A NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE FROM RABBIT LIVER

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ABSTRACT

A NUCLEOSIDE TRIPHOSPHATE PYROPHOSPHOHYDROLASE
FROM RABBIT LIVER

By

Janet K. Wang

A nucleoside triphosphate pyrophosphohydrolase had been purified 600-fold from rabbit liver by a procedure combining heat denaturation, ammonium sulfate solubilization and DEAE-cellulose anion exchange chromatography. The final recovery was approximately 30%. The enzyme catalyzed the hydrolysis of ITP and certain other nucleoside triphosphates with the stoichiometric liberation of inorganic pyrophosphate and the corresponding nucleoside monophosphates as products. The enzyme catalyzed the hydrolysis at an optimum rate at pH 9.75 and required a sulfhydryl compound for activity. dITP was shown to be the most effective substrate for this enzyme, while ITP and XTP were hydrolyzed to a lesser extent. GTP, dGTP, TTP, and UTP were hydrolyzed at less than 10% of the rate of ITP. Neither ATP, CTP, dCTP, nor IMP could serve as substrates. Use of sucrose density centrifugation permitted estimation of the molecular weight

at about 37,000. Pyrophosphohydrolase appears to be a soluble enzyme occurring in the cytoplasm. Crude extracts of rabbit heart, pancreas, kidney, thymus, bone marrow, lung, spleen, brain and muscle possessed similar pyrophosphohydrolase activities. Brain tissue was found to be the most potent source of this enzyme, containing the highest catalytic activity per cell of any tissue tested.

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LIST OF ABBREVIATIONS

IMP, IDP, ITP, inosine 5' mono-, di-, and triphosphates, respectively. GMP, GDP, GTP, guanosine 5' mono-, di-, and triphosphates, respectively. AMP, ADP, ATP, adenosine 5' mono-, di-, and triphosphates. XTP, xanthosine triphosphate. CTP, cytidine triphosphate. UDP, UTP, uridine 5' di-, and triphosphates. dITP, dGTP, dCTP, dATP, deoxyinosine, deoxyguanosine, deoxycytidine and deoxyadenosine triphosphates respectively. TTP, thymidine 5' triphosphate. Pi, PPi, orthophosphate and pyrophosphate. GSH, glutathione. DTT, dithiothreitol. DEAE, diethylaminoethyl. PEI, polyethyleneimine. EDTA, (ethylenedinitrilo)-tetraacetic acid. DNase, deoxyribonuclease. RNase, ribonuclease.

INTRODUCTION

Inosinic acid is centrally located in the metabolic interrelationships of purines. Greenberg (1), and later Schulmand and Buchanan (2) reported that in a soluble enzyme preparation from pigeon liver capable of effecting the rapid synthesis of purines from small molecule precursors, the nucleotide inosine 5' monophosphate was the initial purine compound formed. Both of the primary nucleic acid purines, adenine and guanine, are derived in nucleotide form directly from IMP by amination and oxidation reactions (4,5,6,7,8).

The natural occurrence of inosine polyphosphates is still an open question. The chromatographic analyses for nucleoside polyphosphates in various sources (9,10,11,12) did not show the presence of IDP or ITP, although IMP was often found. It is quite possible that IDP and ITP are rapidly degraded to IMP and thus are found in very low concentration in the sources examined. However, Siekevitz et al. reported that an appreciable amount of ITP was found in rat liver mitochondria (13). Also, ITP is reported to be present in amounts up to 4.5 μg of phosphorus per gram

of hemoglobin in human erythrocytes as determined by high voltage paper electrophoresis (47).

Phosphorylation of nucleosides to nucleotides in the presence of ATP has been considered as an alternative means for addition to the nucleotide pool. Early investigators (14,15,16,17,18) have reported systems which catalyze the transphosphorylation of nucleoside mono- and triphosphate to give two nucleoside diphosphates. Myokinase (adenylate kinase), which catalyzes the reversible reaction ATP + AMP $\rightleftharpoons$ 2ADP, was among the first to be discovered (19,20). Besides AMP, ATP can phosphorylate GMP, CMP, and UMP, but not IMP. However, all the nucleoside triphosphates, including ITP, can phosphorylate AMP. Joklik (21) reported that preparations from yeast carry out a myokinase-like reaction on IDP: 2IDP $\rightleftharpoons$ IMP + ITP. However, the reaction has been tested only in the forward direction. Recently, Agarwal et al. (22) have isolated and identified a guanylate kinase from human erythrocytes that will also act on IMP. The K_m value is much higher for IMP than for GMP, thus the reaction preceeds less efficiently when IMP is used as a substrate. The reaction can be represented as follows: IMP + ATP $\rightleftharpoons$ ADP + IDP.

A transphosphorylation reaction between ATP and IDP to give ITP and ADP was indicated by the findings of Krebs and Hems(23). They reported that in suspensions of pigeon breast muscle both ATP and ITP rapidly incorporated ^{32}P added

as inorganic P^{32}-phosphate, the two terminal phosphate groups of ATP and the terminal phosphate group of ITP interchanged with inorganic phosphate, all at about the same rate. Berg and Joklik (24) obtained an enzyme from yeast and muscle which carries out the same reaction between ATP and IDP and also showed that UDP could participate in the reaction. The reaction is specific for a nucleoside triphosphate as phosphate donor and a nucleoside diphosphate as acceptor, hence the name nucleoside diphosphokinase is given to the enzyme.

The occurrence of the reversible reaction between ITP and AMP to give ADP and IDP was reported by Krebs and Hems (25) in sheep brain and lysed mitochondria of sheep liver. However, no reaction occurred between ATP and IMP incubated under the same conditions.

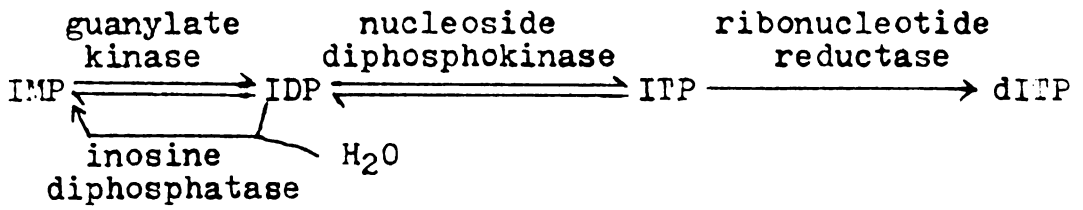
Plaut (26) concluded that a potent inosinediphosphatase existed in rat liver mitochondria which catalyzed the reaction: IDP + $H_2O \rightleftharpoons$ IMP + Pi. The enzyme will also hydrolyse GDP and UDP, but is inactive on ADP and CDP.

Reichard et al. (27,28,29) isolated a ribonucleotide reductase from E.coli which is highly specific for ribonucleoside diphosphates (CDP,UDP,ADP,and GDP), and the rate of their reduction is markedly affected by the presence of low concentrations of nucleoside triphosphates. However, the possibility that IDP may serve as a substrate was not tested. Recently, Blakley et al. (30,31) reported the isolation of a ribonucleotide reductase from Lactobacillus leichmannii which catalyzes

the reduction of GTP, ATP, CTP, ITP and to a much smaller extent, UTP. Although the reduction rate of ITP is small compared to GTP, however, the rate is much enhanced in the presence of dTTP.

Thus for E.coli and Lactobacillus leichmannii (32,33), it was concluded that the exclusive biosynthetic pathway yielding deoxyribonucleotides involves reduction of the corresponding ribonucleotides. That this pathway operates at least to some extent in animal tissues is evident from the results of tracer experiments in various mammalian organs (34), tumor cells (35), and chick embryo (36). Larsson and Neilands (37) have also shown by a labelling experiment that direct ribonucleotide reduction is the exclusive biosynthetic pathway of deoxyribonucleotides in regenerating rat liver.

As a summary of all the above biosynthetic and degradative pathways for inosine polyphosphates, it can be postulated that the formation and transformation of inosine phosphates and deoxyinosine phosphate may occur according to the following scheme:



Wyatt et al. (38) reported that the deoxyribonucleic acid of bacteriophages T2, T4, and T6, in contrast to the deoxyribonucleic acid of their host, has hydromethylcytosine

in place of cytosine. Later, Koerner et al. (39) characterized a dCTP-cleaving enzyme which catalyzes the hydrolysis of dCTP with the release of pyrophosphate and the corresponding deoxymononucleotides, thus excluding dCTP as a substrate for the deoxyribonucleic acid polymerase present in cell-free enzyme preparations of E.coli infected with T2 phage. At about the same time, Zimmerman and Kornberg (40) reported that a single enzyme specific for the deoxycytidyl residue was found in phage infected E.coli which catalyzed the cleavage of both deoxycytidine di- and triphosphates according to the equations:

$$dCDP + H_2O \rightleftharpoons dCMP + Pi$$

$$dCTP + H_2O \rightleftharpoons dCMP + PPi$$

Another similar situation is found in the transformation of uridine and deoxyuridine phosphates. It is known that dUTP can substitute for dTTP in the DNA polymerase reaction (41). However, with rare exceptions, uracil does not occur in DNA. The explanation for this appears to be the existence of a specific and powerful deoxyuridine triphosphatase which specifically cleaves dUTP to dUMP and pyrophosphate (42). Later, Bertani (43) postulated that the formation and transformations of deoxyuridine phosphates occurred according to the following scheme:

$$UMP \rightleftharpoons UDP \longrightarrow dUDP \rightleftharpoons dUTP \rightleftharpoons dUMP + PPi$$

The physiological function of deoxyuridine triphosphatase thus appears to be to prevent the accumulation of dUTP in the cell and involves the same principle as that underlying the action of the phage-induced deoxycytidine triphosphatase

mentioned above.

Recently an inosine triphosphate pyrophosphohydrolase has been purified and characterized in red blood cells of the rabbit (44). The enzyme was found to be most active towards the hydrolysis of ITP, dITP and XTP with the release of inorganic pyrophosphate and the corresponding mononucleotides as products. Since inosine and xanthine do not occur in RNA and deoxyinosine does not occur in DNA, there must be a biological control mechanism which prevents the accumulation of these three nucleotides within the cell. Furthermore, it is known that DNA polymerase isolated from calf thymus gland will accept dITP as a substrate in the synthesis of single-chain polydeoxynucleotides (polydeoxyinosine phosphate) (45). Polyribonucleotides with alternating inosinic acid and cytidylic acid units have also been shown to be synthesized de novo by the Azotobacter vinelandii RNA polymerase (46).

One of the possible reasons that ITP and dITP do not participate in nucleic acid synthesis may involve the existence of a specific pyrophosphohydrolase such as the one isolated from red blood cells of the rabbit. This enzyme may reduce the concentration of dITP and ITP, thus making them unavailable for use as substrate by either DNA or RNA polymerases respectively. Hence it was of interest to investigate the absence or existence of such an enzyme in mammalian tissues other than the erythrocytes.

This thesis reports that a nucleoside triphosphate pyrophosphohydrolase has been detected in rabbit liver and partially purified from that tissue. The enzyme was found to catalyze the hydrolysis of nucleoside triphosphates with the release of inorganic pyrophosphate and the corresponding nucleoside monophosphates according to the following stoichiometric equation: $NTP + H_2O \rightleftharpoons NMP + PPi$

(N: purine or pyrimidine nucleoside)

ITP, dITP and XTP were found to be the most effective substrates. Several other properties of this enzyme isolated from liver were examined and compared to the enzyme of red blood cells.

Efforts have been made to elucidate the cellular distribution of pyrophosphohydrolase. Particle separation by isopycnic sucrose density gradient and the isolation of nuclei by various methods were carried out. Similar pyrophosphohydrolase activity was detected in other tissues of the rabbit, suggesting that liver and the red blood cells were not the only sources of this enzyme.

MATERIALS

I. Reagents

Ribonucleoside mono-, di- and triphosphates and deoxyribonucleoside triphosphates (except dITP) were purchased from P-L Biochemicals, Milwaukee, Wisconsin. Deoxyinosine 5' triphosphate and deoxyribonucleic acid (Type V) were purchased from Sigma Chemical Company, St. Louis, Missouri. Hyflo-supra celite was obtained from Johns-Manville Co., New York. Analytical reagent grade ammonium sulfate was ordered from Mallinckrodt Chemical Works, St. Louis, Missouri. Yeast inorganic pyrophosphate (600 units/mg) was acquired from Nutritional Biochemical Corporation, Cleveland, Ohio. Deoxyribonuclease I (3000 units/mg, RNase free) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. The acrylamide, TEMED (N, N, N', N'-Tetramethylethylenediamine), BIS (N, N'-methylenebisacrylamide) and ammonium persulfate were purchased from Canal Industrial Corporation, Rockville, Maryland. Cellex-D (DEAE cellulose) and Dowex I resin were obtained from Bio-Rad Laboratories, Richmond, California. Nembutal was purchased from Abbott Laboratory, North Chicago, Illinois. Heparin was acquired from Fisher Scientific Company, Chicago, Illinois. Polyethyleneimine cellulose-coated plastic sheets were purchased from Brink-

mann Instruments, Inc., Westburg, New York. Formaldehyde was obtained from Merck Chemical Division, Rahway, New Jersey. Stannous chloride was from Mallinckrodt Chemical Works, New York. Ammonium molybdate was purchased from Baker Chemical Co., Phillipsburg, New Jersey. 2-mercaptoethanol was obtained from Matheson Coleman and Bell, East Rutherford, New Jersey. Indole was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. All other compounds or enzymes were purchased from Sigma Chemical Company, St. Louis, Missouri.

II. Biological Materials

A. Preparation of Crude Enzyme from Rabbit Liver

A New Zealand white male rabbit was injected intravenously with a solution containing 2000 I.U. of heparin and 100 mg of nembutal. The animal was killed by exsanguination by means of a heart puncture and the liver was then perfused through the aorta with one litre of 0.9% saline. The liver was excised, passed through a tissue mincer, and weighed. The minced liver was then homogenized in 3 volumes (w/v) of 50 mM Tris-Cl buffer, pH 7.4 (0°C), and 1 mM GSH with a Potter-Elvehjem homogenizer equipped with a Telfon pestle. This crude homogenate was then centrifuged at 27,000 x g for 20 minutes to remove cell debris. The supernatant solution was then subjected to centrifugation

at 45,000 r.p.m. for two hours in order to sediment the ribosomes. The high speed supernatant fraction thus obtained was used as the starting material from which nucleoside triphosphate pyrophosphohydrolase was purified.

B. Preparation of Crude Homogenates and High Speed Supernatant of Different Organs from Rabbit

The rabbit was killed as described in A. The liver, thymus, bone marrow, lung, heart, spleen, pancreas, kidney, brain and skeletal muscle were removed, kept at 4°C, and weighed. The tissue were chopped with scalpels and then homogenized in 5 volumes (w/v) of 50 mM Tris-Cl, pH 7.4, 1 mM GSH with a telfon pestle in a motor driven Potter-Elvehjem homogenizer. The homogenates were allowed to pass through four layers of cheesecloth. The homogenates obtained at this point were referred to as the crude homogenates and were used for DNA analysis. The crude homogenates and centrifuged at 27,000 x g for 20 minutes. The supernatant fractions obtained were again centrifuged at 37,000 r.p.m. for two hours. The high speed supernatant fractions prepared in this way were used for the analysis of nucleoside triphosphate pyrophosphohydrolase in the respective tissues of the rabbit.

METHODS

I. Assay of Nucleoside Triphosphate Pyrophosphohydrolase

The assay for pyrophosphohydrolase was carried out by using a coupled assay system ultilizing yeast inorganic pyrophosphatase as described by Chern et al. (44). The enzyme was incubated in 1 ml of reaction mixture containing 50 mM β-alanine buffer, pH 9.5 (37°C), 10 mM $MgCl_2$, 1 mM DTT, one unit yeast inorganic pyrophosphatase and 0.5 mM ITP. The reaction was usually initiated by the addition of the substrate and then incubated at 37°C for 20 minutes. A control reaction mixture lacking pyrophosphohydrolase was used to correct for the small amounts of orthophosphate and pyrophosphate found in commercial preparations of nucleoside polyphosphates used as substrates throughout the study. After incubation, the reaction was stopped by the addition of 2.2 ml of 7.27% cold trichloroacetic acid (TCA) at 4°C. The precipitate thus formed was removed by centrifugation at 2.000 x g for 5 minutes. The supernatant solution was then neutralized to pH 4.29 by the addition of a mixture of 37% formaldehyde and 3M acetate buffer (1:10). The solution was then analyzed for inorganic phosphate according to the

precedure of Rathbun et al. (48). Inorganic phosphate produced in the coupled reaction was then estimated colorimetrically by comparison with values obtained using a standard KH_2PO_4 solution.

II. Protein Determination

Protein concentration was determined by the method of Lowry et al. (49) using crystallized bovine serum albumin as the standard protein.

III. Sucrose Density Centrifugation

The method of Martin and Ames (50) for the determination of the sedimentation coefficient and the molecular weight of a protein by sucrose density centrifugation was used. Linear sucrose gradients of 5-20% sucrose in 50 mM Tris-Cl, pH 7.0, 1 mM GSH and 1 mM $MgCl_2$ in a total volume of 4.6 ml were prepared in the cellulose nitrate tubes. These were kept at 4°C for 2-3 hours. A sample of 0.1 ml was carefully layered onto the top of the gradient, and the material was then centrifuged at 50,000 r.p.m. in a SW-50 swinging bucket rotor for 16 hours at 4°C. Rabbit hemoglobin (M. W 65,000) (51) and pancreatic DNase I (M. W. 31,000) (52) were used as reference markers. After centrifugation, the tubes were punctured at the bottom and a volume of 0.2 ml per fraction was collected. The fractions were analyzed for DNase I and pyrophosphohydrolase activities. Hemoglobin was

measured by the absorption at 415 nm. DNase I activity was followed by the increase in UV absorption at 260 nm due to the depolymerisation of DNA by DNase I according to the method of Kunitz (53).

IV. Identification of Reaction Products by Dowex I Chromatography and Polyethyleneimine Cellulose Thin Layer Chromatography

A. Dowex I Chromatography

Chromatographic analysis of the reaction products by pyrophosphohydrolase was performed according to the method of Zimmerman and Kornberg (40), as modified by Chern and Morris (44). A 1 x 12 cm Dowex I resin (100-200 mesh, Cl^- form) column was prepared, washed with 0.3 N HCl and then washed with distilled water until the pH of the effluent was about 5-6. A recording UV analyzer (Instrument Specialties Company, Inc., Lincoln, Nebraska) was used to monitor the absorbance of the column effluent at 254 nm. An automatic fraction collector (ISCO, Nebraska) was used to collect the eluate fractions (8.8 ml each). The sample was applied to the column and elution was started with 0.02 N HCl. Fractions were collected until Pi and IMP were eluted from the column. The eluant was then changed to 0.1 N HCl and elution continued until PPi, IDP and ITP were washed from the column. The pyrophosphate peak was identified with the aid of yeast inorganic pyrophosphatase in producing measurable Pi. The identity

of each nucleotide was confirmed by chromatographic comparison to reference standards on PEI cellulose thin layer chromatography. Fractions containing each nucleotide were pooled and the absorbance at 248.5 nm in 0.1N HCl was determined and compared to ITP in 0.1N HCl as reference standard.

B. PEI (polyethyleneimine) Cellulose Thin Layer Chromatography

Nucleotide separation by ion exchange chromatography on PEI cellulose thin layer was carried out according to the procedure of Randerath and Randerath (54). A 20 x 20 cm PEI cellulose precoated plastic sheet (0.1 mm thick, cellulose MN 300 polyethyleneimine impregnated, Brinkmann Instruments) was washed with water by ascending chromatography and then dried. The samples together with reference standards were spotted onto the plate and developed with 1.6 M LiCl for 2 hours by ascending chromatography. Nucleotides were visualized with the aid of an ultra-violet lamp.

V. Analytical Disc Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out essentially as described by Davis (55). The acrylamide and bisacrylamide were recrystallized from chloroform and acetone as described by Loening (56). The 7% acrylamide gel was prepared as follows: one part of solution A (1N HCl, 48 ml;

Tris, 36.6 gm; TEMED, 0.23 ml; and water to 100 ml) was mixed with two parts solution C (acrylamide, 25 gm; BIS, 0.735 gm; and water to 100 ml) and one part of water. The polymerization reaction was then initiated by the addition of 4 parts of catalyst (ammonium persulfate, 0.14 gm; and water to 100 ml) at 4°. Without delay, the mixture was added to gel tubes (0.6 cm diameter) to a depth of 7.5 cm, and a small volume of water was carefully layered on the top of the gel solution. When polymerization was completed, a stacking gel (large pore gel) with one-quarter of the height of the running gel was polymerized on the top of the running gel. The stacking gel was prepared as follows: one part of solution B (1N HCl, 48 ml; Tris, 5.98 gm; TEMED, 0.46 ml; and water to 100 ml) was mixed with two parts of solution D (acrylamide, 14 gm; BIS, 0.25 gm; and water to 100 ml) and 4 parts of water. Polymerization reaction was started by the addition of 1 part of catalyst (ammonium persulfate, 0.14 gm; and water to 100 ml). The protein sample was prepared in 2.5% of sucrose and 1 mM 2-mercaptoethanol. 3µl of 0.05% bromophenol blue was added as a tracking dye to produce a leading band of dye for locating the front during electrophoresis. The gels were subjected to a current of 5 ma per gel cylinder with 0.025M Tris-glycine (pH 8.6) buffer containing 1 mM 2-mercaptoethanol as electrode buffer.

Electrophoresis in 7% gel using 0.05M Tris-Cl, pH 7.5, 1 mM 2-mercaptoethanol as electrode buffer was also carried

out using the above procedure.

After electrophoresis, gels were stained for proteins with Coomassie brilliant blue in trichloroacetic acid for one hour as described by Chramback et al.(57). Pyrophosphohydrolase in polyacrylamide gel was histochemically stained by a modification of the lead conversion methods developed by Gomori (58,59,60). After electrophoresis, the gel was first incubated in 50 mM β-alanine, 1 mM DTT, 10 mM $MgCl_2$, 20 mM $CaCl_2$, and 0.5 mM ITP for two hours at 4°, it was then transferred to a water bath at 37°. Due to the hydrolysis of ITP by pyrophosphohydrolase, calcium pyrophosphate precipitated as a white band which intensified after 1 hour of incubation.

VI. Separation of Lysosomes, Mitochondria and Peroxisomes by Isopycnic Density Gradient

Particles were separated by isopycnic sucrose density gradient centrifugation in a B-30 zonal rotor as described in detail by Tolbert (61). Female Sprague-Dawley rats of about 150 gm were used and the livers perfused with grinding medium. From 20-50 gm of tissue was chopped into very small pieces and homogenized by one pass in a loose-fitting power-driven Potter-Elvehjem homogenizer with a solution of 7% sucrose and 20 mM glycylglycine at pH 7.5. The homogenate was filtered through 4 layers of cheesecloth. The particles were sedimented between 100 and 10,000 x g for 20 minutes,

and resuspended in grinding medium before application over the sucrose gradient at the core area of the zonal rotor. The gradient were developed for 3 to 4 hours at 30,000 r.p.m. Gradient fractions of 10 ml were collected and analyzed for pyrophosphohydrolase, cytochrome c oxidase (62), catalase (63), and acid phosphatase (64) activities.

VII. Preparation of Rabbit Liver Nuclei

A. Aqueous Medium

The method of Chauveau (65) for the isolation and purification of liver nuclei was performed. 5 gm of rabbit liver was forced through a tissue mincer to remove most of the connective tissue. The extract was then suspended in 50 ml of 2.4M sucrose, 3.3 mM $CaCl_2$ prepared in 50 mM Tris-Cl buffer, pH 7.4, 1 mM GSH, and was homogenized in a power-driven Potter-Elvehjem homogenizer using 6-10 strokes. The suspension was centrifuged at 26,000 r.p.m. for one hour. The nuclei pellet was resuspended in 5 ml of 1M sucrose, 1 mM $CaCl_2$, prepared in 50 mM Tris-Cl, pH 7.4, rehomogenized and then centrifuged at 3,000 x g for 5 minutes. The pellet materials were stained with Wright stain and found to be free of cytoplasmic and intact cells contamination as judged by visual examination in the light microscope.

B. Non-aqueous Medium

Isolation of nuclei in non-aqueous medium was a modification of the method of Allfrey et al.(66). One gram of rabbit liver was forced through a tissue mincer and then suspended in 50 mM Tris-Cl (pH 7.4) in a lyophilizing flask. The cell suspension was rapidly frozen as a thin film on the wall of the lyophilizing flask by immersing and spinning the flask in a CO_2-acetone slurry. The flask was then connected to a lyophilizer (VirTis Research Equipment, New York) for 48 hours. The dried cell mass was then ground in a porcelain dish for 2 minutes with 5 ml of petroleum ether. The cell suspension was then centrifuged at 900 x g for 20 minutes. The sediment was resuspended with the help of a Vortex mixer in 7 ml of cyclohexane-CCl_4 mixture (specific gravity 1.30), and then centrifuged at 2,000 x g for 40 minutes. The sediment obtained in the above step was resuspended in 7 ml of cyclohexane-CCl_4 of specific gravity 1.31, centrifuged at 2,000 x g for 40 minutes. The above process was repeated by suspending the respective sediments in 7 ml of cyclohexane-CCl_4 of specific gravity 1.32, 1.33, and 1.335, and centrifuged as before. The pale white pellet obtained at specific gravity 1.335 was then suspended in 5 ml of cyclohexane-CCl_4 mixture of specific gravity 1.39, and centrifuged at 2,000 x g for 50 minutes. The supernatant fraction, which contained the nuclei, was carefully decanted and dried under vaccumn.

C. Glycerol Medium

Preparation of liver nuclei in a glycerol medium was performed by a modified method of Schneider (67). One gram of minced rabbit liver was suspended in 15 ml of 70% glycerol, 1 mM EDTA at pH 8.4. The suspension was homogenized in a Potter-Elvehjem homogenizer by means of 20 strokes against a stationary pestle. The homogenate was filtered through 4 layers of cheesecloth. Effective separation of the nuclei from the homogenate was accomplished by the use of the following layering technique. The homogenate was simply poured down the side of a 15 ml centrifuge tube containing 1 ml of suspending glycerol medium, and centrifuged at 2,700 x g for 10 minutes. The sidiment of nuclei was washed twice with 10 ml of original medium by centrifuging at 2,700 x g for 10 minutes. The final sediment was suspended in 1 ml of original medium.

D. Citrate Medium

Preparation of nuclei in citrate medium was performed using a modification of the method of Higashi et al.(68). The connective tissue of rabbit liver was removed by passing through a tissue mincer. The tissue was then homogenized with 1:9 volumes (w/v) of 2.5% citrate, 1 mM GSH in a motor-driven Potter-Elvehjem. After centrifuging at 600 x g for 10 minutes, the pellet was again homogenized with 5 ml/gm

of original tissue of 0.25M sucrose containing 1.5% citrate and 1 mM GSH. The homogenate was then layered on 2 volumes of 0.88M sucrose containing 1.5% citrate, 1 mM GSH and centrifuged at 900 x g for 10 minutes.

VIII. Determination of Deoxyribonucleic Acid (69)

This colorimetric procedure is based on a reaction between indole and the deoxysugar of DNA. Two ml of the solution to be tested (in 1N NaOH) was measured into a throughly cleaned test tube. 1 ml of 0.04% indole and 1 ml of concentrated HCl were added and the mixture was shaken well. The test tube was then placed in a boiling water bath for 10 minutes. The tube was then cooled under running water and centrifuged to remove precipitated protein. The solution was then extracted three times with 4 ml of chloroform and centrifiged to give a completely clear water phase. The intensity of the yellow color which developed was measured at 490 nm against a blank treated in an identical manner but using 2 ml of 1N NaOH as the sample. A standard curve was prepared using a commercial preparation of calf thymus DNA analyzed under the same conditions.

RESULTS

I. Purification of Nucleoside Triphosphate Pyrophosphohydrolase

A. Heat Denaturation Step

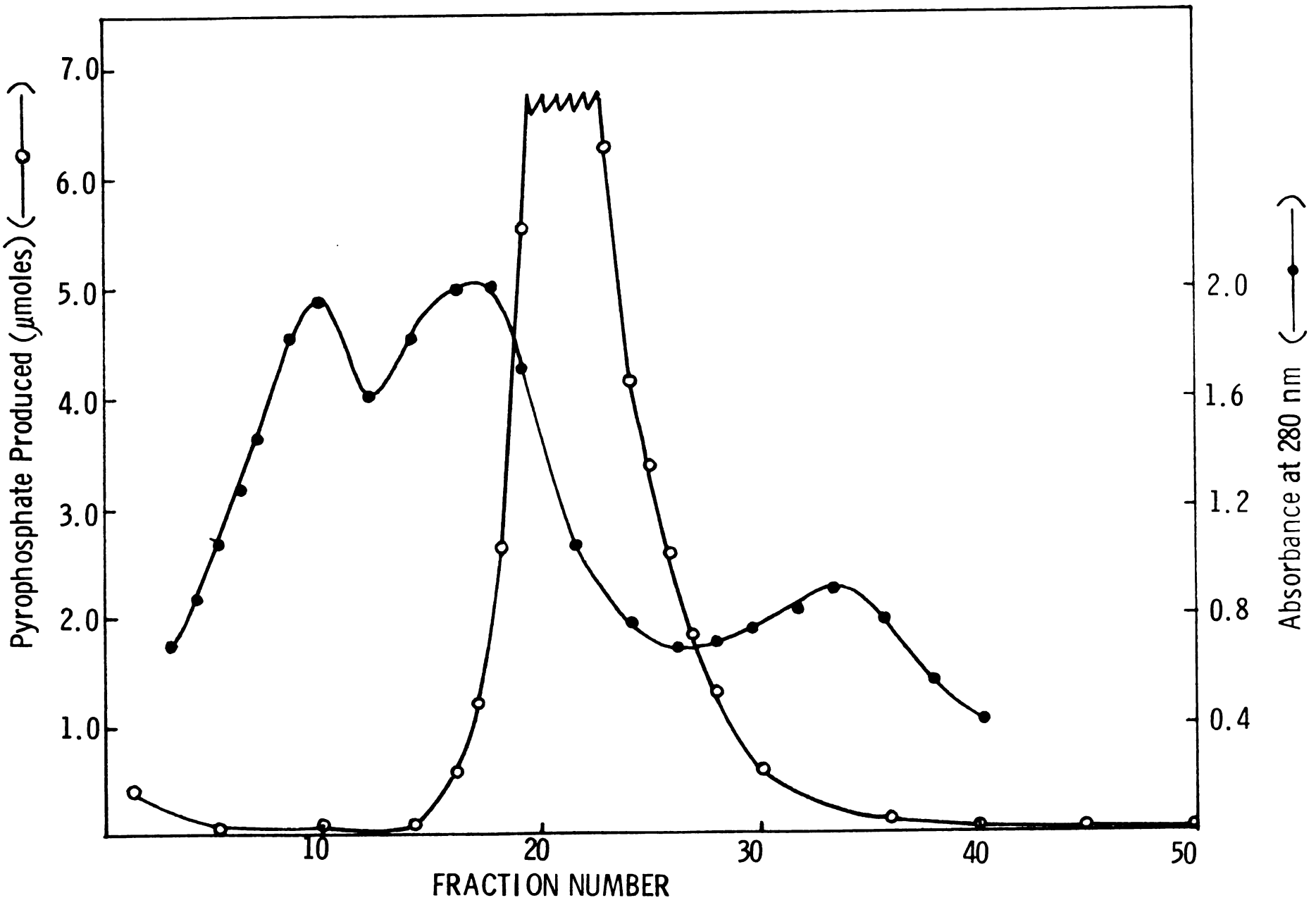
The high speed supernatant fraction prepared from the rabbit liver homogenate as described under the section of "Biological Material" was heat denatured at 65°C for 5 minutes with continuous swirling. The suspension was cooled immediately to 5°C. The precipitate formed was removed by centrifugation at 12,000 x g for 20 minutes. The supernatant fraction was further purified by the ammonium sulfate gradient described in the next section. Most of the inorganic pyrophosphatase was destroyed by this heat denaturation step. As shown in Table 1, a 3-fold increase in specific activity was obtained.

B. Purification by Ammonium Sulfate Solubilization

Further purification of pyrophosphohydrolase by the method of King (70) ultilizing a reverse ammonium sulfate gradient to solubilize the precipitated protein was carried out. The classical method of protein separation by ammonium

sulfate precipitation could be performed in the reverse manner. Namely, the proteins were first precipitated in the presence of a carrier and then they were solubilized with a decreasing salt gradient. An advantage of the reverse procedure is that it makes possible for one to choose the best recovery of the desired protein with the least amounts of contaminants. Hyflo-supra celite was added to the heat denatured supernatant fraction (1 gram celite to 100 mg protein) and mixed thoroughly. The suspension was brought to 80% saturation by the slow addition of powdered ammonium sulfate. After 30 minutes of stirring, the suspension was used to prepare a column of 3 x 2.8 cm. Elution was performed with a decresing gradient of 80%-0% saturation of ammonium sulfate prepared in 50 mM Tris-Cl buffer, pH 7.4 (4°), 1 mM GSH. The flow rate was adjusted to 100 ml per hour. A volume if 15 ml per fraction was collected and aliquots of fractions were analyzed for nucleoside triphosphate pyrophosphohydrolase activity. The enzyme was eluted at the region of 45-50% ammonium sulfate saturation (Figure 1). Fractions around the pyrophosphohydrolase peak were pooled and concentrated by the addition of powered ammonium sulfate to 70% saturation. After 30 minutes of stirring, the precipitate was collected by centrifugation and dissolved in 20 ml of 50 mM Tris-Cl (pH 8.1) containing 1 mM GSH. In some cases, the pooled fractions was concentrated by presure dialysis against the same buffer. As shown in Figure 1, the pyrophosphohydrolase acti-

Figure 1. Ammonium sulfate solubilization of the heat denatured supernatant fraction. The enzyme was eluted with a gradient of 80%-0% saturation of ammonium sulfate in 50 mM Tris-Cl, pH 7.4 and 1 mM GSH. Selected fractions (15 ml per fraction) were analyzed for pyrophosphohydrolase by incubation at 37°C for 20 min. in a reaction mixture containing 50 mM β-alanine (pH 9.5), 10 mM $MgCl_2$, 1 mM DTT, 1 unit of yeast inorganic pyrophosphatase, 0.025 ml of column eluate, 0.5 mM ITP and water to 1 ml. Analysis of inorganic phosphate produced was performed as described in Methods. Alternate fractions were analyzed for absorbance at 280 nm using a Gilford Spectrophotometer.


Pyrophosphate Produced (μmoles) (—o—)
Absorbance at 280 nm (—•—)
FRACTION NUMBER
7.0
6.0
5.0
4.0
3.0
2.0
1.0
2.0
1.6
1.2
0.8
0.4
10
20
30
40
50

vity was separated from the main protein peak and the hemoglobin peak (around fraction 10) as indicated by the profile of absorption at 280 nm.

C. DEAE Cellulose Chromatography

Anion exchange chromatography carried out in DEAE cellulose column was applied to the purification of pyrophosphohydrolase. The enzyme solution from ammonium sulfate gradient was dialysed against 50 mM Tris-Cl (pH 8.1) and 1 mM GSH. The sample was applied to a DEAE cellulose column (2.5 x 1.4 cm) previously equilibrated with the same elution buffer. Elution was performed using a linear NaCl gradient of 0 - 0.2N prepared in the same elution buffer (Figure 2). A volume of 2 ml per fraction was collected. The fractions were observed to be very low in protein concentration which could not be detected by UV absorption at 280 nm. The fraction containing pyrophosphohydrolase activity were pooled and concentrated by pressure dialysis against 50 mM Tris-Cl, pH 7.0 and 1 mM GSH. The enzyme preparations were then stored under liquid nitrogen. The enzyme activity was found to be stable for long periods by this method of storage. The recovery of enzyme in this step was approximately 58%.

The results of each individual purification step are summarized in Table 1. A final specific activity of 130 units of activity/mg protein and an overall purification

Figure 2. DEAE cellulose column chromatography on ammonium sulfate fractions. The sample was eluted with a linear NaCl gradient of 0 - 0.2N in 50 mM Tris-Cl (pH 8.1) and 1 mM GSH. Eluted fractions (2 ml each) were analyzed for pyrophosphohydrolase as described in Figure 1.

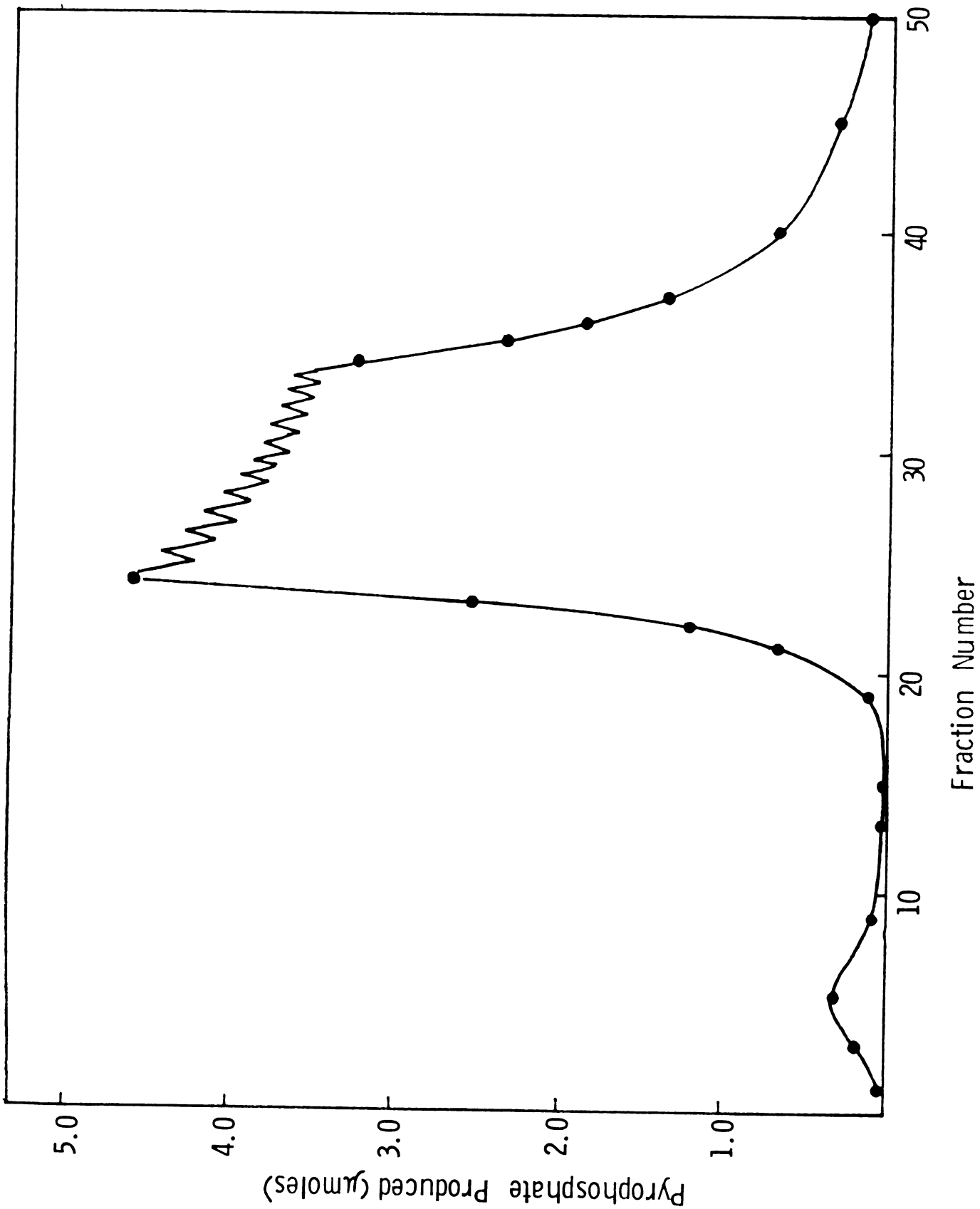

Pyrophosphate Produced (μmoles)
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
Fraction Number

Table 1. Purification of Nucleoside Triphosphate Pyrophosphohydrolase

	volume ml	activity per ml	total activity*	% recovery	protein mg/ml	S.A.**	Fold
Crude Homogenate	350.0	8.1***	2820	100.0	41.2	.197	1.0
High Speed Supernatant Fraction	180.0	21.8***	3920	139.0	19.2	1.14	5.8
Heat Denatured Supernatant Fraction	153.0	12.3	1900	67.8	3.7	3.34	17.0
Ammonium Sulfate Step	19.1	82.6	1580	56.0	4.3	19.20	98.0
DEAE Cellulose Step	12.9	72.1	930	33.0	0.55	130	660.0

* Activity expressed as μmoles of PPi liberated from ITP per 20 minutes under standard assay conditions (see methods).

** S.A. Specific activity expressed as activity per mg protein

*** These two fractions were assayed using both ITP and ATP as substrates, and the activity expressed as the difference between the two values.

from the crude of about 660-fold was obtained. The further studies described below employed this enzyme preparation.

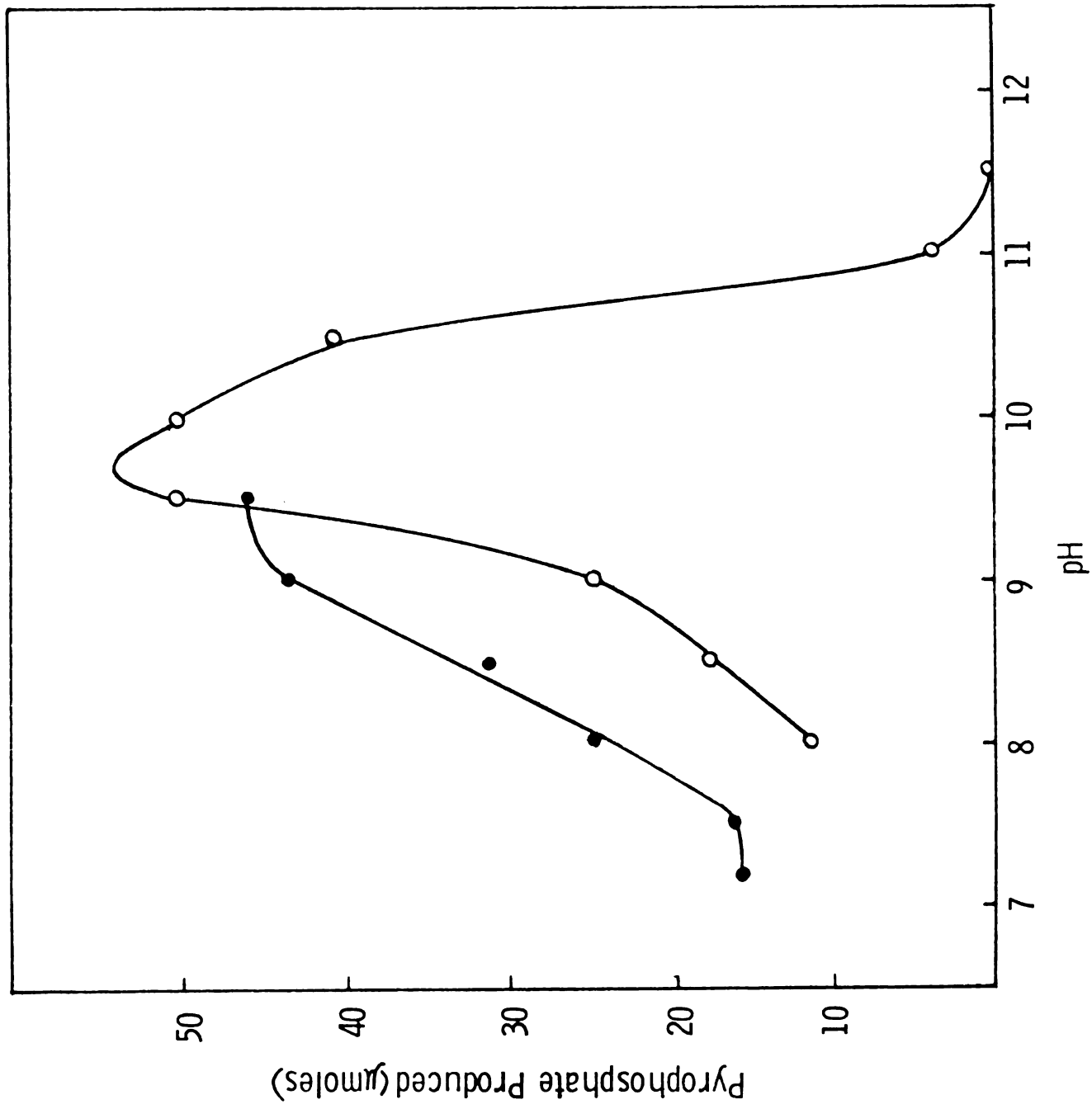
II. Properties of Nucleoside Triphosphate Pyrophosphohydrolase

A. pH optimum

Two buffer system were used to determine the optimum pH of pyrophosphohydrolase activity on ITP hydrolysis. The results are shown in Figure 3. A sharp pH optimum at 9.75 was obtained with β-alanine buffer. No activity was observed above pH 11.0 for β-alanine, but considerable activities were observed between 7.0 and 9.0 for both Tris-Cl and β-alanine buffers.

Since yeast inorganic pyrophosphatase was used in the coupled assay system, it was important to determine that this enzyme was not a limiting factor in the assay system in the range of pH used. Chern (71) had shown that a drastic decrease of activity of inorganic pyrophosphatase above pH 9.5 using sodium pyrophosphate was observed. However, using one unit of yeast inorganic pyrophosphatase (which was the amount used in the coupled enzyme assay), the activity remaining still exceeded that of the total pyrophosphohydrolase activity observed. Hence it was concluded that pyrophosphatase was not limiting in the assay system. Therefore the data obtained are considered to be

Figure 3. The effect of pH on pyrophosphohydrolase activity. Pyrophosphohydrolase activity was determined using 50 mM Tris-Cl (—●—) or 50 mM β-alanine (—○—) buffer. DEAE enzyme was used as described in Figure 1.


Pyrophosphate Produced (μmoles)
10
20
30
40
50
7
8
9
10
11
12
pH

reliable for the pH optimum of pyrophosphohydrolase.

B. Substrate Specificity

The results of different nucleoside mono-, di- and triphosphates tested as substrates of pyrophosphohydrolase are shown in Table 2. Of all the compounds tested, dITP was shown to be the most effective substrate, while ITP and XTP were hydrolyzed to a lesser extent. GTP, dGTP, TTP, UTP and IDP were hydrolyzed at less than 10% of the rate of ITP, while ATP, CTP, dCTP, and IMP do not serve as substrates for pyrophosphohydrolase. All assays were corrected for the small amounts of Pi and PPi present in commercial preparation of nucleoside polyphosphates used as substrates.

C. Substrate Concentration Curve

The result of variation of substrate concentrations on pyrophosphohydrolase activity was shown in Figure 4. A substrate concentration of 5×10^{-4}M was found to be optimum. At higher concentration of substrates, inhibition was observed.

D. Requirement for a Sulfhydryl Compound

The effect of a sulfhydryl compound--dithiothreitol, on the hydrolysis of ITP by pyrophosphohydrolase was studied. It was shown in Figure 5 that the activity of enzyme was dependent on the presence of a sulfhydryl compound in the

Figure 4. Effect of ITP concentration upon the observed pyrophosphohydrolase activity. Analyses were performed as described in Figure 1 using ITP as substrate.

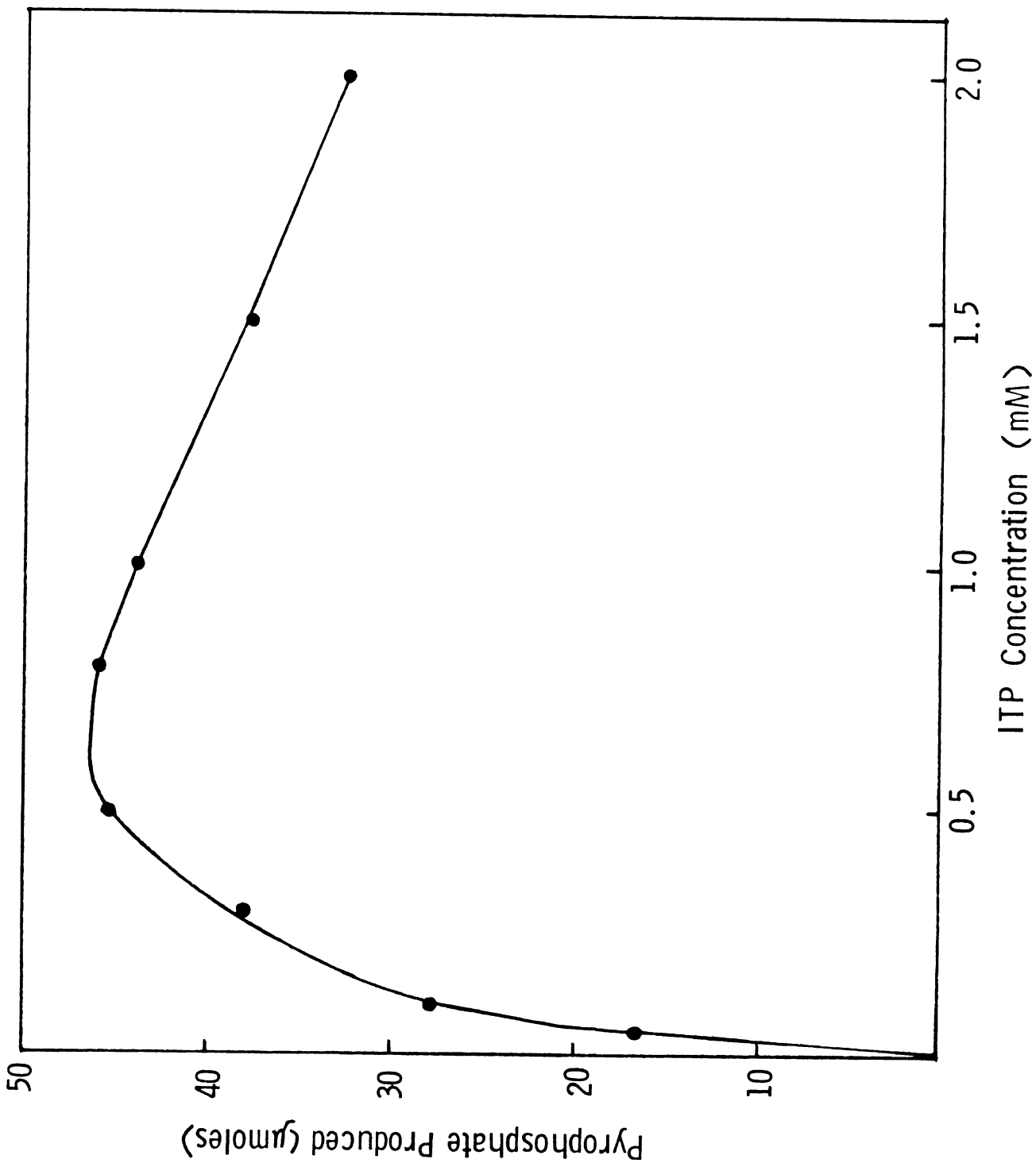

Pyrophosphate Produced (μmoles)
50
40
30
20
10
0.5
1.0
1.5
2.0
ITP Concentration (mM)

Figure 5. Effect of sulfhydryl compound on the observed pyrophosphohydrolase activity.

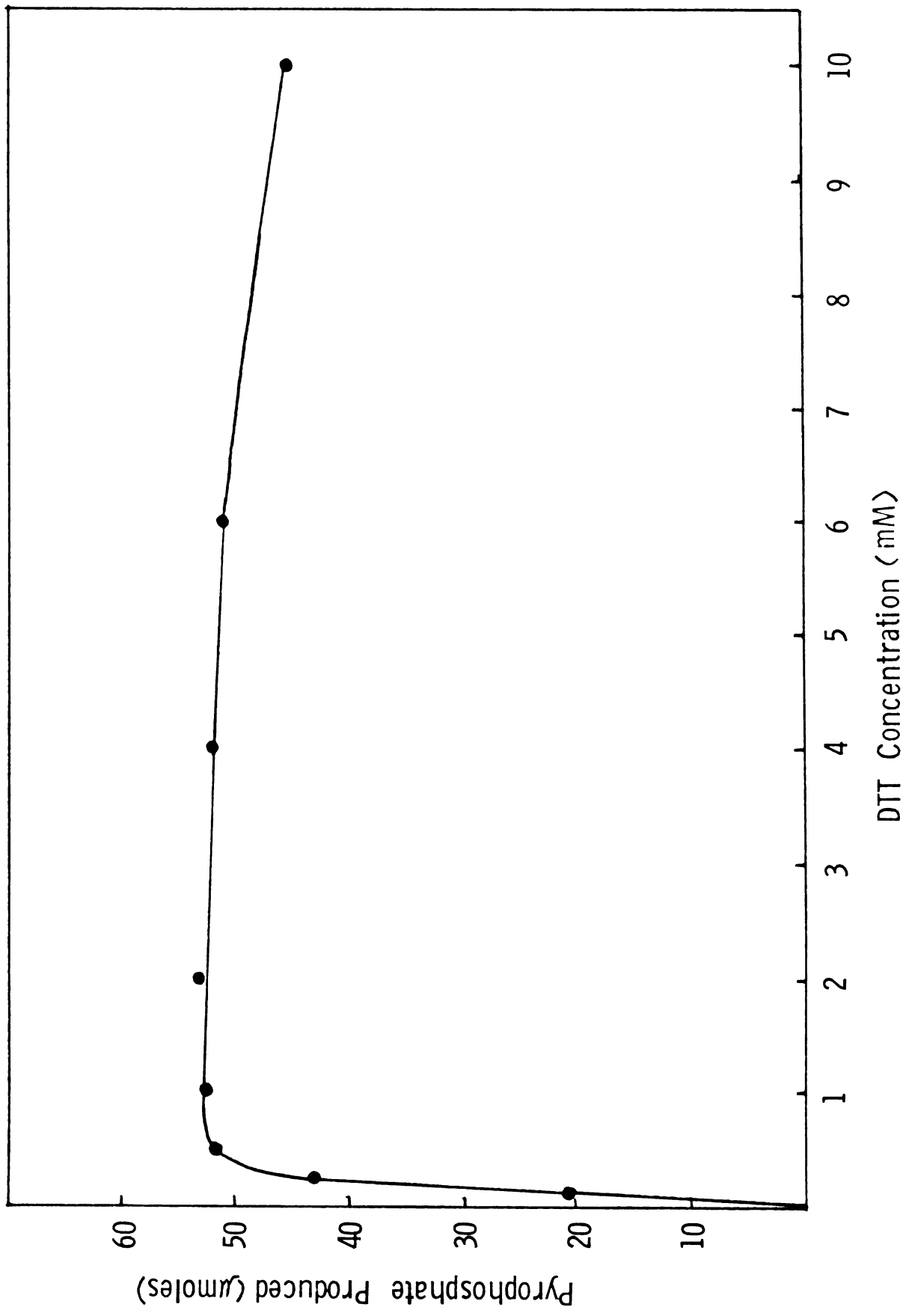

Pyrophosphate Produced (μmoles)
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9
10
DTT Concentration (mM)

Table 2. Substrate Specificity. All substrates analyzed were 0.5 mM in the assay mixture (see Figure 1).

Substrate	Relative Activity
ITP	100
dITP	152
XTP	99
ATP	0.3
CTP	1.1
dCTP	0.7
GTP	8.0
dGTP	6.0
TTP	3.0
UTP	9.0
IDP	4.0
IMP	0.6

assay mixture. Hence at all stages of purification and analyses of pyrophosphohydrolase, a sulfhydryl compound, either DTT or GSH, was present in the buffer or the reaction mixture.

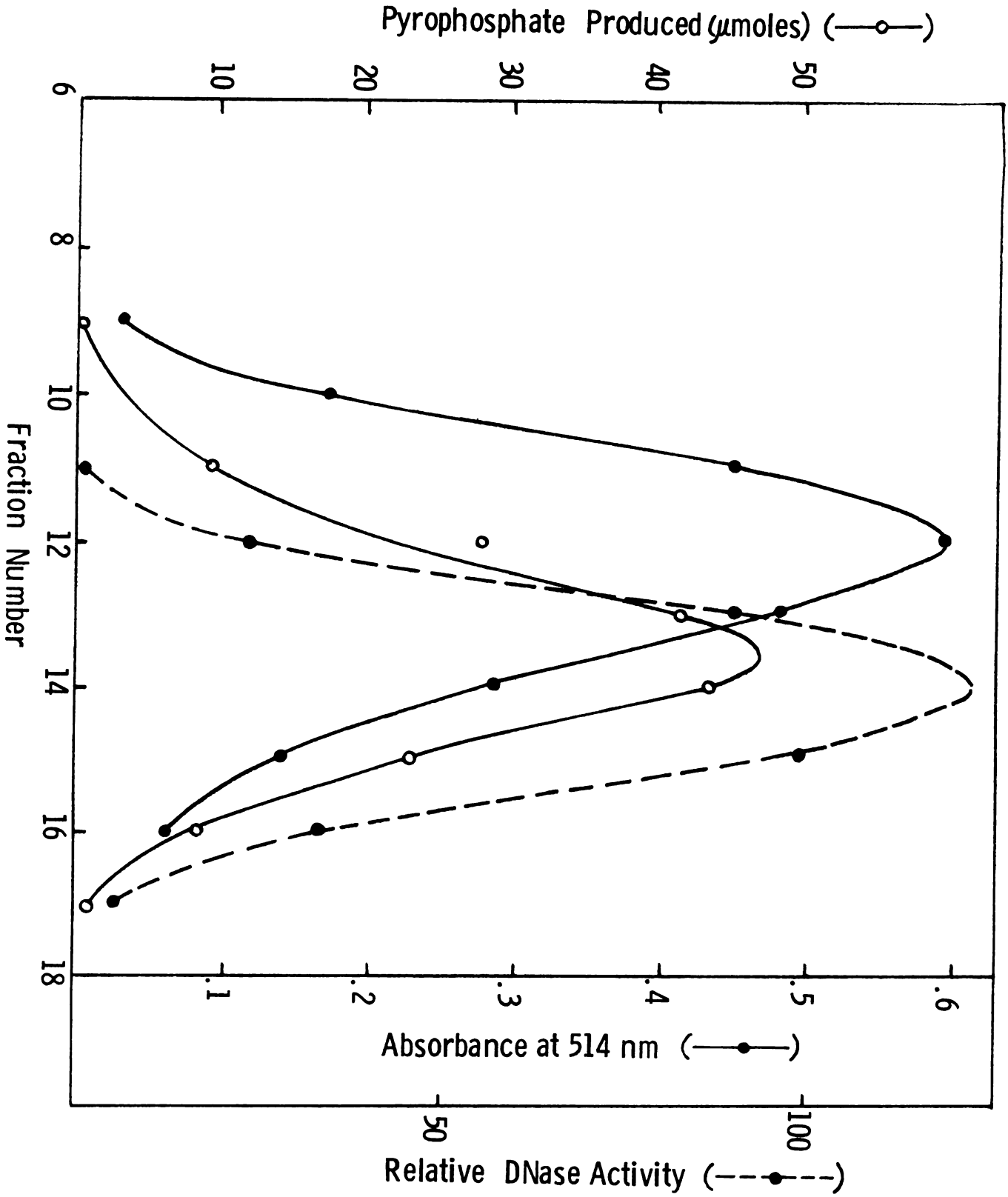
E. Determination of Molecular Weight by Sucrose Density Centrifugation

The procedure for the estimation of molecular weight of pyrophosphohydrolase was described under the section of "Methods". The sedimentation profile of the activities of nucleoside triphosphate pyrophosphohydrolase and the two reference markers--rabbit hemoglobin and pancreatic DNase I are shown in Figure 6. An estimated molecular weight of 37,000 to 39,000 was obtained for pyrophosphohydrolase using the relationship that the sedimentation distance is proportional to the $(\text{molecular weight})^{2/3}$. This molecular weight is based on the assumption that all three protein molecules have similar molecular shapes in the solutions used for centrifugation.

F. Stoichiometry and Reaction Products of ITP Hydrolysis by Nucleoside Triphosphate Pyrophosphohydrolase

A large scale reaction mixture (50 ml) containing 50 mM β-alanine, pH 9.5 (37°), 10 mM $MgCl_2$, 1 mM DTT, 1.5ml of DEAE purified enzyme, and 40 μmoles of ITP was incubated at 37°C for 20 minutes, and the reaction was stopped by heating to 80°C for 3 minutes. The sample was then applied

Figure 6. Sucrose density centrifugation of the pyrophosphohydrolase obtained from DEAE cellulose column chromatography. Centrifugation was carried out for 16 hours at 50,000 r.p.m. with a 5-20% sucrose gradient. (see Methods for details)


Pyrophosphate Produced (μmoles) (—o—)
10
20
30
40
50
6
8
10
12
14
16
18
Fraction Number
.1
.2
.3
.4
.5
.6
Absorbance at 514 nm (—●—)
50
100
Relative DNase Activity (---●---)

to a Dowex I column. Chromatography and characterization of the reaction products were carried out as described in the section of "Methods". The results of the analysis are shown in Figure 7A. Inorganic phosphate and pyrophosphate are well separated from each other, as are IMP, IDP and ITP. The peak for ITP was absent in this analysis indication complete hydrolysis of ITP by pyrophosphohydrolase. The unknown was thought to be a degradation product of IMP which also absorbed at 254 nm. Figure 8 shows the result of PEI cellulose thin layer chromatography developed in 1.6M LiCl as described in the section of "Methods". The inosine mono-, di-, and triphosphates are well resolved from each other. A control reaction mixture lacking pyrophosphohydrolase was subjected to the same procedure of analysis in order to correct for some of the impurities presented in the commercial ITP preparation and any possible degradation products of ITP introduced by the treatment. The results are shown in Figure 7B. In this control analysis, no IMP was found to form from ITP. The difference between these two analyses gives the stoichiometry of the pyrophosphohydrolase reaction with ITP as substrate. The result is summarized in Table 3. The reaction products of ITP hydrolysis by pyrophosphohydrolase are IMP and PPi. For each μmole of ITP hydrolysed, 1 μmole of IMP and 1 μmole of PPi are formed.

Figure 7. Analysis of reaction products by Dowex I chromatography following ITP hydrolysis by pyrophosphohydrolase. Figure 7A illustrates the elution profile of the reaction products following incubation for 20 min. in the presence of pyrophosphohydrolase. Figure 7B illustrates the elution profile of a control incubation (with no enzyme) (see Methods for details).

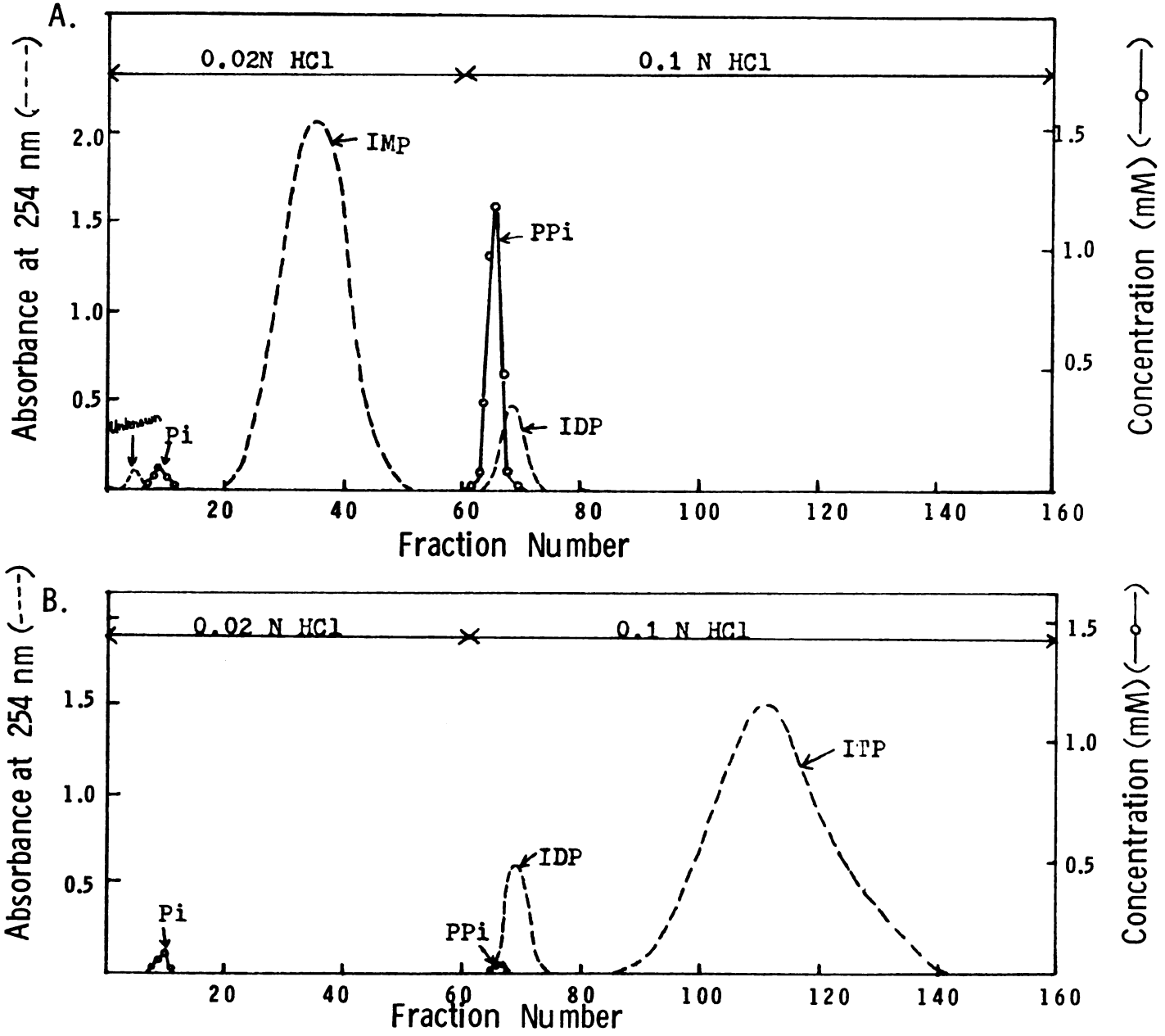

A.
0.02N HCl
0.1 N HCl
IMP
PPi
IDP
Pi
Absorbance at 254 nm (----)
Concentration (mM) (—o—)
Fraction Number
B.
0.02 N HCl
0.1 N HCl
ITP
IDP
PPi
Pi
Absorbance at 254 nm (----)
Concentration (mM) (—o—)
Fraction Number

Figure 8. Comparison of reaction products of ITP hydrolysis by pyrophosphohydrolase to the reference standards on PEI cellulose thin layer by ascending chromatography developed in 1.6M LiCl for 2 hours. Nucleotides were identified using an UV lamp.

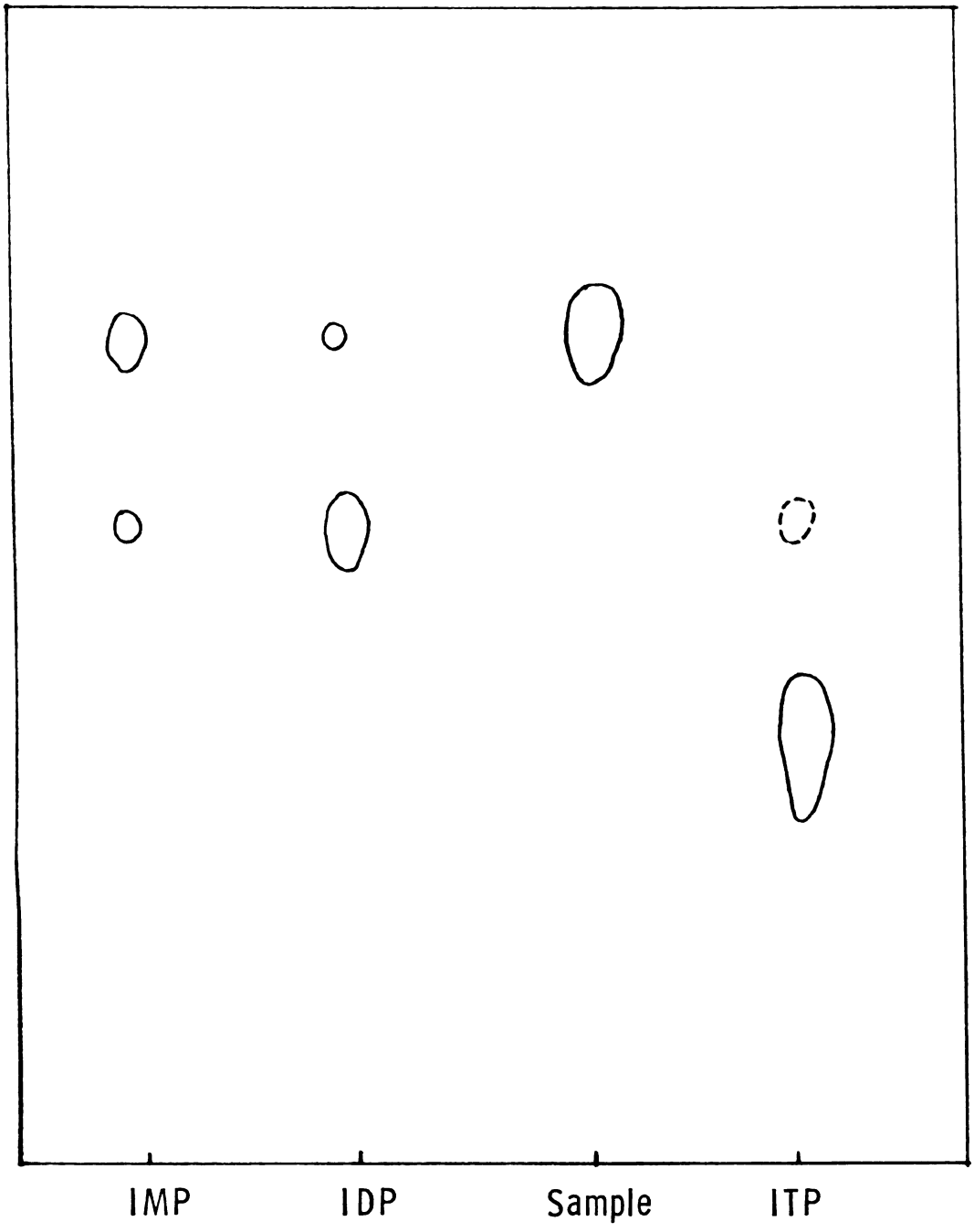

IMP
IDP
Sample
ITP

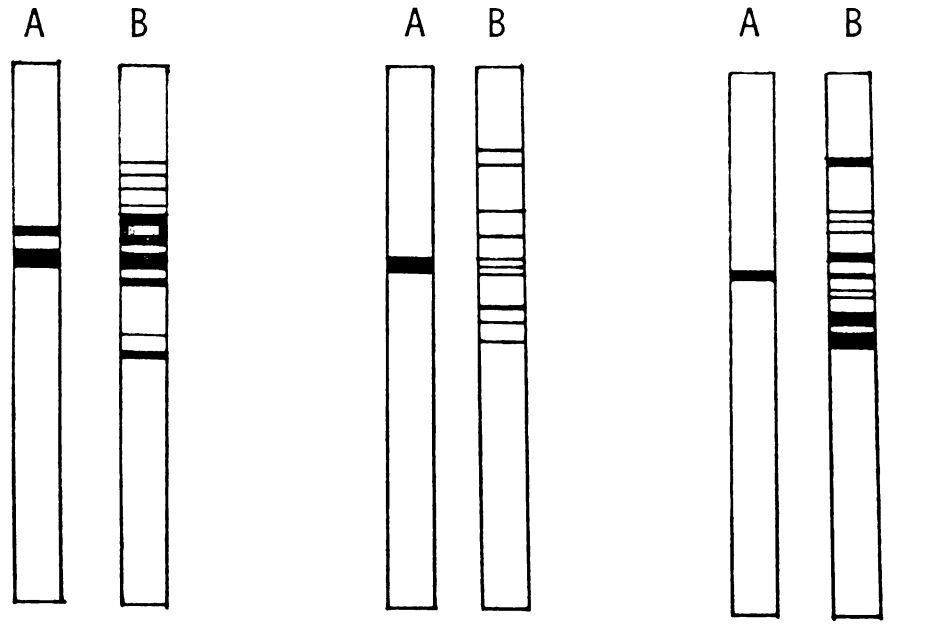
Table 3. Products and stoichiometry of ITP hydrolysis by Nucleoside Triphosphate Pyrophosphohydrolase.

Compound	μmoles (plus enzyme)	μmoles (minus enzyme)	μmoles (difference)
IMP	36.20	0	+ 36.20
IDP	3.11	3.16	- 0.05
ITP	0	35.80	- 35.80
PPi	36.59	0.45	+ 36.14
Pi	1.69	1.76	- 0.065
Unknown	0.69	0	+ 0.69

G. Comparison of Rabbit Liver, Rabbit Red Blood Cell and Human Red Blood Cell Nucleoside Triphosphate Pyrophosphohydrolase by Analytical Disc Gel Electrophoresis

The rabbit red blood cell pyrophosphohydrolase used was prepared by the method of Chern et al.(44). The human red cell pyrophosphohydrolase of over 1,000 fold purification was prepared by the procedure of Morris (72). Polyacrylamide gel were prepared and samples were subjected to electrophoresis as described under the section of "Methods". Figure 9 shows the profiles of protein bands stained with Coomassie brilliant blue and the corresponding pyrophosphohydrolase activity bands in the polyacrylamide gel with Tris-glycine buffer at pH 8.6. All three enzymes were shown to be non-homogeneous with more than eight protein bands. The rabbit and human red blood cell enzymes show only one pyrophosphohydrolase activity band as indicated by the white Ca-pyrophosphate precipitate (see "Methods"). However, for the rabbit liver pyrophosphohydrolase tested, two white bands were observed. The lower band can be visualized after 20 minutes of incubation, but the upper band can only be seen on longer incubation at 37°C. It was also found that when gels prepared in the same manner were incubated in reaction mixtures containing ATP, GTP, or glucose-1-phosphate as substrates, only the upper band was visible. However, it should be pointed out that using the coupled enzyme assay system for nucleoside triphosphate pyrophosphohydrolase, very little activity was found when ATP, GTP or glucose 1-phosphate were used as substrates instead of ITP. One may thus

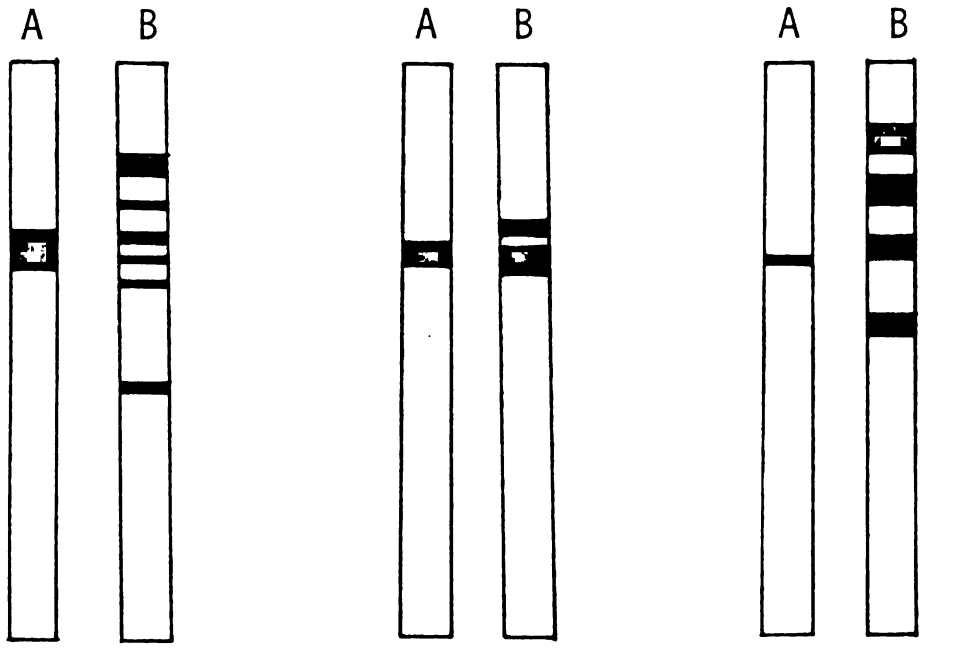
Figure 9. Disc gel electrophoresis of pyrophosphohydrolase preparations from rabbit liver, rabbit red blood cell, and human red blood cell at pH 8.6. The protein bands were visualized with Coomassie brilliant blue (see Methods). Pyrophosphohydrolase activity was identified by the lead conversion method of Gomori as white bands of Ca-pyrophosphate (see Methods for details)



A. Pyrophosphohydrolase Activity Stain

B. Coomassie Brilliant Blue Stain

Figure 10. Disc gel electrophoresis of pyrophosphohydrolase preparations from rabbit liver, rabbit red blood cell and human red blood cell at pH 7.5. (see Methods for details)



A. Pyrophosphohydrolase Activity Stain

B. Coomassie Brilliant Blue Stain

conclude that the upper band is not the result of hydrolysis of ITP by a contaminating phosphatase in the pyrophosphohydrolase preparation. Rather, it is probably some binding between a protein and the phosphate group of the substrates tested. Figure 10 shows the profiles of the protein bands and the pyrophosphohydrolase activity band of all three enzymes in polyacrylamide gels with Tris-Cl at pH 7.5 as electrode buffer. The resolution of the protein bands is less distinct and the mobility of the protein molecules is much slower as compared to electrophoresis at pH 8.6 (1.1 cm/hour at pH 7.5 as compared to 5.4 cm/hour at pH 8.6). As shown in Figures 9 and 10, the pyrophosphohydrolase activity bands of rabbit liver, rabbit red blood cell and human red blood cell migrated at the same rate during electrophoresis.

III. Occurence of Nucleoside Triphosphate Pyrophosphohydrolase within the Cell

A. Consideration of the Possibility that Pyrophosphohydrolase is a Lysosomal Enzyme

Since nucleoside triphosphate pyrophosphohydrolase is a hydrolytic enzyme, it might be located in the lysosome of the cell where most hydrolytic enzymes are located to carry out their degradative functions. Investigations were undertaken to elucidate this possibility. Lysosomes were separated from mitochondria and peroxisomes and other cell elements as described under the section of "Methods". The results are

shown in Figure 11. The mitochondria band, marked by cytochrome c oxidase, and the peroxisomal band, marked by catalase are well separated. The lysosomal activity, marked by acid phosphatase, is located between the mitochondria and peroxisomes. From this gradient profile of rat liver homogenate, it is observed that a major portion of pyrophosphohydrolase activity was found in the cytosol or top soluble fraction of the gradient. A small portion of pyrophosphohydrolase was also presented in the lysosomal band and followed closely in distribution with acid phosphatase activity.

However, further analysis on the gradient fractions using ATP as substrate (Figure 12) revealed a similar distribution of activity around the lysosomal region which was absent in the cytosol. Also, fractions around the lysosomal region were found to be active towards hydrolysis of GTP, UTP, CTP, and XTP, while fractions from the cytosol were found to hydrolyze only ITP and XTP to a significant extent (dITP not tested). In view of the fact that the pyrophosphohydrolase characterized has little effect on the hydrolysis of other nucleoside triphosphates except ITP, XTP and dITP, it was concluded that the activity which coincides in distribution with acid phosphatase is the product of some nonspecific phosphatase located in the lysosome. It was concluded from these results that pyrophosphohydrolase is located in the cytosol.

Figure 11. Separation of particles by isopycnic sucrose density gradient centrifugation. The gradients were developed for 3 to 4 hours at 30,000 r.p.m. Gradient fractions of 10 ml were collected. Pyrophosphohydrolase activity was analyzed as described in Figure 1. Acid phosphatase, cytochrome c oxidase and catalase were used as marker enzymes for lysosome, mitochondria and peroxisome respectively. (see Methods for more details).

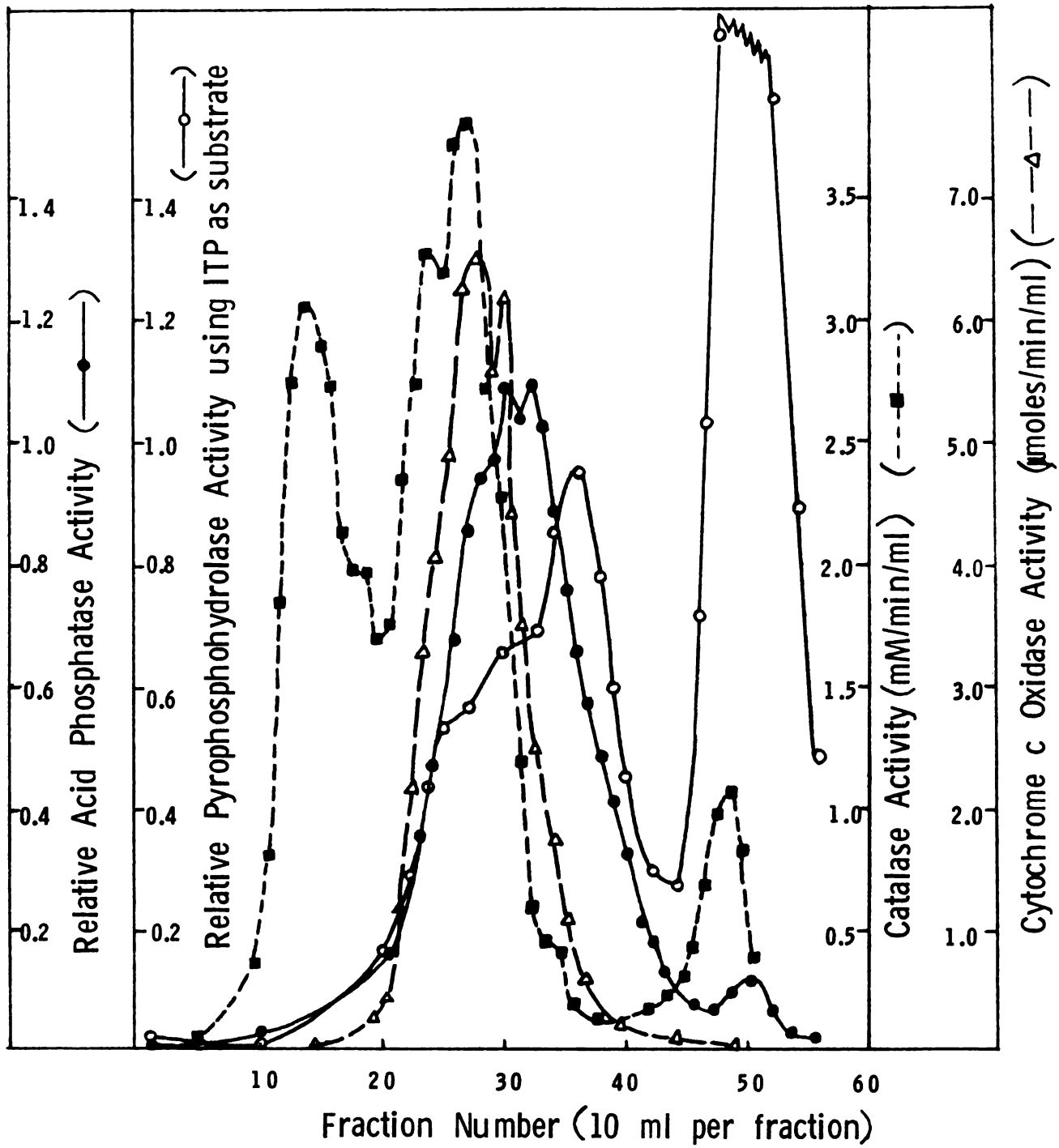
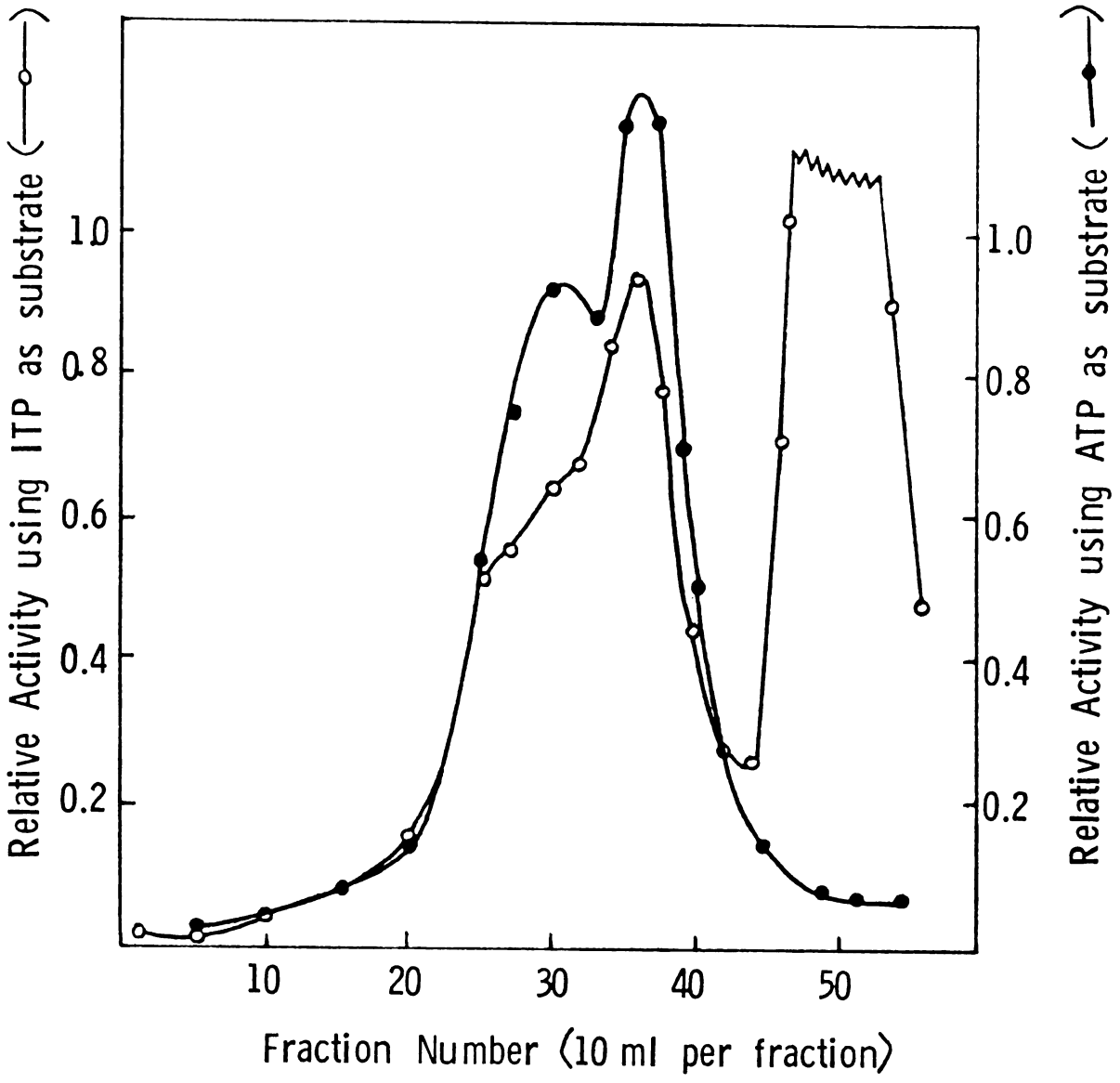

Relative Acid Phosphatase Activity (—●—)
Relative Pyrophosphohydrolase Activity using ITP as substrate (—○—)
Catalase Activity (mM/min/ml) (- - ■ - -)
Cytochrome c Oxidase Activity (μmoles/min/ml) (— —△— —)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
3.5
3.0
2.5
2.0
1.5
1.0
0.5
7.0
6.0
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
Fraction Number (10 ml per fraction)

Figure 12. Activity profiles using ITP and ATP as substrates on fractions from isopycnic density gradient as described in Figure 11. The same assay conditions as described in Figure 1 were used for both ITP and ATP.


Relative Activity using ITP as substrate (—○—)
Relative Activity using ATP as substrate (—●—)
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
Fraction Number (10 ml per fraction)

B. Is Pyrophosphohydrolase Located in the Nucleus?

Several methods of preparation of liver nuclei were carried out as described in the section of "Methods". However, no conclusive result can be drawn due to the difficulty of obtaining a clean preparation of nuclei without any cytoplasmic contamination.

In the preparation of liver nuclei in aqueous medium, the nuclei isolated are morphologically distinct and free of cytoplasmic contamination, but the activity was found entirely in the cytosol fraction. However, the fact that pyrophosphohydrolase activity was not found in the nuclei may be due to one of the following reasons: when cells are broken in an aqueous medium, there is the possibility of loss of water soluble components from the nucleus, or there may be an exchange reaction of soluble enzyme between the nucleus and the soluble phase of the homogenate. Hence a non-aqueous method of isolation of nuclei which retains the water soluble components of nuclei was carried out.

In the non-aqueous preparation of nuclei, pyrophosphohydrolase activity was found in both cytoplasmic and nuclear fractions. However, nuclei prepared in organic solvents were broken into fragments and morphologically non-identifiable. There may have been cytoplasmic and whole cell contaminations which were not distinguishable. The total pyrophosphohydrolase activity in the nuclei fraction was 5 units as compared to about 200 units in the cytoplas-

mic fraction. Glucose-6-phosphate dehydrogenase was chosen as a cytoplasmic enzyme marker (73) and was found mostly in the cytoplasmic fraction as was predicted (total activity of 2.5 units in cytoplasmic fraction as compared to 0.05 units in nuclear fraction). Thus, it is possible that the 5 units of pyrophosphohydrolase activity is due to cytoplasmic contaimination.

Two other methods of preparation of nuclei were tried. The nuclei isolated from both the glycerol medium and citrate medium were found to be contaiminted with cell membranes and cytoplasmic constituents, and pyrophosphohydrolase activity was found to be much higher in the cytoplasmic fraction than was in the nuclear fraction. Hence, no conclusion can be drawn as to whether pyrophosphohydrolase indeed occurs in the nucleus from these experiments.

IV. Survey of Nucleoside Triphosphate Pyrophosphohydrolase Activity in Different Organs of Rabbit

Crude homogenates and high speed supernatants of different organs of rabbit were prepared as described under the section of "Methods". They were analyzed for pyrophosphohydrolase activity and DNA content. Taking the value of 7.2×10^{-12} gm of DNA per cell for rabbit liver (74), the amount of pyrophosphohydrolase per cell was calculated. The results were summarized in Table 4.

Table 4. Survey of Nucleoside Triphosphate Pyrophosphohydrolase Activity in Different Organs of Rabbit

Organ	μmoles PPi formed/mg DNA	μmoles PPi formed/cell (x 10^{-9})
Liver	58.0	424.0
Thymus	32.7	236.0
Bone marrow	5.2	37.6
lung	37.5	271.0
Heart	28.3	211.0
Spleen	9.4	68.0
Pancreas	30.8	223.0
Kidney	51.5	372.0
Brain	91.0	660.0
Muscle	20.8	150.0
Red Blood Cell		9.6*

* This value was obtained by counting the number of cells directly and then analyzing the pyrophosphohydrolase activity by lysing the cells.

DISSCUSSION

A nucleoside triphosphate pyrophosphohydrolase which is most active in catalyzing the hydrolysis of ITP, dITP and XTP with the release of inorganic pyrophosphate and the corresponding nucleoside monophosphates was first detected and purified in rabbit erythrocytes (44).

An investigation to detect the possible occurrence of a similar enzyme in rabbit liver is the subject of this report. It has been possible to demonstrate that such an enzyme does exist in rabbit liver. Furthermore, crude extracts of rabbit heart, pancreas, kidney, thymus, bone marrow, lung, spleen, brain and skeletal muscle were all found to possess similar pyrophosphohydrolase activity, with brain having the highest activity on a per cell basis. The significance of the latter finding cannot be defined at this stage.

The nucleoside triphosphate pyrophosphohydrolase was purified to 600-fold from rabbit liver by a procedure combining heat denaturation, ammonium sulfate solubilization and DEAE-cellulose chromatography. The crude homogenate of rabbit liver was found to be contaminated with phosphatase and pyrophosphatase which interfere with the coupled enzyme assay system used. The fraction was therefore assayed using both

ITP and ATP as substrates and the difference between the two values was taken as pyrophosphohydrolase activity. However, it was found that the total activity obtained by this method for crude homogenate was less than the total activity in the high speed supernatant fraction obtained using the same procedure (Table 1). This has been taken to indicate that the difference in activity produced by ITP and ATP probably gives only a relative estimate of pyrophosphohydrolase activity in the crude homogenate. The activity assayed in the high speed supernatant fraction was found to be mostly pyrophosphohydrolase. Heat denaturation of the high speed supernatant fraction at 65°C removed most of the inorganic pyrophosphatase activity, the specific activity of the pyrophosphohydrolase was increased 3-fold with a 50% recovery. The reverse ammonium sulfate gradient produced a 6-fold purification with 80% yield, the pyrophosphohydrolase activity was efficiently separated from the hemoglobin peak and other preteins by this procedure. DEAE-cellulose chromatography was used to further purify the enzyme to give a final yield of 33%.

The partially purified nucleoside triphosphate pyrophosphohydrolase from rabbit liver was found to possess similar specificities and characteristics to that of red blood cell enzyme. Studies of the effect of pH on pyrophosphohydrolase activity shows that they both possess a pH optimum at 9.75. They both have an absolute requirement of sulfhydryl compound for activity. Substrate concentration

of 5×10^{-4}M was found to be optimum for both enzymes when ITP is used as substrate.

Identification and quantitation of the reaction products on Dowex I column show that the liver and red cell enzymes catalyze the same reaction, that is, they catalyze the pyrophosphorolytic cleavage of ITP to yield stoichiometric amounts of IMP and PPi.

As far as substrate specificity is concerned, ITP, dITP and XTP are found to be the most effective substrates for both enzymes, although the degree of hydrolysis was somewhat different: for red blood cell enzyme, ITP, 100%; dITP, 103%; XTP, 71%; for liver enzyme (Table 2), ITP, 100%; dITP, 152%; XTP, 99%. Hydrolytic rates towards GTP, dGTP, UTP, TTP, CTP, and ATP are about the same for both pyrophosphohydrolase.

An estimated molecular weight of 37,000 using the method of sucrose density centrifugation with two reference markers was obtained for both liver and red cell pyrophosphohydrolase, which adds one more line of evidence that they are the same protein.

Disc gel electrophoresis at pH 8.6 and the histochemical identification method for pyrophosphohydrolase activity on polyacrylamide gel revealed that the rabbit liver and red blood cell pyrophosphohydrolase migrate through the gel at the same rate, as does the human red blood cell pyrophos-

phohydrolase. Thus each of these protein has the same electrophoretic mobility at pH 8.6 and 7.5.

The separation of lysosomes from mitochondria and peroxisomes revealed the fact that pyrophosphohydrolase is not a lysosomal enzyme, but that it is primarily located in the cytosol.

One possible function of pyrophosphohydrolase is that it catalyzes the hydrolysis of ITP, dITP and XTP in order to prevent the accumulation of these nucleotides so that they will not be available for nucleic acid synthesis. If this hypothesis is correct, then there is the possibility that this enzyme is a nuclear enzyme. However, as shown in the results, attempts to identify the pyrophosphohydrolase as a nuclear enzyme were unsuccessful and the results were inconclusive. Even though the enzyme is not found exclusively in the nucleus, where nucleic acid synthesis occurs, this does not exclude the proposed function of pyrophosphohydrolase since the site of pyrophosphohydrolase action could be more related to the site of ITP and dITP synthesis than to the site of RNA and DNA synthesis.

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