, 72). In this study, substantial amounts of inorganic pyrophosphatase were also detected in rabbit reticulocytes. The nucleoside triphosphate pyrophosphohydrolase from rabbit reticulocytes apparently represents an enzyme which has not been identified, purified and characterized previously.

dCTP-cleaving enzyme of phage infected Echerichia coli (47) and nucleoside triphosphate pyrophosphohydrolase of the plasma membrane of the liver cell (67) are among those enzyme possessing certain properties similar to the red cell pyrophosphohydrolase. Each has a high pH optimum (9.0, 9.8 and 9.75 respectively) and a requirement for Mg^{++} in order to release PP_i from a nucleoside triphosphate, although the substrate specificities of all three enzymes are different. The formation of pyrophosphate from ATP in the presence of a snake venom has been reported (68, 69).

Almost simultaneously with the publication of the existence of the pyrophosphohydrolase described in this thesis (20), a nucleoside pyrophosphohydrolase from red

cells was reported by Hersko et al. (70). This enzyme degraded nucleoside triphosphates to nucleoside monophosphates and inorganic pyrophosphate. Its pH optimum was 8.7. The Km values for ITP and GTP were estimated at 3×10^{-5}M and 8×10^{-4}M, respectively. Apparently, the enzyme is identical to the one reported here. However, several properties they observed are at odds with our own observations. For example, there was no substrate inhibition observed in their case and IMP was found by them to inhibit the splitting of GTP but not of ITP. Crude enzymes and different assay conditions (different pH, for example) were used for their investigation. Studies conducted using the assay conditions described by Hersko et al. revealed that substrate inhibition was present in their assays. Considering the range of substrate concentrations used to establish the values, the Km values reported by these workers must be considered as being in doubt.

The pyrophosphohydrolase from rabbit reticulocytes, described in this thesis, catalyzed the hydrolysis of the purine nucleoside triphosphates, ITP, dITP and XTP preferentially, but degraded GTP and dGTP to a lesser extent. The hydrolysis of UTP and dUTP demonstrated that the substrate specificity of pyrophosphohydrolase was not limited strictly to purine nucleotides. Deoxyribo- and ribonucleoside triphosphates of either purine or pyrimidine

base possessed similar rates of hydrolysis, which implied that the 2' hydroxyl groups of the sugar moiety was not critical for recognition of the substrate by the enzyme. A keto group of the position 6 in the purine ring was the most important structural requirement noted for pyrophosphohydrolase activity. Substitution by an amino group in the 6 position resulted in complete loss of ability to act as a substrate for the pyrophosphohydrolase.

It appears that the ability of nucleoside triphosphate pyrophosphohydrolase to hydrolyze the various substrates is inherent in the same enzyme molecule. This conclusion was obtained by the study of catalytic activity ratio of ITP/GTP/XTP at different stages of purification, the additive activity study and the histochemical staining of pyrophosphohydrolase activity in polyacrylamide gel using different substrates.

As was already mentioned, the pyrophosphohydrolase can hydrolyze ITP or GTP. In this respect, it may be worth mentioning that the derivatives of hypoxanthine and guanine have been noted to serve alternately for the substrate of several different enzymes such as hypoxanthine-guanine phosphoribosyl transferase, nucleoside phosphorylase, succinyl CoA synthetase and phosphoenolpyruvate carboxylase. The relative activities of guanosine and ionsine triphosphates in the phosphenolpyruvate carboxylase reaction have been shown to depend on the concentration of

nucleotides (73). At low concentrations (5×10^{-6} to 1×10^{-5} M) GTP was approximately five times as active as ITP in catalyzing the $^{14}CO_2$ exchange reaction, but at higher concentration (1×10^{-3} M) ITP was somewhat more effective than GTP. It appears that hypoxanthine and guanine may possess a similar molecular structure and may be used as substrate alternately in the same enzymatic pathway of metabolism.

The discrepancy between the molecular weights determined from sucrose density centrifugation and observed from gel filtration of Sephadex G-100 column may be attributed to the molecular shape of the protein as already mentioned. As hemoglobin was known not to be a good standard for the determination of molecular weight on gel filtration by virtue of the effects of dilution, oxygenation and oxidation (59, 62), other reference standard must be used for the gel filtration study. Amino acid analysis of the purified enzyme, SDS (sodium dodecyl sulfate) gel electrophoresis and ultracentrifugation studies (if the amounts of the enzyme allow) of the homogeneous enzyme may supply a more clear answer concerning the true molecular weight of the pyrophosphohydrolase.

From the view of the energetics of the hydrolysis of ITP by pyrophosphohydrolase, overall reversal of ITP hydrolysis would not be expected. For example, Figure 21A indicated the failure of the accumulating product to prevent the reaction from proceeding to completion.

Preliminary experiments were conducted in order to ascertain whether a pyrophosphohydrolase catalyzing exchange of $^{32}PP_i$ into ITP could be demonstrated. Under the conditions of the experiment similar to those used in the standard enzyme assay, no detectable exchange between $^{32}PP_i$ and ITP was found (unpublished data). However, the results could not rule out the existence of the possible exchange between PP_i and ITP in the presence of a suitable acceptor (other than H_2O) for ITP.

There are two technical problems which have been inherent in the study of pyrophosphohydrolase. First, the amount of nucleoside triphosphate pyrophosphohydrolase present in rabbit red blood cells is low (approximately 0.4 mg pyrophosphohydrolase from the preparation of DEAE cellulose chromatography per rabbit). Second, the standard assay for enzymatic activity is an end-point assay which imposed limitations on the kinetic studies of pyrophosphohydrolase reaction. True initial velocity only could be obtained at low O.D. reading with a colorimeter. Modifications of the assay system have been attempted. Direct quantitation of phosphate-molybdate complex with a spectrophotometer, or coupling of the pyrophosphohydrolase reaction with inosine monophosphate dehydrogenase (74) proved unsatisfactory. The requirement of IMP dehydrogenase for a high concentration of KCl makes it incompatible for coupling to the pyrophosphohydrolase. However, methods for the purification

of and kinetic studies of IMP dehydrogenase from different sources have been reported more recently. Therefore, the coupling of pyrophosphohydrolase with IMP dehydrogenase still is a potentially promising spectral assay. UDP-glucose pyrophosphohydrolase is far from an ideal enzyme to be coupled to pyrophosphohydrolase reaction because of the UTP produced in the coupled reaction being reused by pyrophosphohydrolase. The use of radioactive substrates for pyrophosphohydrolase will render great sensitivity for the assay as we know it. With the success of the synthesis of γ-labeled ITP recently and the known method for the synthesis of GTP by spinach chloroplasts (75) and others, the possibility of developing a sensitive assay for pyrophosphohydrolase with a low cost is greatly enhanced.

The assignment of a biological role to the highly potent red cell pyrophosphohydrolase is still an important question. ITP has been reported as a normal constituent of human erythrocytes (76, 77) only recently, probably because of its very low concentration (76, 78). ITP is present in amounts up to 4.5 μg of phosphorus per g of hemoglobin in human erythrocytes as determined by high voltage paper electrophoresis (i.e., approximately 1 μmole per 100 ml of packed red cells). ITP has been reported to be synthesized from inosine in some samples of fresh human erythrocytes (78). A 0.07% yield of ITP synthesis from 100 ml of human erythrocyte lysate incubated with 10 mM inosine was also reported (79). Bishop et al. (2) failed

to find ITP in normal human blood by anion exchange resin chromatography, probably because of its extremely low concentration in red cells. Several similar studies (80, 81) have indicated that ITP was not detected before or after the incubation of stored ACD (acid-citrate-dextrose) human blood with inosine. In addition, the high concentrations of ITP in human erythrocytes of some subjects has been related to a genetic deficiency in "ITPase" and a Mendelian autosomal trait has been suggested (77, 82).

GTP has been reported by Mendel et al. (4) to be present in human erythrocyte cells at a concentration of 5.7 $\pm$ 0.16 μmoles of GTP per 100 ml of cells. The possibility exists that the physiological substrate of nucleoside triphosphate pyrophosphohydrolase is also GTP, despite the fact that its reaction velocity and enzyme affinity constant are 10 fold lower than the corresponding parameters for ITP. The different levels of ITP and GTP in red blood cells may be resulted from the action of nucleoside triphosphate pyrophosphohydrolase in those cells.

From the pathways of purine nucleotide metabolism summarized in the "Introduction," it was recognized that IMP occupied an important position between biosynthetic and catabolic pathways. The role of IMP in the purine nucleotide catabolism was further emphasized by the inability of the red cells to carry out phosphorylation of

IMP to IDP and ITP. Consequently, IMP exposed permanently to the action of 5'-nucleotidase initiating the catabolic pathway and leading to the freely diffusible hypoxanthine.

The involvement of pyrophosphohydrolase in the turnover of purine nucleotides and the control of nucleotide metabolism are speculated. Its function in the electron transport system of mitochondrion may be ruled out, because the occurrence of the enzyme is only limited to the soluble portion of reticulocyte lysates.

It may be worthwhile to study the action of pyrophosphohydrolase on m-RNA with guanosine or inosine triphosphate at the 5' end. The enzymatic activity responsible for the removal of pyrophosphate of the 5'-end of m-RNA has not been characterized or identified except that Mitra et al. (83) have noted that two enzymatic activities from extracts of E. Coli catalyzed the following two reactions:

$$(1)\quad {}^{*}PPP\ G_pX_pY_pZ \text{-----} \xrightarrow[+\ \text{ribosome}]{\text{GTP terminum enzyme}} (?)\ PP_i\ G_pX_pY_pZ \text{ ---} + {}^{*}P_i$$

$$(2)\quad {}^{*}PPP\ A_pX_pY_pZ \text{-----} \xrightarrow[\text{enzyme}]{\text{ATP terminus}} PPPA_pX_pY_pA \text{ ---} + {}^{*}P_i$$

However, the reaction products of first reaction has not been characterized.

The findings of nucleoside tirphosphate pyrophosphohydrolase and the presence of ITP in red blood

cell provide a stimulation for studies of the pathway for the biosynthesis of ITP--a problem which has been under consideration in our laboratory for several years.

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