Purification and Properties of Nucleoside Tetraphosphate Hydrolase from Rabbit Muscle*

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ABSTRACT: An enzyme that catalyzes the hydrolysis of adenosine 5′-tetraphosphate to adenosine triphosphate and inorganic phosphate (P_i) has been purified about 1000-fold from an extract of rabbit muscle. The enzyme also hydrolyzes inosine tetraphosphate to inosine triphosphate and P_i and tripolyphosphate to pyrophosphate and P_i, but shows little or no activity with other nucleotides and polyphosphates tested. It has an absolute requirement for a divalent cation: cobalt is the most effective for adenosine tetraphosphate hydrolysis while nickel is best for tripolyphosphate hydrolysis. Both adenosine tetraphosphate and tripolyphosphate hydrolyses are inhibited by the sulfhydryl binding reagents *p*-mercuribenzoate (hydroxy) and *N*-ethylmaleimide but not by iodoacetamide. The K_m values for adenosine tetraphosphate and inosine tetraphosphate are 2.7×10^{-5} and 3.4×10^{-5} M, respectively. The K_i value for tripolyphosphate as an inhibitor of adenosine tetraphosphate hydrolysis is 2×10^{-5} M. The phosphate released from adenosine tetraphosphate and tripolyphosphate results from a cleavage between the terminal phosphorus and the bridge oxygen. An enzyme exhibiting similar properties has been partially purified from rabbit liver, kidney, and heart.

Adenosine 5′-tetraphosphate was discovered by Marrian as a contaminant of commercial adenosine triphosphate (ATP)[1] prepared from ox muscle (Marrian, 1953, 1954). It was subsequently reported to be present in horse muscle (Lieberman, 1955), salmon liver (Tsuyuki *et al.*, 1956), and as a contaminant of commercial ATP presumably obtained from yeast (Sacks, 1955). Nothing is known concerning the metabolism of this nucleotide. During preliminary experiments aimed at finding a biological system that would synthesize adenosine 5′-tetraphosphate, it was found that under certain conditions added adenosine tetraphosphate was hydrolyzed, yielding ATP as the major product. This report summarizes our studies on the purification and properties of the enzyme from rabbit muscle. For convenience the term hydrolase will be used to describe the enzyme, and adenosine tetraphosphatase, inosine tetraphosphatase, and tripolyphosphatase to describe the individual hydrolytic activities.

Experimental Procedures

Measurement of Enzyme Activities

Phosphate Release Assay. Tripolyphosphatase and adenosine tetraphosphatase activities were determined by measuring the amount of orthophosphate released. The following components were present in a final incubation volume of 1.0 ml: 40 μmoles of Tris, pH 8.0, 5 μmoles of $MgSO_4$, 5 μmoles of 2-mercaptoethanol, 1–2 μmoles of adenosine tetraphosphate or tripolyphosphate, and 0.05 enzyme unit of hydrolase. The tubes were incubated 10–20 min at 37° and the reaction was terminated with 1.0 ml of cold 20% trichloroacetic acid. Denatured protein was removed by centrifugation and a 1.0-ml aliquot was used for the determination of orthophosphate by the method of Fiske and Subbarow as modified by Lindberg and Ernster (1956). The reaction is proportional to time up to the point when about three-fourths of the substrate is hydrolyzed, and the amount of orthophosphate released is directly proportional to the amount of hydrolase added (0.01–0.10 enzyme unit customarily used).

Spectrophotometric Assay. Adenosine tetraphosphatase activity was also determined with a spectrophotometric assay. The assay medium contained 40 μmoles of Tris, pH 8.0, 5 μmoles of $MgSO_4$, 5 μmoles of 2-mercaptoethanol, 0.4 μmole of NADP, 0.5 μmole of adenosine tetraphosphate, 2 units (U) of hexokinase, 25 μmoles of glucose, 0.7 U of glucose 6-phosphate dehydrogenase, and hydrolase in a final volume of 1.0 ml. All components except the hydrolase were preincubated 2 min at 37°, and the reaction was followed at 340 mμ. After obtaining a blank rate (typically about 0.005 optical density unit/min), the reaction was started

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[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ITP, inosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; AP_4, adenosine tetraphosphate; IP_4, inosine tetraphosphate; PPP_i, tripolyphosphate; $PPPP_i$, tetrapolyphosphate; PHMB, *p*-mercuribenzoate (hydroxy); NEM, *N*-ethylmaleimide.

by the addition of the hydrolase. The assay was used with 0.005–0.02 enzyme unit of hydrolase and was proportional to enzyme concentration. The spectrophotometric assay cannot be used before the cellulose phosphate step in the purification procedure because of the presence of myokinase which interferes with the assay. Inosine tetraphosphatase activity was determined by the same assay except that twice as much hexokinase was added. The determination of adenosine tetraphosphatase activity by the spectrophotometric and phosphate release methods agreed to within about 5%.

An enzyme unit of hydrolase is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmole of substrate/min at 37°. Specific activity is calculated as enzyme units/mg of protein with adenosine tetraphosphate as substrate and magnesium as the activating cation.

Nucleoside Triphosphatase Assay. The assay medium contained 40 μmoles of Tris, pH 8.0, 5 μmoles of $MgSO_4$, 5 μmoles of 2-mercaptoethanol, 10 μmoles of phosphoenolpyruvate, 4 U of lactic dehydrogenase, 2 U of pyruvate kinase, 0.4 μmole of NADH, 50 μmoles of KCl, 1–2 μmoles of a nucleoside triphosphate, and hydrolase in a final volume of 1.0 ml. The nucleoside triphosphates were usually contaminated with a small amount of the corresponding nucleoside diphosphate which resulted in an initial decrease in absorption at 340 mμ. After this initial decrease there was no oxidation of NADH, and the hydrolase was added. If a decrease in absorption at 340 mμ did not occur within a few minutes, about 1 μmole of the corresponding nucleoside diphosphate was added to ensure that the pyruvate kinase and lactic dehydrogenase were both active.

For the determination of the dephosphorylation of all other substrates, assay conditions exactly like those outlined for the phosphate release method were used except that 2–3 μmoles of the appropriate substrate were added.

Determination of Protein

Protein was determined by the method of Lowry *et al.* (1951) or the spectrophotometric method of Warburg and Christian (1941).

Ion-exchange Chromatography of Nucleotides and Polyphosphates. All separations were made with Dowex 1 (Cl^-), 0.8 × 11 cm, columns.

The various compounds were eluted as follows: AMP with 60 ml of 0.01 N HCl; P_i with 150 ml of 0.01 N HCl; ADP with 80 ml of 0.01 N HCl–0.05 M KCl; PP_i with 100 ml of 0.01 N HCl–0.1 M KCl or 50 ml of 0.06 N HCl–0.09 M KCl; ATP with 180 ml of 0.01 N HCl–0.1 M KCl or 30 ml of 0.06 N HCl–0.06 M KCl; PPP_i with 150 ml of 0.01 M HCl–0.15 M KCl or 25 ml of 0.2 N HCl–0.2 M KCl; adenosine tetraphosphate with 30 ml of 0.2 N HCl–0.2 M KCl; and $PPPP_i$ and inosine tetraphosphate with 25 ml of 0.2 N HCl–0.2 M KCl.

Test for Exchange of [^{32}P]Orthophosphate into Adenosine Tetraphosphate and Tripolyphosphate. The possible exchange of [^{32}P]orthophosphate into adenosine tetraphosphate and tripolyphosphate was tested for by looking for radioactivity not extractable with isobutyl alcohol–benzene (Walters and Cooper, 1965).

Determination of the Incorporation of ^{18}O into Phosphate after Hydrolysis of Adenosine Tetraphosphate and Tripolyphosphate in $H_2{}^{18}O$. The contents of the incubation tubes (25 ml) were placed on 0.8 × 11 cm Dowex 1 (Cl^-) columns that had been dried at 100° overnight. A small portion was added, and the top part of the column bed allowed to swell, then the remainder was added. The first 10 ml of effluent was discarded. The next 10 ml was saved and the atom per cent excess ^{18}O in the water determined. After all the sample was on, the columns were washed with about 10 ml of water and the appropriate fractions were separated as described above. The P_i, PP_i, and PPP_i fractions were evaporated to near dryness, then heated 15 min at 100° in 1 N HCl to hydrolyze the compounds to orthophosphate. The ATP and adenosine tetraphosphate were adsorbed on 300 mg of charcoal (Norit A boiled in 1 N HCl and washed with water). Four milliliters of 1 N HCl was added to the charcoal and the suspension was heated for 10 min at 100° to hydrolyze the pyrophosphate linkages. The charcoal was removed by centrifugation and washed twice with water. The washings were combined with the original supernatant and filtered.

The various orthophosphate fractions were precipitated as $MgNH_4PO_4$ and converted to H_3PO_4 using Dowex 50 in the hydrogen form. The H_3PO_4 was isolated as KH_2PO_4 as described by Dempsey *et al.* (1963). The ^{18}O content of the KH_2PO_4 was determined by the guanidine hydrochloride method of Boyer *et al.* (1961) using a Consolidated Electrodynamics Corp. Model 21-130 mass spectrometer.

DEAE-Cellulose and Cellulose Phosphate Chromatography. DEAE-cellulose was purchased from the Brown Company, Boston, Mass., and the cellulose phosphate was a product of the Mann Chemical Company, New York, N. Y. Both were prepared for use by washes in the following solutions: 0.1 N NaOH, H_2O, 95% ethanol, H_2O, 0.1 N HCl, H_2O, and 0.001 M EDTA. The chromatography columns of designated size were packed at room temperature using 3 psi of air pressure.

Gel Filtration. Sephadex G-100 was a product of Pharmacia Fine Chemicals, Inc., New Market, N. J. The gel was allowed to swell in 0.01 M Tris, pH 7.4, for 24 hr and the column equilibrated at 3° by passing buffer through it overnight.

Chemicals. Adenosine 5′-tetraphosphate was purchased from the Sigma Chemical Co. For certain studies the commercial product was purified by ion-exchange chromatography to remove contaminating ATP. Adenosine tetraphosphate (520 mg) was dissolved in 10 ml of water, and 2 ml was placed on each of five Dowex columns. The adenosine tetraphosphate was separated from ATP as previously described. The eluate was chilled to 0° and immediately neutralized to pH 7 with 2 N KOH. Adenosine tetraphosphate was precipitated as the barium salt by the addition of 5 ml of 2 M barium acetate and 400 ml of 95% ethanol. The

suspension was allowed to stand at 0° for several hours and centrifuged, and the supernatant solution was discarded. The precipitate was washed with 20 ml of water and dissolved in cold 1 N HCl, the barium was removed with Na_2SO_4, and the supernatant was neutralized with KOH. The concentration of adenosine tetraphosphate was calculated from its absorption at 260 mμ using a value of ϵ_{mM} 15.4.

Inosine 5′-tetraphosphate was prepared by the deamination of adenosine tetraphosphate with nitrous acid. To a glass-stoppered 40-ml tube were added 0.780 g of sodium acetate, 76 mg of adenosine tetraphosphate, 6.7 ml of 60% $NaNO_2$, and 3.0 ml of glacial acetic acid. The pH at this point was about 4. The tube was kept stoppered and allowed to stand at room temperature for 5 hr. Then the tube was chilled, the pH adjusted to 7 by the addition of about 2.3 ml of saturated KOH, and the inosine tetraphosphate isolated as the barium salt and converted to the sodium salt as described above. This was purified by chromatography on a Dowex column to remove ITP that was formed by the deamination of the ATP contaminating the adenosine tetraphosphate. The eluate was chilled and immediately neutralized, and the inosine tetraphosphate was reisolated as the barium salt and converted to the sodium salt. Inosine tetraphosphate was calculated from its absorption at 248.5 mμ using a value of ϵ_{mM} 12.2. All other nucleotides were obtained from the Sigma Chemical Co. or Pabst Laboratories.

Potassium tripolyphosphate was purchased from Alfa Inorganics, Beverly, Mass., and purified by ion-exchange chromatography on Dowex 1 (Cl^-) columns. Two milliliters of 0.1 M $K_5P_3O_{10}$ was applied to each of five Dowex columns. The tripolyphosphate eluates were pooled, chilled, and immediately neutralized. The tripolyphosphate was then concentrated by precipitation with barium and converted to the sodium salt as described for adenosine tetraphosphate. The concentration of tripolyphosphate was calculated from the amount of inorganic orthophosphate liberated after heating 10 min at 100° in 1 N HCl.

Sodium trimetaphosphate and sodium tetrametaphosphate were generous gifts of Dr. David Lipkin, Chemistry Department, Washington University, St. Louis, Mo. Sodium tetrapolyphosphate was prepared by the alkaline hydrolysis of sodium tetrametaphosphate. $Na_4P_4O_{12}\cdot 4H_2O$ (480 mg) was dissolved in 10 ml of 0.5 N NaOH and the solution was heated 75 min at 65°. After cooling, 40 ml of absolute ethanol was added and the mixture was cooled to −20° overnight. The precipitate was removed by centrifugation, dissolved in water, and transferred to a Dowex 1 (Cl^-) column. The tetrapolyphosphate eluate was neutralized and concentrated by precipitation with barium and converted to the sodium salt as described for adenosine tetraphosphate. Two-dimensional paper chromatography of this product by the method described by Karl-Kroupa (1956) showed contamination by tetrametaphosphate, but no trace of tripolyphosphate. The concentration of tetrapolyphosphate plus tetrametaphosphate was determined from the amount of orthophosphate liberated after heating 10 min at 100° in 1 N HCl.

$[^{32}P]H_3PO_4$ was obtained from Oak Ridge National Laboratories. The preparation was hydrolyzed in 1 N HCl for 90 min at 100° to hydrolyze any polyphosphate.

^{32}P-Labeled pyrophosphate was prepared by the pyrolysis of $[^{32}P]K_2HPO_4$. Five μmoles of neutralized potassium phosphate and about 1.5 mcuries of $[^{32}P]$-orthophosphate were evaporated to dryness in a platinum crucible, and the crucible was heated to redness for 2.5 min. The residue was dissolved in water and transferred to a Dowex 1 (Cl^-) column. The pyrophosphate was eluted with 0.06 N HCl–0.09 M KCl following removal of residual P_i. The tubes with the highest radioactivity were neutralized by the addition of solid Tris.

ATP labeled with ^{32}P in the terminal position was prepared according to the method described by Glynn and Chappell (1964). The radioactive ATP was separated from the other nucleotides on Dowex 1 (Cl^-). The ATP was eluted with 0.06 N HCl–0.06 M KCl and the tubes having the most radioactivity were neutralized with solid Tris. It is important not to use high chloride concentrations to elute the ATP as a small amount of adenosine tetraphosphate is synthesized in this system (Small and Cooper (1966)).

$[^{18}O]H_2O$ was purchased from Yeda Research and Development Co. Ltd, Rehovoth, Israel, and was distilled prior to use. Hexokinase, glucose 6-phosphate dehydrogenase, pyruvate kinase, and lactic dehydrogenase were obtained from California Foundation for Biochemical Research.

Results

Purification of Nucleoside Tetraphosphate Hydrolase from Rabbit Muscle

Step 1. Preparation of the Crude Enzyme. The muscle from the hind legs and back of two freshly killed rabbits was removed and placed in ice. The remaining operations were conducted at 0–5°. The muscle was ground, suspended in two volumes (w/v) of 0.005 M 2-mercaptoethanol, and homogenized for 30 sec at low speed in a 4-l. Waring blendor. The homogenate was centrifuged 20 min at 14,000 × *g*. The resulting supernatant solution is the crude enzyme.

Step 2. Acid Precipitation. The pH of the crude extract (usually about 6.5) was adjusted to 5.4 by the addition of 0.5 N acetic acid over a 15-min period. The precipitate was immediately removed by centrifuging 15 min at 14,000 × *g* and the supernatant solution was neutralized with solid Tris.

Step 3. Batch DEAE-Cellulose. A volume of gravity-packed DEAE-cellulose, previously equilibrated with 0.01 M Tris, pH 7.0, equal to the volume of the crude enzyme, was filtered on a Büchner funnel. The enzyme was diluted with an equal volume of water and the DEAE-cellulose cake was added and thoroughly stirred. The mixture was allowed to stand with intermittent stirring for 30–40 min and filtered on a Büchner

TABLE I: Rabbit Muscle Nucleoside Tetraphosphate Hydrolase Purification Summary.[a]

Fraction	Volume (ml)	Protein (total mg)	ATPase (total U)	PPase (total U)	PPPase (total U)	AP_4ase (total U)	AP_4ase (U/mg)	PPPase/ AP_4ase
1a, Crude	2190	44,500	872	4070	475	680	0.015	
2a, pH 5.4	2180	37,700	260	3600	349	384	0.010	
3a, DEAE	1970	2,320	112	808	158	256	0.110	
3a plus 3b		4,460	282	1750	376	576	0.129	
4, $(NH_4)_2SO_4$ + dialysis	114		58	890	240	351		
5, Cellulose phosphate	335	245	0	162	98	192	0.785	
6, $(NH_4)_2SO_4$ extraction								
60%		31.3				0.3	0.011	
56%		84.8				0.6	0.008	
52%		38.8				3.0	0.076	
48%		16.0				10.9	0.683	
44%[b]		10.7				26.5	2.26	
40%[b]		15.2				62.0	4.08	
36%[b]		9.2				33.8	3.68	
32%[b]		9.8				23.3	2.38	
28%		6.2				9.2	1.47	
Pooled fractions		45		12.7	49.5	145	3.24	0.34
7, Sephadex	43	23.2			46.3	103	4.43	0.45
8, DEAE	126	6.3		4.4	25	63	10.0	0.40

[a] The enzymes were assayed by the phosphate release method with magnesium as the activator. Protein was estimated by the method of Lowry *et al.* (1951) for steps 1, 2, and 3 and spectrophotometrically for the remaining steps. A total of approximately 2 kg of muscle was used for the complete preparation. [b] Fractions pooled.

funnel. The filtrate was used as a source of 3-phosphoglycerate kinase (Small and Cooper, 1966). The DEAE-cellulose cake was suspended in 0.01 M Tris–0.005 M 2-mercaptoethanol–0.15 M KCl, pH 7.0, using 1.3 times the volume of the crude enzyme, and the mixture was stirred for 30 min. The suspension was filtered and the filtrate was discarded. The enzyme was then eluted by suspending the DEAE-cellulose in one volume (referred to the crude enzyme) of 0.01 M Tris–0.005 M 2-mercaptoethanol–0.3 M KCl, pH 7.0, stirring for 15–20 min, and then filtering. The protein was concentrated by slow addition of 560 g of ammonium sulfate/l. of filtrate. After standing overnight at 0°, the precipitate was removed by centrifuging 15 min at 37,000 × *g*. The precipitate can be stored at −20° for at least a week without significant loss of activity. Two preparations (from four rabbits) were combined for the next step.

Step 4. Dialysis. The combined precipitates were dissolved in 80–100 ml of 0.01 M potassium citrate–0.005 M 2-mercaptoethanol, pH 6.0, and dialyzed 10–11 hr against 4 l. of the same solution. The precipitate that formed during dialysis was removed by centrifugation.

Step 5. Cellulose Phosphate Column. The dialyzed solution was transferred to a cellulose phosphate column, 5.8 × 25 cm, previously equilibrated with 0.01 M potassium citrate–0.005 M 2-mercaptoethanol, pH 6.0. The column was washed with 50 ml of the citrate–mercaptoethanol solution and elution was begun with 0.01 M potassium citrate–0.005 M 2-mercaptoethanol–0.04 M KCl, pH 6.0. Fractions of 25 ml were collected. The enzymatic activity was found in fractions 65–80. The active fractions were pooled and the protein concentrated by the addition of 610 g of solid ammonium sulfate/l. Special Mann enzyme grade ammonium sulfate was used for this and all subsequent steps. After standing at 0° for several hours, the precipitate was collected in one 40-ml centrifuge tube by centrifuging at 37,000 × *g*.

Step 6. Ammonium Sulfate Extraction. The precipitate from the previous step was extracted with ammonium sulfate–0.001 M mercaptoethanol, pH 7.0, solutions. One milliliter of 60% saturated ammonium sulfate was added to the precipitate for every 20 enzyme units, the suspension was stirred for 15 min and then centrifuged, and the process was repeated using the same volume of 56% saturated, then 52%, 48%, etc., down to and including 28%. The supernatant solutions

TABLE II: Stoichiometry of the Hydrolysis of Adenosine Tetraphosphate.[a]

	μmoles				
	P_i	AMP	ADP	ATP	AP_4
Zero time	0.38	0.04	0.06	0.28	1.60
15-min incubation	1.84	0.04	0.12	1.71	0.16
Net change	+1.46	0	+0.06	+1.43	−1.44

[a] The conditions were those described for the orthophosphate release assay except that the reaction was terminated by heating at 100° for 2.5 min. Enzyme (18 μg) having a specific activity of 5.6 was used, and the incubation time was 15 min. The nucleotides were separated as described in the text.

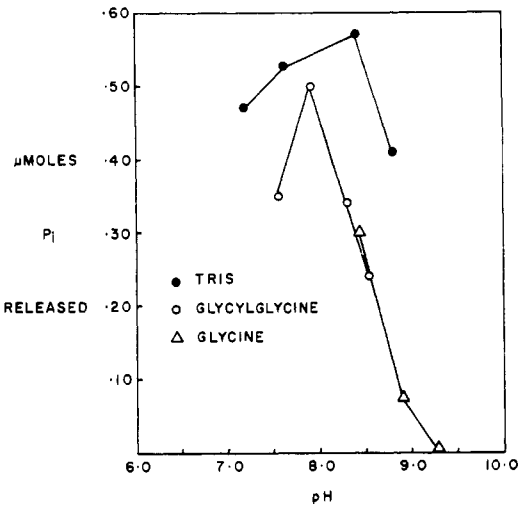

FIGURE 1. Effect of pH on magnesium-activated adenosine tetraphosphatase. About 8 μg of enzyme (specific activity of 5 U/mg) was used per tube. Each tube contained 70 μmoles of buffer in a final volume of 1.0 ml. All components of the incubation tubes except the magnesium were added, and the pH was checked with a Radiometer pH meter. The reaction was then started by the addition of magnesium. Incubation was at 37° for 15 min.

from the 44–32% extractions were pooled, and solid ammonium sulfate was added until the solution was 0.9 saturated. After standing several hours at 0°, the precipitate was collected by centrifugation.

Step 7. Sephadex G-100 Column. The precipitate from the preceding step was dissolved in a minimal volume of 0.02 M Tris–0.005 M 2-mercaptoethanol–0.001 M potassium tripolyphosphate, pH 7.4, and transferred to a 3 × 35 cm column of Sephadex G-100 equilibrated with the same solution. The column was then developed with the same solution and 10-ml fractions were collected. The initial fractions containing adenosine tetraphosphatase activity had a low specific activity and were discarded. The remaining active fractions (tubes 13–22) were combined.

Step 8. DEAE-Cellulose Column. The pooled fractions from the Sephadex column were transferred to a 1.8 × 22 cm DEAE column previously equilibrated with 0.01 M Tris–0.005 M 2-mercaptoethanol, pH 7.4. Convex gradient elution (Peterson and Sober, 1962) was carried out at a flow rate of 4 ml/min with 500 ml of 0.01 M Tris–0.005 M 2-mercaptoethanol–0.1 M KCl, pH 7.4, in the mixing vessel and 0.01 M Tris–0.005 M 2-mercaptoethanol–0.35 M KCl, pH 7.4, in the reservoir. The reservoir was above and connected to the top of the mixing vessel. Fractions of 10 ml were collected. The peak of enzymatic activity was found in fractions 23–34. The enzyme is very unstable at this point and has a half-life of about 2 hr.

Step 9. Ammonium Sulfate Precipitation. The active fractions from the DEAE-cellulose column were pooled and 610 g of solid ammonium sulfate was added per liter. This step was performed immediately after the preceding one. After standing several hours at 0° the suspension was centrifuged at 105,000 × *g* for 30 min and the precipitate was stored at −20°. The enzyme loses about 50% of its activity in 1 month under these conditions. A purification summary is given in Table I. The purified hydrolase does not behave as a homogeneous protein, as judged by its sedimentation and electrophoretic behavior.

Stoichiometry of the Reaction. The stoichiometry of the hydrolysis of adenosine tetraphosphate to ATP and orthophosphate was determined by measuring the change in adenosine tetraphosphate and ATP after separation of the nucleotides on Dowex 1 (Cl^-) columns and by measuring the amount of orthophosphate formed in duplicate incubation tubes. The results shown in Table II prove that the reaction proceeds as follows.

$$\text{adenosine tetraphosphate} + H_2O \rightarrow \text{ATP} + P_i$$

A similar experiment showed that pyrophosphate and orthophosphate were formed in equal amounts by the hydrolysis of tripolyphosphate (Table III).

Effect of pH on Magnesium-Activated Adenosine Tetraphosphatase and Tripolyphosphatase. Figure 1 shows that the optimal pH for adenosine tetraphosphate hydrolysis is about 8 in both Tris and glycyl-

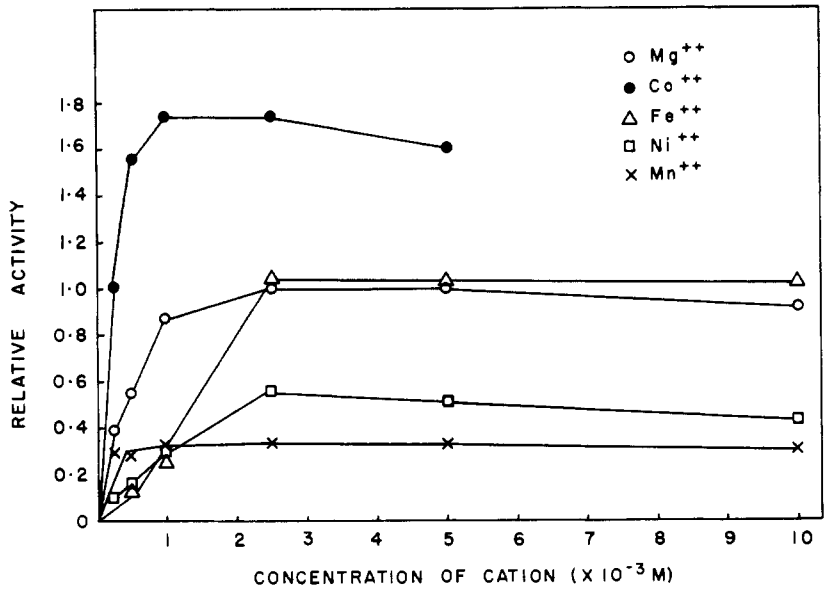

FIGURE 2: Activation of adenosine tetraphosphatase by divalent cations. About 8 to 10 μg of enzyme (5 U/mg) was used per tube. The adenosine tetraphosphatase activity was determined by measuring the orthophosphate released after a 15-min incubation at 37°. Relative activity was calculated by setting the amount of phosphate released from adenosine tetraphosphate with 5 μmoles of $MgSO_4$ equal to 1.00. The following salts of the cations were used: $MgSO_4$, $MnCl_2$, $Ni(CH_2H_3O_2)_2$, $CoCl_2$, $Fe(NH_4)_2(SO_4)_2$. An independent experiment showed that the different anions had no effect on the enzymatic activity at the concentrations used in this experiment.

TABLE III: Pyrophosphate and Orthophosphate as Products of the Enzymatic Hydrolysis of Tripolyphosphate.[a]

	μmoles	
	P_i	PP_i
Zero time	0.10	0.10
20-min incubation	1.33	1.41
Net change	+1.23	+1.31

[a] Each tube contained 40 μmoles of Tris, pH 8.0, 2 μmoles of tripolyphosphate, 2 μmoles of $Ni(C_2H_3O_2)_2$, and 13 μg of enzyme (4 U/mg) in a final volume of 1.0 ml. The reaction was terminated by heating at 100° for 2 min. The orthophosphate and pyrophosphate were separated on a Dowex column. The pyrophosphate was determined by the amount of orthophosphate released after heating 15 min at 100° in 1 N HCl.

glycine buffers with magnesium as the activating cation. Similar results were obtained with PPP_i as the substrate. All subsequent determinations of the enzyme activities were carried out in Tris buffer, pH 8.0.

Requirement for a Divalent Cation. The enzyme exhibits an absolute requirement for a divalent cation for the hydrolysis of adenosine tetraphosphate and tripolyphosphate. There is an interesting difference in the V_{max} obtained with various cations, as illustrated in Figures 2 and 3. For adenosine tetraphosphate hydrolysis the order of effectiveness is $Co^{2+} > Fe^{2+} \sim Mg^{2+} > Ni^{2+} > Mn^{2+}$, whereas for tripolyphosphate hydrolysis the order is $Ni^{2+} > Co^{2+} > Fe^{2+} \sim Mg^{2+} > Mn^{2+}$. Ca^{2+}, Zn^{2+}, Cd^{2+}, and Cu^{2+} showed no significant activation for either adenosine tetraphosphatase or tripolyphosphatase. No simple correlation exists between relative activity of the cations and their ionic size or electronegativity.

Substrate Specificity. The specificity studies summarized in Table IV show that adenosine tetraphosphate and inosine tetraphosphate are the only nucleotides of those tested that are hydrolyzed at appreciable rates. The purest preparations always hydrolyze GDP at a slow rate, but the fact that the ratio of the rate of hydrolysis of GDP to that of adenosine tetraphosphate decreases from over 1 to about 0.03 during the purification suggests that a separate, contaminating enzyme is responsible for GDP hydrolysis. The same argument applies in the case of pyrophosphate hydrolysis (see Table I). Other preparations of the enzyme have shown a much lower rate of hydrolysis of pyrophosphate.

In the case of tripolyphosphate, however, the following evidence suggests that its hydrolysis is catalyzed by the same enzyme that hydrolyzes adenosine tetraphosphate. (a) The ratio of the rate of tripolyphosphate hydrolysis to adenosine tetraphosphate hydrolysis remains fairly constant through the last three purification steps (Table I). No valid comparison can be made at any of the earlier steps since the pyrophosphate formed upon hydrolysis of tripolyphosphate is further cleaved

TABLE IV: Substrate Specificity of the Purified Rabbit Muscle Enzyme.[a]

P_i Release Assay		Spectrophotometric Assays	
Substrate	Relative Rate of Hydrolysis	Substrate	Relative Rate of Hydrolysis
AP_4	100	AP_4	100
IP_4	70	ATP, GTP, UTP, CTP, ITP	0
PPP_i	39		
PP_i	7		
GDP	3		
UDP, IDP	1		
ADP, AMP, trimetaphosphate, and tetrametaphosphate	0		

[a] Enzyme having a specific activity of about 10 U/mg was used. Enzyme (4–6 μg) was used for assaying adenosine tetraphosphate, inosine tetraphosphate, and tripolyphosphate by the phosphate release method. Enzyme (40–60) μg was used for assaying the other substances. Magnesium was the activating cation in all cases.

by the contaminating pyrophosphatase. (b) The tripolyphosphatase activity migrates at the same rate as the adenosine tetraphosphatase on a DEAE-cellulose column. (c) Increasing amounts of the sulfhydryl-binding reagents *p*-mercuribenzoate (hydroxy) and *N*-ethylmaleimide inhibit both activities to the same degree (Table V). Neither activity is significantly inhibited by iodoacetamide at concentrations as high as 0.002 M. (d) The two activities are inactivated at the same rate when allowed to stand in dilute solution at 0° (Table VI). (e) Tripolyphosphate is a competitive inhibitor of adenosine tetraphosphate hydrolysis as shown in Figure 4, suggesting that the two compounds combine at the same active site on the enzyme.

It seems reasonable to assume that both inosine tetraphosphate and inorganic tetrapolyphosphate are also used as substrates for the hydrolase. Table VII shows that the rate of tetrapolyphosphate hydrolysis with different activators is much slower than that of nickel-activated tripolyphosphatase. Two-dimensional paper chromatography of the tetrapolyphosphate revealed the presence of tetrametaphosphate. However, the data in Table VII show that the presence of 0.002 M tetrametaphosphate does not inhibit the hydrolysis of tripolyphosphate. Therefore, the slow hydrolysis of tetrapolyphosphate does not result from an inhibition by tetrametaphosphate. Cobalt is the best activator of the three cations tried with tetrapolyphosphate as substrate and also for adenosine tetraphosphate hydrolysis, in contrast to tripolyphosphate where nickel serves as the best activator. The results indicate that the presence of the adenosine moiety esterified to the tetraphosphate chain in some manner enhances its ability to be hydrolyzed by the muscle enzyme. It should be noted that the enhancement is not specific for adenosine since inosine tetraphosphate is also hydrolyzed more rapidly than tetrapolyphosphate.

Kinetics. The effect of the concentration of adenosine tetraphosphate or inosine tetraphosphate was studied using the spectrophotometric assay. Figure 5 illustrates

TABLE V: Inhibition of Hydrolase by *p*-Mercuribenzoate and *N*-Ethylmaleimide.[a]

		AP_4 as Substrate		PPP_i as Substrate	
Expt	Inhibitor	μmoles P_i Released	% Inhibition	μmoles P_i Released	% Inhibition
1	None	0.42	0.0	0.59	0.0
	0.0004 M NEM	0.28	34.5	0.37	37.0
	0.0006 M NEM	0.20	53.5	0.26	56.0
	0.001 M NEM	0.14	68.0	0.18	69.0
	0.002 M NEM	0.04	92.0	0.06	90.0
2	None	0.30	0.0	0.38	0.0
	1×10^{-6} M PHMB	0.18	40.0	0.27	28.0
	2×10^{-6} M PHMB	0.02	93.0	0.04	90.0

[a] All incubation tubes contained 40 μmoles of Tris, pH 8.0, 3–4 μg of enzyme (8 U/mg), 5 μmoles of $MgSO_4$, and 12 μmoles of adenosine tetraphosphate or 2 μmoles of $CoCl_2$ and 2 μmoles of tripolyphosphate in a final volume of 1.0 ml. The enzyme was incubated 10 min at room temperature with the inhibitor and all components of the incubation mixture present except the substrates. The concentrations of the inhibitors given are the final concentrations after all components have been added. The concentrations of inhibitors were about 10% higher during the preincubation. The reaction was started by addition of the appropriate substrate. Incubation was for 15 min at 37°.

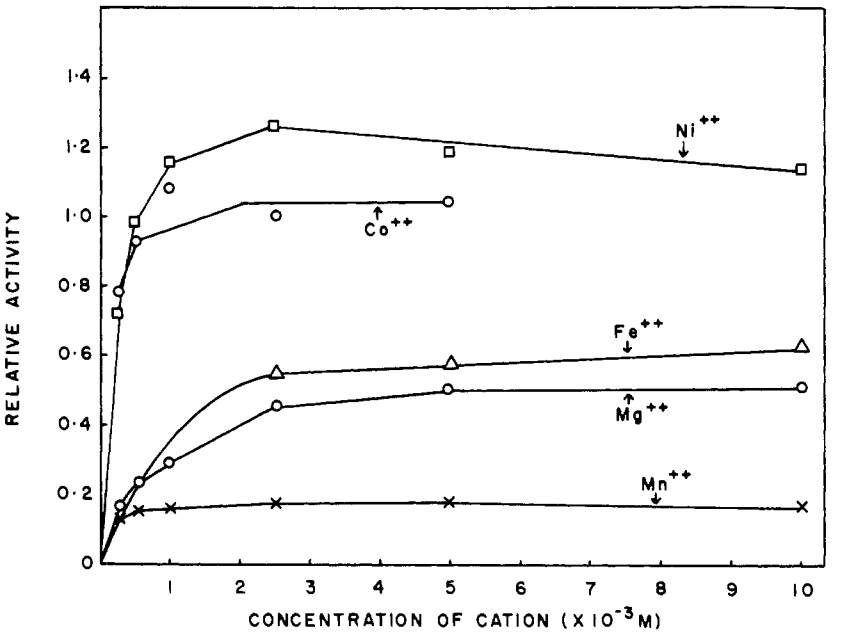

FIGURE 3: Activation of tripolyphosphatase by divalent cations. The experimental conditions were exactly as described for Figure 2.

a determination of the K_m values for these two substrates by plotting *S vs. S/v* (Dixon and Webb, 1964). The K_m values obtained in this manner are 2.7×10^{-5} M for adenosine tetraphosphate and 3.4×10^{-5} M for inosine tetraphosphate. The ratio of the V_{max} of inosine tetraphosphate to the V_{max} of adenosine tetraphosphate is 0.70, in agreement with the value obtained using the phosphate release method of assay.

A sensitive assay similar to the spectrophotometric assay for adenosine tetraphosphatase and inosine tetraphosphatase was not available for tripolyphosphate hydrolysis, and therefore an accurate determination of K_m for tripolyphosphate was very difficult. However, the ability of tripolyphosphate to bind to the enzymatic site was determined by using tripolyphosphate as an inhibitor of adenosine tetraphosphate hydrolysis which was assayed spectrophotometrically. Figure 4 shows a plot of *S vs. S/v* for the hydrolysis of adenosine tetraphosphate alone and in the presence of 1.7×10^{-5} M tripolyphosphate. The parallel lines are consistent with tripolyphosphate acting as a competitive inhibitor of adenosine tetraphosphate hydrolysis. The same results were obtained with larger amounts of hexokinase,

TABLE VI: Inactivation of Hydrolase upon Standing at 0°.[a]

Assay Time (hr)	Substrate		PPP_i/ AP_4
	AP_4	PPP_i	
0	4.15	3.84	0.92
24	1.25	1.05	0.84

[a] Assay tubes contained 40 μmoles of Tris, pH 8.0, and either 1.1 μmoles of adenosine tetraphosphate and 2 μmoles of $CoCl_2$ or 2 μmoles of tripolyphosphate and 2 μmoles of $Ni(C_2H_3O_2)_2$ were used. The results are expressed as μmoles of orthophosphate released/min per ml of enzyme solution. About 13 μg of enzyme was used for the assay of zero time, and about 16 μg for the assay at 24 hr.

TABLE VII: Hydrolysis of Inorganic Polyphosphates by Rabbit Muscle Enzyme.[a]

Exptl Conditions	μmoles P_i Released
$PPPP_i$ + $MgSO_4$	0.05
$PPPP_i$ + $CoCl_2$	0.17
$PPPP_i$ + $Ni(C_2H_3O_2)_2$	0.10
PPP_i + $Ni(C_2H_3O_2)_2$	0.41
PPP_i + TetrametaP + $Ni(C_2H_3O_2)_2$	0.41
TetrametaP + $Ni(C_2H_3O_2)_2$	0.01

[a] All incubation tubes contained 0.04 M Tris, pH 8.0, 0.002 M substrate, 0.002 M cation, and 10 μg of enzyme (4 U/mg) in a final volume of 1.0 ml. The tubes were incubated 15 min at 37°.

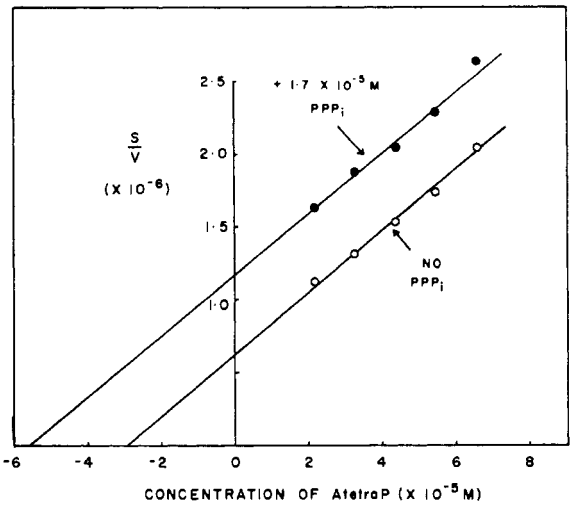

FIGURE 4: Inhibition of adenosine tetraphosphatase by tripolyphosphate. Enzyme (5–6 μg) (5 U/mg) was used per assay. The spectrophotometric assay was used with magnesium as the activating cation.

which indicates that tripolyphosphate is truly inhibiting the hydrolysis reaction and not the hexokinase used in the detector reaction. If it is assumed that the amount of tripolyphosphate broken down during the assay time is insignificant, one can calculate K_i, the inhibitor constant, to be 2×10^{-5} M. Thus, the enzyme has a very high affinity for tripolyphosphate and presumably a low K_m. The question of whether tripolyphosphate or adenosine tetraphosphate is the "true" substrate for this enzyme obviously cannot be answered on the basis of their relative K_m values or affinities to the enzyme.

Adenosine, AMP, and ADP each at a concentration of 0.003 M failed to inhibit significantly the hydrolysis of adenosine tetraphosphate at concentrations ranging from 2.2 to 5.5×10^{-5} M using the spectrophotometric assay. Even 0.003 M ATP gave no inhibition of adenosine tetraphosphate hydrolysis as measured by the phosphate release assay. With the same amount of adenosine tetraphosphate but using the spectrophotometric assay, 0.0017 M tripolyphosphate inhibited the hydrolysis of adenosine tetraphosphate by 65%. Pyrophosphate will also competitively inhibit adenosine tetraphosphate hydrolysis, but the K_i is 8×10^{-3} M, indicating that pyrophosphate has a lower affinity than does tripolyphosphate. Thus, the adenosine moiety esterified to either the pyrophosphate chain or the tripolyphosphate chain decreases their ability to inhibit the hydrolysis of adenosine tetraphosphate.

Effect of Temperature on Adenosine Tetraphosphatase. Figure 6 shows an Arrhenius plot of the adenosine tetraphosphatase reaction. From the slope of the plot one can calculate an activation energy of 8450 cal/mole.

Hydrolysis of Adenosine Tetraphosphate and Tripolyphosphate in $[^{18}O]H_2O$. Hydrolysis of adenosine tetraphosphate and tripolyphosphate in $[^{18}O]H_2O$ resulted in the incorporation of ^{18}O exclusively into the orthophosphate (Table VIII). Cleavage between the terminal phosphorus and the bridge oxygen is common to all transphosphorylase-type reactions and all phosphatase-catalyzed reactions that have been studied (Cohn, 1959). The data also show that there is no exchange between the $[^{18}O]H_2O$ and the substrates.

Test for Exchange Reactions Catalyzed by the Purified Enzyme. Attempts to show incorporation of $[^{32}P]$-orthophosphate into adenosine tetraphosphate and tripolyphosphate yielded negative results. Similarly, attempts to show an exchange between $[^{32}P]$ATP and

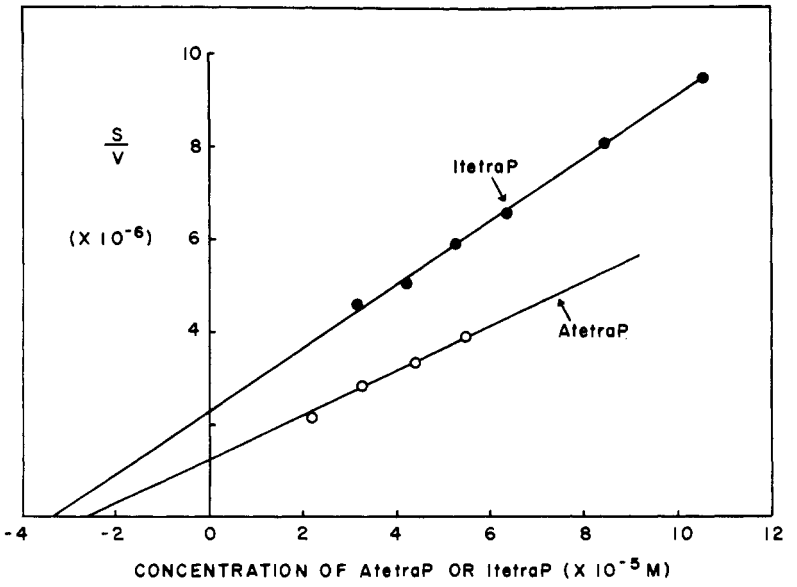

FIGURE 5: Comparison of K_m values for adenosine tetraphosphate and inosine tetraphosphate. Enzyme (3–4 μg) (5 U/mg) was used per assay.

TABLE VIII: Hydrolysis of Adenosine Tetraphosphate and Tripolyphosphate in $H_2{}^{18}O$.[a]

Tube	Fraction	Atom % Excess ^{18}O	μatom ^{18}O/ Molecule of Product
1	AP_4	0.000	0.00
2	AP_4	0.000	0.00
2	ATP	0.000	0.00
2	P_i	0.321	0.98
3	PPP_i	0.000	0.00
4	PPP_i	0.000	0.00
4	PP_i	0.000	0.00
4	P_i	0.306	0.95

[a] The incubation medium contained 0.04 M Tris, pH 8.0, 0.002 M $CoCl_2$, and 0.002 M adenosine tetraphosphate or 0.002 M $Ni(C_2H_3O_2)_2$ and 0.002 M tripolyphosphate, 13.3 enzyme units, in a final volume of 25 ml. The medium water contained 1.407 atoms % excess ^{18}O. Tubes 1 and 3 were controls with no enzyme. All tubes incubated for 20 min at 37°. The reaction was terminated by heating at 100° for 2 min. Approximately 60% of the substrate was hydrolyzed in tubes 2 and 4.

adenosine tetraphosphate or [^{32}P]pyrophosphate and tripolyphosphate also yielded negative results.

Detection of Nucleoside Tetraphosphate Hydrolase in Other Tissues. It was of interest to determine whether this enzyme is unique to muscle or if it occurs in other tissues as well. Rabbit liver, kidney, and heart were examined with the most extensive work being done with liver. The presence of ATPase, pyrophosphatase, and nonspecific phosphatases in extracts from these tissues made it rather difficult to identify the enzyme unequivocally. Most of the ATPase is associated with particulate matter which can be removed by high speed centrifugation. The presence of the specific hydrolase can then be detected by its rather unique specificity for divalent cation activators.

Rabbit liver was homogenized in three volumes of 0.005 M 2-mercaptoethanol and centrifuged 15 min at 37,000 × *g*, and the resulting supernatant solution centrifuged 30 min at 105,000 × *g*. The supernatant solution from the last centrifugation was dialyzed against 0.005 M 2-mercaptoethanol for 3.5 hr with a change of the dialysate at the end of 2 hr. The bright red precipitate that formed during dialysis was removed by centrifugation. The dialyzed material is fraction 1.

The enzyme was adsorbed and eluted from DEAE-cellulose in exactly the same manner as described for the muscle enzyme. The material (fraction 2) eluted off the DEAE-cellulose with 0.01 M Tris–0.005 M 2-mercaptoethanol–0.3 M KCl was concentrated by the addition of 560 g of ammonium sulfate/l. of solution. The precipitate was removed by centrifugation after standing at 0° for several hours and extracted twice with 10-ml portions of 50% saturated ammonium sulfate solution containing 0.001 M 2-mercaptoethanol in the manner described for the purification of the muscle enzyme. The nonextracted material was dissolved in 0.01 M Tris, pH 8.0 (fraction 3). This was transferred to a Sephadex G-100 column, 1.8 × 50 cm, previously equilibrated with 0.01 M Tris–0.005 M 2-mercaptoethanol–0.1 M KCl, pH 7.4. The column was developed with the same solution, and 3.5-ml fractions were collected. The tripolyphosphatase, adenosine tetraphosphatase, and pyrophosphatase activities migrated through the column at the same rate, but the peak ATPase activity was slightly in front of the other activities. The fractions containing the majority of the tripolyphosphatase and adenosine tetraphosphatase activity (tubes 11–19) were combined, and an equal volume of saturated ammonium sulfate solution, pH 7.0, was slowly added. After standing at 0° for several hours the precipitate was collected by centrifugation and dissolved in 0.01 M Tris, pH 8.0 (fraction 4). Table IX summarizes the purification steps. Cobalt was used as activator for all the enzymatic activities measured during the purification steps.

The enzyme (fraction 4) hydrolyzes tripolyphosphate about six times faster than pyrophosphate and adenosine tetraphosphate about thirteen times faster than ATP. This is suggestive evidence that the hydrolyses of tripolyphosphate and adenosine tetraphosphate are

TABLE IX: Rabbit Liver Nucleoside Tetraphosphate Hydrolase Purification Summary.

Fraction	Vol (ml)	Protein (total mg)	PPPase (total U)	PPase (total U)	ATPase (total U)	AP_4ase (total U)	AP_4ase (U/mg)
1	140	3360	66	100	15	35	0.010
2	285	985	25	50	12.5	21	0.021
3	8	744	12.6	19.2	4.2	15.2	0.020
4	4.5	34	1.9	0.3	0.2	2.6	0.076

[a] The hydrolysis of the various substrates were assayed by the phosphate release method except that 2 μmoles of $CoCl_2$ was used as the activating cation in each case and the 2-mercaptoethanol was omitted. The enzyme unit is the amount of enzyme that hydrolyzes 1 μmole of substrate/min under these conditions.

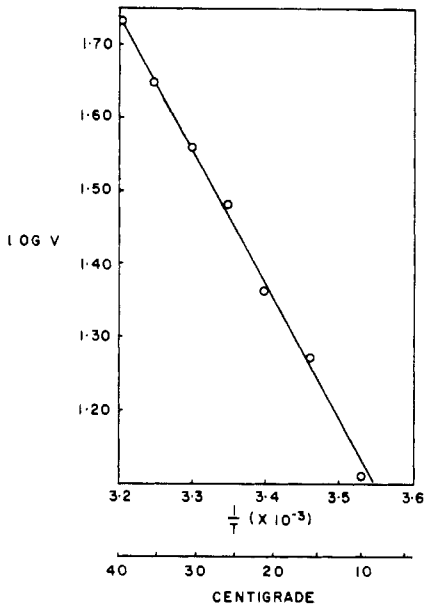

FIGURE 6: Arrhenius plot for adenosine tetraphosphatase reaction. Enzyme (6–9 μg) (5 U/mg) was used per assay. The spectrophotometric assay was used with magnesium as the activating cation.

not due to nonspecific pyrophosphatases but are probably due to an enzyme similar to the one purified from muscle. More convincing support for this conclusion is obtained when the activating metal specificity is studied (Table X). As was found with the muscle enzyme, the best activator for adenosine tetraphosphate hydrolysis is cobalt, whereas nickel is the best for tripolyphosphate hydrolysis. The relative effectiveness of the other metals in general is also the same as was found for the muscle enzyme.

Another similarity between the liver enzyme and the muscle enzyme is that both are inhibited by the sulfhydryl-binding reagents, *p*-mercuribenzoate (hydroxy) and *N*-ethylmaleimide. The inhibition of the liver enzyme is shown in Table XI.

TABLE X: Activation of Rabbit Liver Adenosine Tetraphosphatase and Tripolyphosphatase by Divalent Cations.[a]

Metal Added	μmoles P_i Released			
	AP_4	ATP	PPP_i	PP_i
None	0.06	0.06	0.07	0.17
$MgSO_4$	0.64	0.07	0.70	1.00
$Fe(NH_4)_2SO_4$	0.59	0.08	0.33	0.12
$CoCl_2$	0.91	0.14	0.69	0.26
$Ni(C_2H_3O_2)_2$	0.57	0.06	0.96	0.12
$MnCl_2$	0.32	0.11	0.33	0.18

[a] About 0.8 mg of protein (fraction 4), 2.5 μmoles of substrate, and 2.5 μmoles of cation were used. Incubation was at 37° for 15 min. The phosphate release assay was used.

TABLE XI: Inhibition of Rabbit Liver Adenosine Tetraphosphatase and Tripolyphosphatase by *p*-Mercuribenzoate (hydroxy) and *N*-Ethylmaleimide.[a]

Additions	μmoles P_i released	
	AP_4	PPP_i
None	0.76	0.79
1×10^{-5} M PHMB	0.70	0.78
1×10^{-4} M PHMB	0.22	0.09
1×10^{-3} M NEM	0.41	0.33
5×10^{-3} M NEM	0.08	0.08

[a] About 0.8 mg of protein (fraction 4) and either 2.5 μmoles of $CoCl_2$ and 2.5 μmoles of adenosine tetraphosphate or 2.5 μmoles of $Ni(C_2H_3O_2)_2$ and 2.5 μmoles of tripolyphosphate were used. The enzyme was preincubated for 10 min at room temperature with all components present except the substrates. The concentrations of the inhibitors given are the final concentrations after all components have been added. The concentrations of inhibitors were about 10% higher during the preincubation. The reaction was begun by the addition of substrate and was for 12 min at 37°.

The crude extracts of rabbit kidney and heart were prepared and dialyzed in exactly the same manner as described for liver except the homogenization was carried out for 1 min in a Waring blendor. The dialyzed crude fractions were made 50% saturated with respect to ammonium sulfate by the addition of solid ammonium sulfate (300 g/l. of solution). The suspension was centrifuged and the precipitate dissolved in 0.01 M Tris, pH 8.0.

The results obtained with the crude extracts and the 0–50% ammonium sulfate precipitates of both the heart and kidney extracts are summarized in Table XII. With crude extracts from both heart and kidney, tripolyphosphate is hydrolyzed three to four times faster than pyrophosphate with nickel as the activating cation. The 0–50% saturated ammonium sulfate precipitates of both the heart and kidney extracts do not hydrolyze any significant amount of pyrophosphate. The small amount of pyrophosphate broken down by the crude enzymes is most likely attributed to the presence of some magnesium which was not completely removed by the dialysis. The results are not quite as good with adenosine tetraphosphate as substrate, especially with the enzyme extract from heart, but there appears to be good evidence that an enzyme similar to the muscle nucleoside tetraphosphate hydrolase exists in rabbit kidney and heart as well as in liver.

TABLE XII: Hydrolysis of Adenosine Tetraphosphate, ATP, Tripolyphosphate, and Pyrophosphate by Rabbit Heart and Kidney Extracts.[a]

Additions	Kidney	Heart
A. Dialyzed Crude Extracts		
AP_4, Mg^{2+}	0.21	0.11
ATP, Mg^{2+}	0.11	0.14
PPP_i, Mg^{2+}	0.27	0.16
PP_i, Mg^{2+}	3.5[b]	3.5[b]
AP_4, Co^{2+}	0.25	0.12
ATP, Co^{2+}	0.05	0.15
PPP_i, Ni^{2+}	0.18	0.10
PP_i, Ni^{2+}	0.05	0.03
B. 0–50% Saturated $(NH_4)_2SO_4$ Precipitates		
AP_4, Mg^{2+}	1.27	0.64
ATP, Mg^{2+}	0.48	0.53
PPP_i, Mg^{2+}	1.27	0.51
PP_i, Mg^{2+}	5.70	1.65
AP_4, Co^{2+}	1.23	0.69
ATP, Co^{2+}	0.36	0.56
PPP_i, Ni^{2+}	1.28	0.73
PP_i, Ni^{2+}	0.00	0.01

[a] Five μmoles of $MgSO_4$, 2 μmoles of $CoCl_2$, 2 μmoles of $Ni(C_2H_3O_2)_2$, and 2 μmoles of substrate were used. Dialyzed crude kidney extract contained 15.8 mg of protein/ml, the heart 12.8. The 0–50% saturated ammonium sulfate precipitate of the kidney extract contained 46.4 mg/ml, the heart 20.0. Results are expressed as μmoles of orthophosphate released/min per ml of enzyme solution. [b] Values given are minimal values as all the pyrophosphate added was hydrolyzed.

Discussion

From the available data it is not possible to decide whether adenosine tetraphosphate or tripolyphosphate is the physiological substrate for this hydrolytic enzyme. The maximal rate of adenosine tetraphosphate hydrolysis with cobalt as the activating cation is only slightly greater than the maximal rate of tripolyphosphate hydrolysis with nickel as the activating cation. The K_m determined for adenosine tetraphosphate as substrate and the K_i determined for tripolyphosphate as an inhibitor of adenosine tetraphosphate hydrolysis are about the same, suggesting that the two substrates may be bound equally well to the enzymatic site.

One might speculate that the physiological function of the enzyme is to hydrolyze tripolyphosphate that is a product of certain enzyme-catalyzed reactions. For example, tripolyphosphate has been shown to be a product during the biosynthesis of vitamin B_{12} coenzyme by *Clostridium tetanomorphum* (Peterkofsky and Weissbach, 1963). An enzyme is present in *Escherichia coli* that cleaves deoxyguanosine triphosphate to deoxyguanosine and tripolyphosphate (Kornberg *et al.*, 1958). However, neither of these reactions have been shown to occur in mammals. Muscle adenylate kinase has been shown to form tripolyphosphate from ADP and pyrophosphate (Lieberman, 1956). The rate with pyrophosphate as acceptor in the latter reaction is only about 0.1% the rate of the enzyme-catalyzed dismutation of ADP to ATP and AMP, and rather high levels of pyrophosphate are required to attain maximal rates, so there is considerable doubt whether significant reaction occurs under *in vivo* conditions.

The hydrolase may be a component of a multienzyme system. For example, Mudd has shown that the methionine-activating enzyme isolated from yeast will hydrolyze added tripolyphosphate (Mudd, 1962). It is not known whether adenosine tetraphosphate will also be hydrolyzed by this enzyme. If the muscle enzyme that hydrolyzes tripolyphosphate and adenosine tetraphosphate is merely the methionine-activating enzyme, then one should be able to show a release of orthophosphate from ATP that is dependent upon L-methionine as shown in the equation

$$\text{L-methionine} + \text{ATP} \rightarrow \textit{S}\text{-adenosylmethionine} + PP_i + P_i$$

To test this possibility, assay tubes were set up containing 40 μmoles of Tris, pH 8.0, 5 μmoles of $MgSO_4$, 5 μmoles of 2-mercaptoethanol, 4.5 μmoles of ATP, 100 μmoles of KCl, 4.5 μmoles of L-methionine, and 16 μg of the purified hydrolase, specific activity about 5 U/mg. No significant orthophosphate was released after 20-min incubation at 37° with or without the methionine. Therefore, tripolyphosphate and adenosine tetraphosphate are hydrolyzed by a muscle enzyme that is distinct from the methionine-activating enzyme.

It is possible that the hydrolysis of tripolyphosphate or adenosine tetraphosphate is merely a trace reaction catalyzed by the enzyme analogous to the ATPase activity of hexokinase (Trayser and Colowick, 1961).

References

Boyer, P. D., Graves, D. J., Svelter, C. H., and Dempsey, M. E. (1961), *Anal. Chem. 33*, 1906.

Cohn, M. (1959), *J. Cellular Comp. Physiol. 54*, 17.

Dempsey, M. E., Boyer, P. D., and Benson, E. S. (1963), *J. Biol. Chem. 238*, 2708.

Dixon, M., and Webb, E. C. (1964), Enzymes, 2nd ed., New York, Academic, Chapter 8.

Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.

Karl-Kroupa, E. (1956), *Anal. Chem. 28*, 1091.

Kornberg, S. R., Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A. (1958), *J. Biol. Chem. 233*, 159.

Lieberman, I. (1955), *J. Am. Chem. Soc. 77*, 3373.

Lieberman, I. (1956), *J. Biol. Chem. 219*, 307.

Lindberg, O., and Ernster, L. (1956), *Methods Biochem. Analy. 3*, 1.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and

Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marrian, D. H. (1953), *Biochim. Biophys. Acta 12*, 492.
Marrian, D. H. (1954), *Biochim. Biophys. Acta 13*, 278.
Mudd, S. H. (1962), *J. Biol. Chem. 237*, PC1372.
Peterkofsky, A., and Weissbach, H. (1963), *J. Biol. Chem. 238*, 1491.
Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.
Sacks, J. (1955), *Biochim. Biophys. Acta 16*, 436.
Small, G. D., and Cooper, C. (1966), *Biochemistry 5*, 26 (this issue; following paper).
Tsuyuki, H., Mayah, H., and Idler, D. R. (1956), Abstracts, 130th National Meeting of the American Chemical Society, New York, N. Y., p. 37C.
Trayser, K. A., and Colowick, S. P. (1961), *Arch. Biochem. Biophys. 94*, 161.
Walters, E., and Cooper, C. (1965), *Anal. Biochem. 10*, 370.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Studies on the Occurrence and Biosynthesis of Adenosine Tetraphosphate*

Gary D. Small† and Cecil Cooper

ABSTRACT: Adenosine 5′-tetraphosphate has been detected in extracts of rabbit and horse muscle in amounts corresponding to 0.03–0.04% of the total adenosine mononucleotide content. The adenosine 5′-tetraphosphate was determined with a specific enzyme that hydrolyzes only the terminal phosphate of the nucleotide. Adenosine 5′-tetraphosphate is formed by a reaction between 1,3-diphosphoglycerate and adenosine triphosphate (ATP) catalyzed by yeast 3-phosphoglycerate kinase. The rate of this reaction is about 10^{-4} times the rate of the transfer of a phosphoryl group from 1,3-diphosphoglycerate to ADP catalyzed by the same enzyme. The analogous enzyme was purified from rabbit muscle and found not to catalyze this reaction. The possible significance of the small amount of adenosine 5′-tetraphosphate detected in muscle is discussed.

Adenosine 5′-tetraphosphate has been reported as a contaminant of adenosine triphosphate (ATP[1]) prepared from various biological sources (Marrian, 1954; Lieberman, 1955; Sacks, 1955), but there is no published data concerning the level of the nucleotide in tissues or its biological synthesis. The purification of an enzyme that cleaves only the terminal phosphate (Small and Cooper, 1966) provided a convenient and sensitive assay for adenosine 5′-tetraphosphate in extracts of various tissues. This paper reports the detection of adenosine 5′-tetraphosphate in rabbit and horse muscle and summarizes studies concerning the biological synthesis of the nucleotide.

Experimental Procedures

Measurement of Enzyme Activities. 3-Phosphoglycerate kinase was assayed by coupling it to glyceraldehyde 3-phosphate dehydrogenase. The assay medium contained 40 μmoles of Tris, pH 7.4, 10 μmoles of cysteine or 2-mercaptoethanol, 2.5 μmoles of $MgSO_4$, 6 μmoles of 3-phosphoglycerate, 0.3 μmole of NADH, and 4 U of glyceraldehyde 3-phosphate dehydrogenase in a final volume of 1.0 ml. The reaction was started by the addition of 3-phosphoglycerate kinase and followed by measuring the decrease in optical density at 340 mμ.

Myokinase was assayed by coupling it with the pyruvate kinase–lactic dehydrogenase system. The assay medium contained 40 μmoles of Tris, pH 7.4, 15 μmoles of $MgSO_4$, 50 μmoles of KCl, 4 μmoles of phosphoenolpyruvate, 6 μmoles of ATP, 6 μmoles of AMP, 0.6 μmole of NADH, 5 U of pyruvate kinase, and 7 U of lactic dehydrogenase in a final volume of 3.0 ml. The reaction was started by the addition of myokinase and followed by measuring the decrease in optical density at 340 mμ. An enzyme unit in all cases is defined

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† The data are from the thesis presented by G. D. Small in partial fulfillment of the requirements for the Ph.D. degree from Western Reserve University. Present address: Department of Biochemistry, University of Washington, Seattle, Wash.

[1] Abbreviations used are: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AP_4, adenosine 5′-tetraphosphate; CTP, cytidine 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.