

# Enzyme-Catalyzed Synthesis of Nucleoside Triphosphates from Nucleoside Monophosphates

## ATP from AMP and Ribavirin 5'-triphosphate from Ribavirin 5'-monophosphate

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### ABSTRACT

Syntheses of adenosine 5'-triphosphate (ATP) from adenosine 5'-monophosphate (AMP) and ribavirin 5'-triphosphate (RTP) from ribavirin 5'-monophosphate (RMP) (1) were performed using enzymes as catalysts. Synthesis of ATP is based on acetyl phosphate as the phosphate donor, and acetate kinase (*Bacillus stearothermophilus*, EC 2.7.2.1), adenylate kinase (porcine muscle, EC 2.7.4.3), and inorganic pyrophosphatase (yeast, EC 2.6.1.1) as the catalysts. Three reactions on a 150-mmol scale provided ATP as its barium salt in 82% yield and 67% purity. Synthesis of RTP used phosphoenol pyruvate (PEP) as the phosphate donor, and pyruvate kinase (rabbit muscle, EC 2.7.1.40) and adenylate kinase (rabbit muscle) as the catalysts. A gram-scale reaction provided RTP as its barium salt in 93% yield and 97% purity. This work demonstrates the utility of the autoxidation-resistant acetate kinase from *B. stearothermophilus*, the value of pyrophosphatase in controlling the level of pyrophosphate in the reactions and the ability of adenylate kinase to accept at least one substrate other than a derivative of adenosine.

**Index Entries:** Acetate kinase, catalyst for synthesis of nucleoside triphosphates; adenylate kinase, catalyst for synthesis of nucleoside triphosphates; ATP, synthesis from AMP; enzymes, catalysts in organic synthesis; nucleoside triphosphates, synthesis from monophosphates; ribavirin 5'-triphosphate, synthesis from ribavirin 5'-monophosphate.

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## INTRODUCTION

This paper describes enzyme-catalyzed procedures for the conversion of adenosine 5'-triphosphate (AMP) to adenosine 5'-triphosphate (ATP) and ribavirin 5'-monophosphate (RMP) to ribavirin 5'-triphosphate (RTP). This work is a part of our continuing program in the development of practical-scale procedures for organic synthesis based on enzymes (2-9).

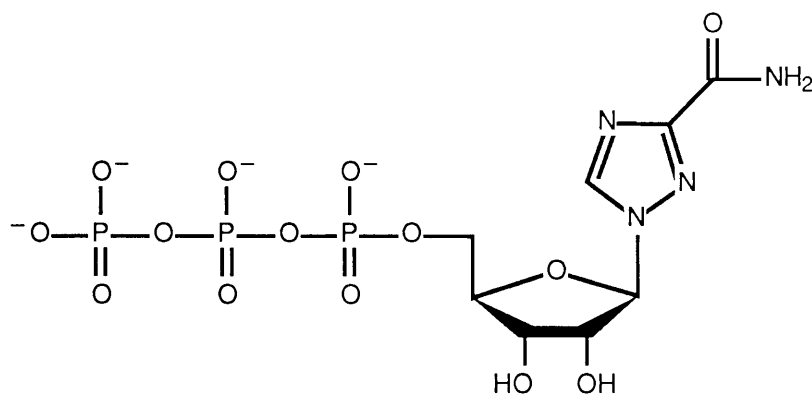
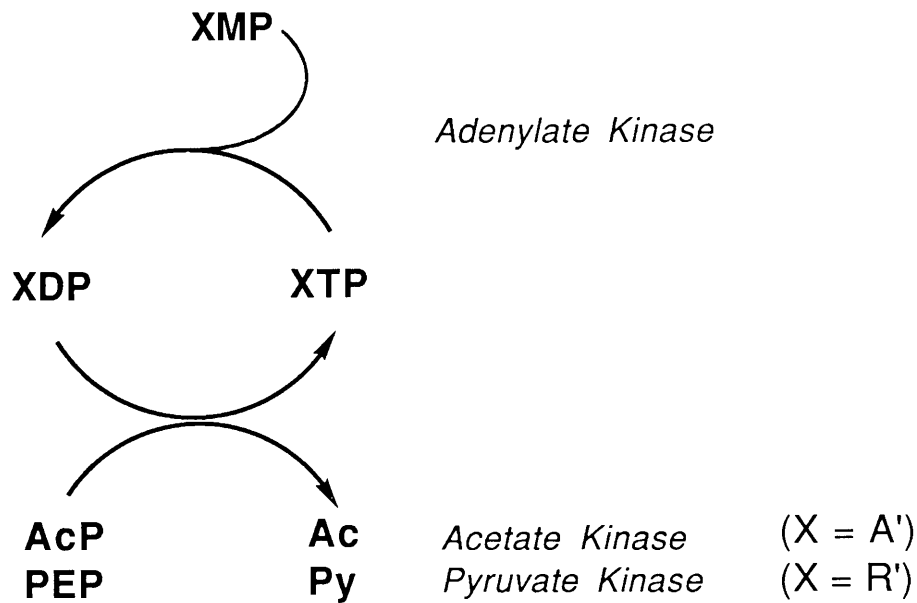
Our interest stems in the synthesis of ATP stem from its central role in biosynthesis, and from the opportunity its synthesis presents to develop techniques for ATP regeneration without interference from coupled reactions consuming ATP (2). Several procedures are already available for the synthesis of ATP, including chemical methods (10,11), fermentation (using whole cells and organelles) (12-15), and enzymatic catalysis (using cell-free enzymes) (16-18). The previously reported enzymic syntheses have two disadvantages. First, they are based on an acetate kinase from *E. coli* that contains a thiol group close to the active site (19). This enzyme is rapidly deactivated. Second, they use diammonium acetyl phosphate as the phosphate donor. This compound is difficult to prepare on large scale (20). Moreover, ammonium ion reacts with acetyl phosphate in solution and forms an insoluble precipitate of magnesium ammonium phosphate. This precipitate both removes from the solution the magnesium ion that is required for activity of the kinases and occludes particles of immobilized enzymes. The procedure reported here uses readily available and inexpensive AMP as the starting material, disodium acetyl phosphate as the phosphate donor, and acetate kinase (*B. stearothermophilus*)/adenylate kinase as the catalyst (Scheme I). Disodium acetyl phosphate can be easily prepared on mol scale (21,22). Acetate kinase from *B. stearothermophilus* is commercially available and no more expensive than the *E. coli* enzyme. It does not contain a thiol group and is not sensitive to autooxidation (23). Adenylate kinase from porcine muscle is commercially available, inexpensive, and stable (if protected against autooxidation) (24).

Ribavirin and its phosphorylated derivatives are interesting for their antiviral properties (25-28). We tested two systems for the synthesis of RTP and RMP: one based on acetyl phosphate, acetate kinase, and adenylate kinase (AcP/AcK/AdK); the second on phosphoenol pyruvate, pyruvate kinase, and adenylate kinase (PEP/PK/AdK) (Scheme I). Although both systems were successful, we chose the system based on PEP/PK/AdK because the slow rate of enzyme-catalyzed reaction requires a hydrolytically stable phosphorylating agent.

## RESULTS

### ATP from AMP

Enzymes were used immobilized in PAN gels (29). We compared the oxidative and thermal stability of the dioxygen-sensitive acetate kinases



Scheme 1. Conversion of nucleoside monophosphates to nucleoside triphosphates (A' = adenosine; R = ribavirin).

from *E. coli* with that of the dioxygen-insensitive *B. stearothermophilus* at 50°C (Fig. 1). The *B. stearothermophilus* enzyme was significantly more stable. We note that the *B. stearothermophilus* enzyme is *more stable*, even in the absence of DTT (an autooxidant), than is the *E. coli* enzyme in the presence of DTT, and believe that the *B. stearothermophilus* enzyme is gen-

erally superior for use in regeneration of ATP in enzyme-catalyzed synthesis. We used three different analytical methods for analysis of nucleotides: HPLC provided the most accurate information concerning the amounts of these substances present;  $^{31}\text{P}$  NMR spectroscopy allowed analysis of all phosphate-containing species; enzymatic assay was generally inferior to the other methods, but was useful for the analysis of starting materials and products (30).

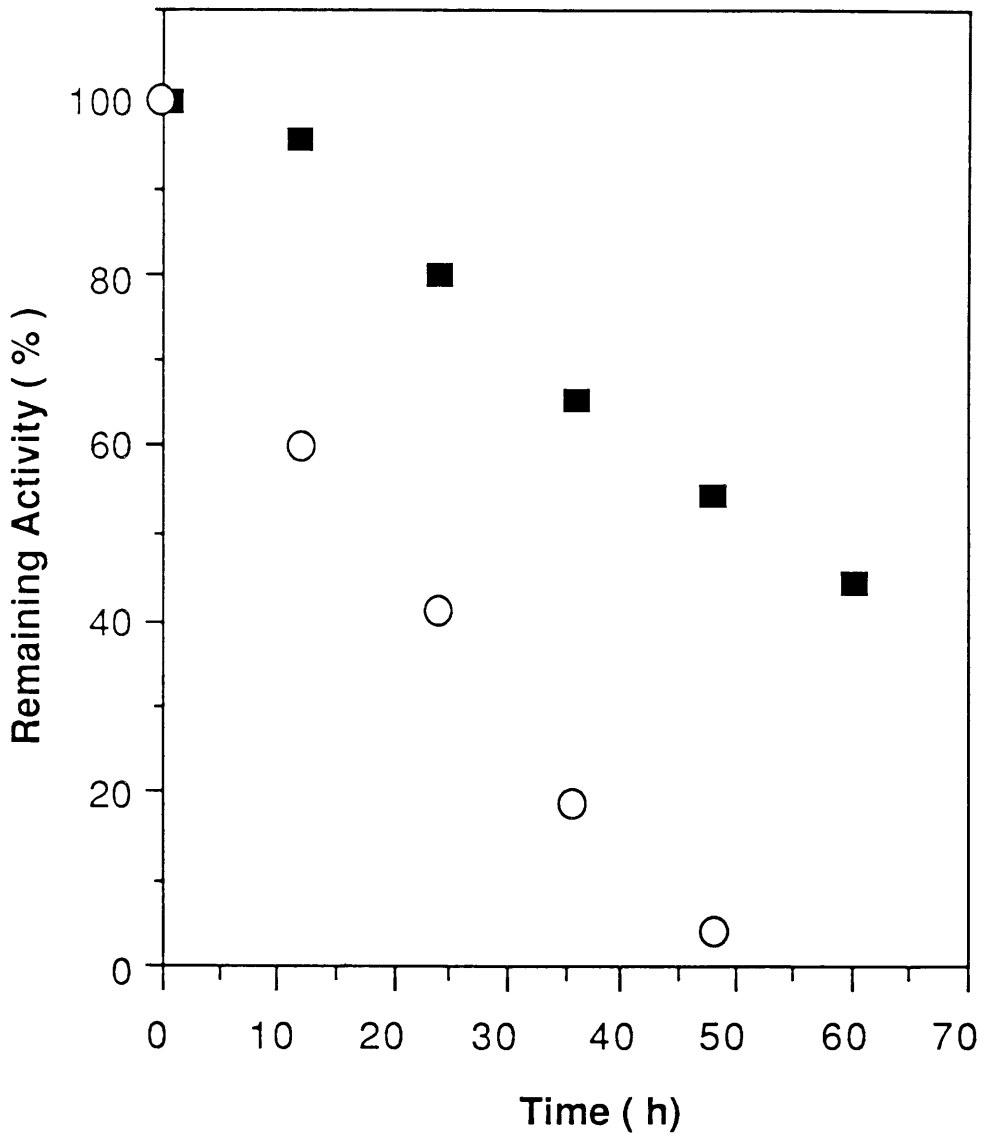


Fig. 1. Stability of acetate kinases from *E. coli* and *B. stearothermophilus*, immobilized in PAN. The enzyme-containing solutions were stirred at pH 7.5 and 50°C under air with DTT for the *E. coli* enzyme (○) and without DTT for the *B. stearothermophilus* enzyme (■).

We performed a number of small-scale reactions to establish conditions for larger-scale syntheses. Some useful observations from these preliminary experiments follow. First, AcP contains inorganic pyrophosphate (PP<sub>i</sub>) as a minor impurity; this compound forms an insoluble magnesium salt during the reaction. Addition of inorganic pyrophosphatase (PPase) prevents this precipitation by hydrolyzing pyrophosphate (31). The magnesium concentration in the solution should be controlled during the reaction. At the beginning of the reaction, the concentration should be low (~20 mM) to minimize the inhibition of acetate kinase and increased, as the reaction proceeds, to its optimal amount: one equiv per equiv of total nucleotides at the end of the reaction (e.g., 100 mmol of magnesium for a reaction conducted using 100 mmol of AMP). Second, AcP should be kept at 4°C and added continuously to the reaction mixture to minimize its hydrolytic decomposition. Third, the maximum practical conversion of AMP to ATP using this system seems to be approximately 90%; this conversion can be increased only at the expense of economy, yield, and purity. The amount of AcP required to obtain this conversion ranges from 2.1 per equiv of AMP at low concentrations (30 mM AMP) to 3.0 equiv at high concentrations (100 mM). Higher conversions of AMP to ATP are thermodynamically possible with AcP as phosphate donor, but concentrations of phosphate (and acetate) become high in the reaction mixture, and rates become slow (for reasons we have not established in detail). Fourth, the mercaptoethanol required to protect adenylate kinase against autooxidation does not seriously affect the activities of other enzymes.

Based on these observations, we conducted successive reactions for conversion of AMP to ATP on the 50-mmol scale, with the final concentration of ATP approximately 0.1 M. Details are given in the Experimental Section; the course of two representative reactions is given in Fig. 2. These reuses of the enzyme resulted in only small losses of AcK and AdK; PPase was less stable. The ATP from the three combined reactions (0.125 mmol) was isolated as its barium salt in 82% yield and 67% purity (32).

## RMP to RTP

Two systems of enzymes, AcP/AcK/AdK and PEP/PK/AdK, were tested in qualitative experiments on assay scale as potential catalysts for the synthesis of ribavirin 5'-triphosphate (RTP) from ribavirin monophosphate (RMP). The reactions were followed by <sup>31</sup>P NMR spectroscopy. Independent assay experiments demonstrated that adenylate kinase catalyzes the phosphorylation of RMP to RDP by ATP, that pyruvate kinase catalyzes the phosphorylation of RDP to RTP by PEP, and that acetate kinase catalyzes the phosphorylation of RDP to RTP by AcP. The most important of these observations is that adenylate kinase accepts RMP and RTP as substrates and provides RDP as product. Adenylate kinase has been reported to be highly specific for adenosine nucleotides (24); apparently by using large quantities of enzyme and by accepting slow rates, it is possible

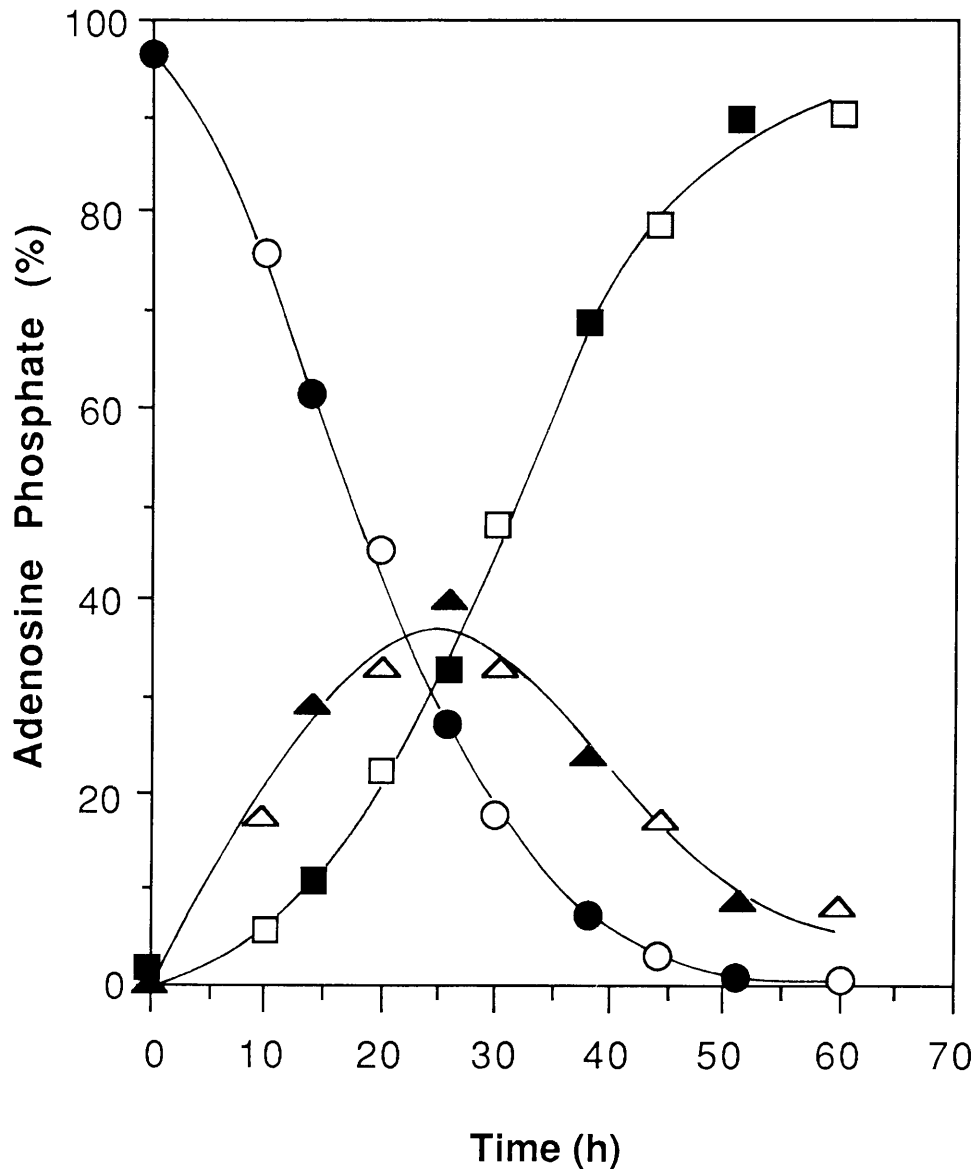


Fig. 2. Reaction progress for the enzyme-catalyzed conversions of AMP to ATP on a 50-mmol scale (runs 1 and 2). (●), run 1, AMP; (▲), run 1, ADP; (■), run 1, ATP; (○), run 2, AMP; (△), run 2, ADP; (□), run 2, ATP.

to observe useful activity with at least one other nucleoside (RMP). This new activity for AdK, combined with the broad substrate acceptability of AcK<sup>33</sup> provides the basis for the conversion of RMP to RTP (34).

Although our comparison of the AdK/PK/PEP and AdK/AcK/AcP systems for conversion of RMP to RTP were qualitative and based on <sup>31</sup>P spectroscopic results, it was clear that both were capable of catalyzing the conversion. We selected the AdK/PK/PEP system for two reasons: PK is less expensive than AcK; PEP is stable in solution and a strong phos-

phorylating agent. It shifts the equilibrium of reaction far toward the production of RTP and contributes to a high yield and purity for the RTP. Because RMP is a poor substrate for AdK, the stability of the phosphate donor is important: the spontaneous hydrolysis of AcP experienced during the long reaction times required generated high levels of  $P_i$ , and resulted in loss of  $Mg^{+2}$  from solution and even slower rates. Reaction was conducted using soluble enzymes and starting with 1.7 mmol of RMP; at completion, the conversion of RMP to RTP was 98% (Fig. 3), with no detectable RDP in solution. This distribution of products almost certainly reflects kinetic limitations: The conversion of RMP to RDP by AdK is the slow step overall in the sequence; conversion of RDP to RTP by PK is probably

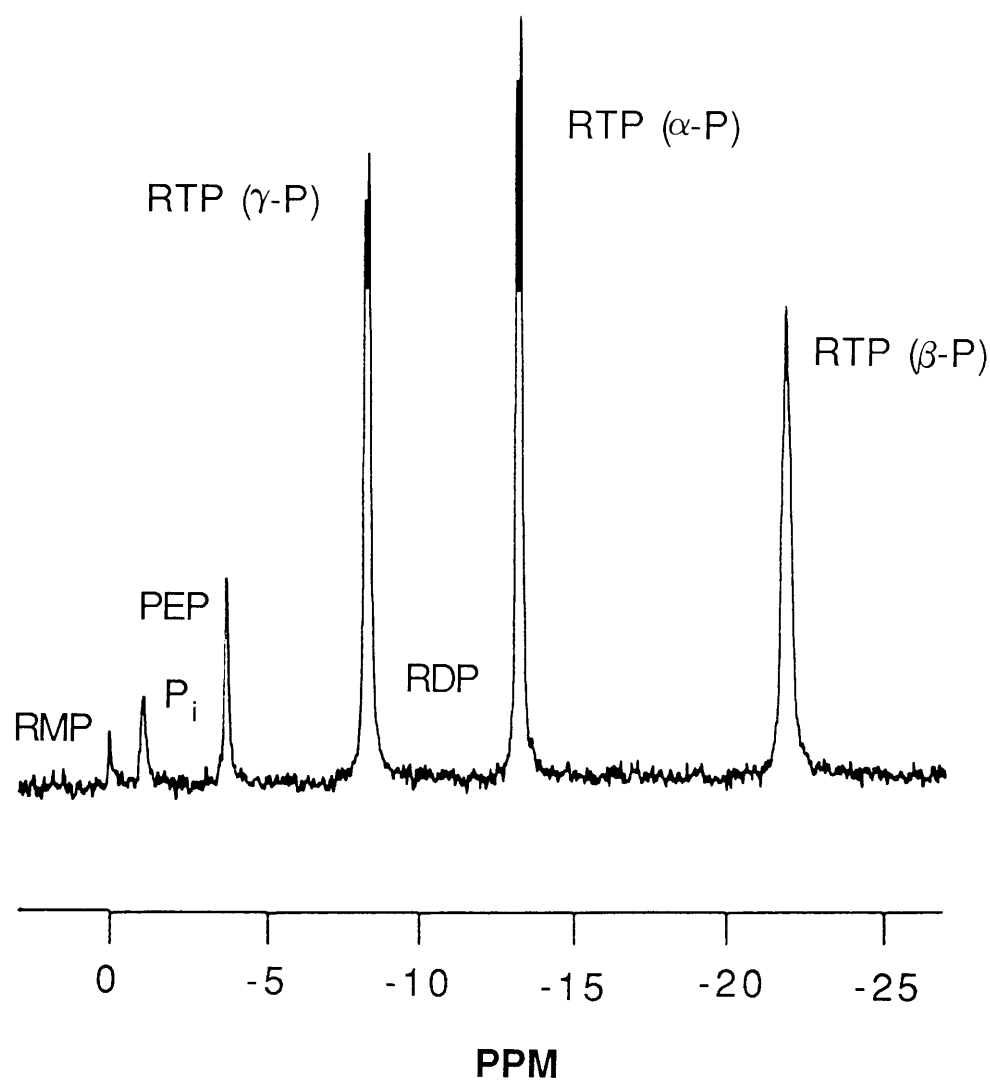


Fig. 3. Decoupled  $^{31}P$  NMR spectrum for the enzyme-catalyzed conversion of RMP to RTP after 140 h of reaction. Three strong peaks from RTP, a tiny peak from RMP, and no detectable peaks from RDP are observed.

fast. RTP was isolated as its barium salt (1.6 mmol) in 93% yield and 97% purity.

## CONCLUSIONS

The procedure described here for the conversion of AMP to ATP solves several problems encountered in previous enzyme-catalyzed syntheses. Substitution of the air-stable AcK from *B. stearothermophilus* for the autoxidation sensitive enzyme from *E. coli* permits the enzymes to be reused several times without significant loss in activity. Introducing PPase into the system minimizes the troublesome precipitates of insoluble magnesium pyrophosphate. The remaining disadvantages of the system originate in the choice of AcP as the ultimate phosphorylating agent. The hydrolytic instability of this substance requires cooling and results in the production of inorganic phosphate in the reaction mixture. This phosphate is the major impurity in the ATP product. The inorganic pyrophosphate present as a minor impurity in AcP necessitates use of another enzyme PPase—an enzyme that is neither especially inexpensive nor stable—to prevent the formation of insoluble precipitate; this requirement makes the procedure more complicated. Nonetheless, we believe that this procedure should be useful when ATP is required as a stoichiometric reagent. The problems stemming from use of AcP might, of course, be circumvented by using PEP/PK, but at the increased difficulty of preparing PEP.

The synthesis of RTP establishes a practical procedure using enzymes for laboratory-scale preparation of RTP from RMP. It uses PEP as the phosphate donor; pyruvate kinase and adenylate kinase as the catalysts. Its advantages are that it is straightforward, and provides RTP in high yield and purity. Its disadvantages are that it requires relatively large quantities of enzymes to achieve useful rates. The particular procedure followed here used soluble enzymes that were discarded. Both the synthesis of ATP and RTP would benefit from application of the MEEC technique (35). The observation that AdK accepts RMP and CMP (34) (both with low rates) suggests that this system may be applicable to other nucleotide monophosphates. We have not yet examined these systems in sufficient detail to understand their kinetics. In particular, in this system, we do not know if ATP is required as a phosphate donor, or if RTP can also serve in this capacity. We have assumed that the presence of ATP in the reaction mixture is beneficial, but we have not established this assumption experimentally.

## EXPERIMENTAL SECTION

### Materials

Acetate kinase (*E. coli* and *B. stearothermophilus*, EC 2.7.2.1), pyruvate kinase (rabbit muscle, EC 2.7.1.40), and inorganic pyrophosphatase (bakers



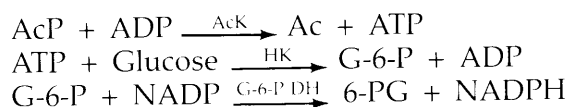
yeast, EC 2.6.1.1) were obtained in lyophilized form from Sigma. Adenylate kinases (porcine muscle, rabbit muscle, and *B. stearothermophilus*, EC 2.7.4.3) were obtained as lyophilized powder or a crystalline suspension in ammonium sulfate solution from Sigma. Other enzymes and biochemicals were obtained from Sigma. All enzymes obtained in lyophilized form were used as received and those in ammonium sulfate solution desalted by dialysis before use; absolute purities were not established. AMP sodium salt was obtained in a Kg quantity from Kyowa Haako. Ribavirin 5'-monophosphate triethylammonium salt was provided by Dr. Steve Tam of Hoffman LaRoche. Acetyl phosphate sodium salt in aqueous solution (22) and phosphoenol pyruvate potassium salt (7) were prepared as described. Other biochemicals were obtained from Sigma and used as received. Chemicals were reagent grade and used without further purification unless otherwise indicated.

## Methods

UV spectrophotometric measurements were performed at 25°C. <sup>31</sup>P NMR spectra were recorded using D<sub>2</sub>O as external NMR lock with the accumulation parameters of pulse angle 45° and pulse delay 15 s (completely proton decoupled). The pH of reaction solutions was controlled with a Fisher pencil combination electrode and Chemtrix pH controller.

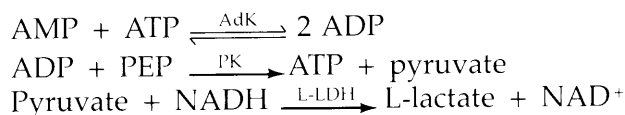
## Enzymatic Assay

Acetate kinases were assayed in the direction of formation of ATP using the following coupled reactions



The assay solution contained buffer (pH 7.5, 175 mM Hepes), 25.5 mM AcP, 9.5 mM ADP, 26.3 mM MgCl<sub>2</sub>, 5 mM glucose, 0.6 mM NADP, 10 U/mL of hexokinase (HK), 5 U/mL of G-6-P dehydrogenase (G-6-P DH), and about 0.02 U/mL of acetate kinase (AcK). Here, 1 U of enzymes catalyze the formation of 1 μmole of ATP per min at pH 7.5 and 25 °C.

Adenylate kinases were assayed in the direction of formation of ADP using the following coupled reactions



The assay solutions contained buffer (pH 7.5, 182 mM TEA), 5.4 mM AMP, 4.3 mM ATP, 10 mM MgCl<sub>2</sub>, 1.8 mM PEP, 0.2 mM NADH, 7 U/mL of pyruvate kinase (PK), 10 U/mL of L-lactate dehydrogenase (L-LDH), and about 0.02 U/mL of adenylate kinase (AdK).

Inorganic pyrophosphatase (PPase) was assayed using a modified Fiske Subbarow method (36). The assay solution (3 mL) contained pH

7.0, 40 mM Tris, 1 mM  $PP_i$ , 33.3 mM  $MgCl_2$ , and about 0.3 U/mL of PPase. Several assay solutions were incubated in the water bath kept at 25°C. Periodically, an aliquot of solution was removed, 7 mL of phosphate assay reagent were added, and the mixture was allowed to stand for 30 min. The corresponding quantities of  $P_i$  were read from the standard curve.

### Enzyme Immobilization

Acetate kinases, adenylate kinases and inorganic pyrophosphatases from several different sources were immobilized as described elsewhere (29). A large-scale immobilization of acetate kinase (*B. stearothermophilus*) was performed with 7 mg of enzyme and 7 g of PAN-1000 in the presence of 0.1 M Hepes (pH 7.5), 10 mM ADP, 20 mM AcP, and 30 mM  $MgCl_2$  (46% immobilization yield). A large-scale immobilization of adenylate kinase (porcine muscle) was carried out with 10 mg of enzyme and 5 g of PAN-1000 in the presence of 0.1 M Hepes (pH 7.5), 20 mM ADP, 20 mM  $MgCl_2$ , and 2 mM DTT (66% immobilization yield). A medium-scale immobilization of inorganic pyrophosphatase (yeast) was performed with 2 mg of enzyme and 2 g of PAN-1000 in the presence of 0.1 M TEA (pH 7.5), 3 mM  $PP_i$ , 3 mM  $MgCl_2$ , and 5 mM DTT (41% immobilization yield).

### Stability of PAN-Immobilized Acetate Kinases

The *E. coli* enzymes were suspended in an aqueous solution containing 50 mM Hepes (pH 7.5), 10 mM  $MgCl_2$ , and 10 mM DTT, and the *B. stearothermophilus* enzymes were suspended in an aqueous solution containing 50 mM Hepes (pH 7.5) and 10 mM  $MgCl_2$ . Each suspension contained 2-3 U/mL of enzymes. The solutions were incubated in the water bath kept at 50°C. Periodically, a small aliquot was removed from each solution and assayed as described in the section on Enzymatic Assay.

### Analysis of Nucleotides

Enzymatic assays for the determination of purities of nucleotides as starting materials and products were performed as described elsewhere (30). Spectra for the analysis by  $^{31}P$  NMR spectroscopy were obtained as described in the Methods section. The assay solutions for  $^{31}P$  NMR spectroscopic measurements were prepared as follows. Periodically, a small aliquot (1-1.5 mL) was removed from a reaction mixture, the enzyme-containing gels removed by centrifugation, and the mother liquor diluted to 1.5 mL with distilled water. The diluted solution was transferred into a 10-mm NMR tube and a 5-mm NMR tube containing deuterium oxide was inserted into the NMR tube for NMR lock. Analysis by HPLC was performed with the following conditions: reverse-phase C18 Radial-Pak column, 5 mM tetrabutylammonium phosphate in 11% MeOH-water, flow rate of 2 mL/min, 10  $\mu$ L of injection volume, and 254 nm for detection wavelength. The samples for HPLC were prepared as follows. Using a disposable syringe (1 mL-size) with a long needle, 50-100  $\mu$ L of liquor

was withdrawn from an enzyme reactor, transferred to a microcentrifuge tube, and diluted to about 1 mL with distilled water. The enzyme-containing gels were removed by centrifugation. The mother liquor was filtered through a 0.45-micron Gelman Acro LC3A disposable filter assembly and diluted 5–10 times with distilled water. A small volume (10  $\mu$ L) of the diluted solution was injected into the HPLC. Each analysis was complete within 15 min after injection.

### Synthesis of ATP

ATP was prepared by conducting a 50-mmol scale reaction three times. The first run is described as a representative example. A reactor system was composed of a three-necked, one-L flask (flask A) with a large magnetic stirbar and three septa, a Fisher pencil combination electrode connected to a Chemtrix pH controller, a 300-mL round flask (flask B), two 50-mL measuring cylinders (cylinders A and B), three LKB peristaltic pumps, an argon supply, a gas bubbler, and an ice bath. Degassed  $\text{MgCl}_2$  (1.1 N, 40 mL) was placed in cylinder A. Degassed NaOH (2 N, 50 mL) was placed in cylinder B. Degassed AcP (0.75 N, 200 mL) was placed in flask B insulated by the ice bath. The solutions placed in the cylinders and flask B were connected separately using a silicon tube to flask A via a peristaltic pump. The pump for the addition of NaOH was connected to the pH controller for its automatic controlling. An aqueous solution (200 mL) containing 50 mmol of AMP, 1 mmol of ATP, 6 mmol of  $\text{MgCl}_2$ , and 1 mmol of mercaptoethanol was transferred into flask A, followed by the addition of immobilized-enzyme suspension (80 mL) containing 200 U of AcK, 200 U of AdK, and 50 U of PPase. The electrode connected to the pH controller was immersed in the solution. Finally, the argon supply and mineral oil bubbler was connected to flask A. The pH of the solution was adjusted to 7.5 with 2 N NaOH and argon was bubbled through the enzyme-containing solution for more than 30 min after flask A was well-capped with septa. Reaction was initiated by the addition of AcP and  $\text{MgCl}_2$  via the peristaltic pumps. AcP was added continuously at a rate of 3 mmol per h for 50 h and magnesium chloride at a rate of 1.1 mmol per h for 40 h. The pH was kept between 7.4 and 7.6 by the controlled addition of 2 N NaOH. All solutions were maintained under argon. The progress of reaction was followed by periodic analysis by HPLC. The reaction was complete when the fractions of AMP/ADP/ATP reached 0.007/0.079/0.913 (60 h). At that time the solution volume was 530 mL. Enzyme-containing gel particles were removed by centrifugation, washed twice with 200-mL portions of degassed redistilled water, assayed by UV, and reused for the next run. The recovered activity for AcK, AdK, and PPase was 99 (100%), 250 (125%), and 31 U (62%), respectively. The mother liquor was stored below 0°C. The second run was performed as above with the recovered enzyme plus 20 U of fresh PPase. The ratio of AMP/ADP/ATP at the end of reaction (60 h) was 0.006/0.093/0.901. The activity recovery for AcK, AdK, and PPase was 145 (73%), 240 (96%), and 30 U (60%), respectively.

The third run was performed as above with the recovered enzymes plus 55 U of fresh AcK and 20 U of fresh PPase. The ratio of AMP/ADP/ATP at the end of reaction (60 h), was 0.007/0.098/0.895. The recovered activity was 190 (95%), 250 (125%), 33 U (66%) each for AcK, AdK, and PPase. The combined mother liquors from the three reactions totaled 1.71 L and contained 138 mmol of ATP, 14 mmol of ADP, and 1 mmol of AMP. The solution in 4 L beaker was cooled to 4°C followed by the slow addition of an aq BaBr<sub>2</sub> solution (1.1 M, 300 mL) with stirring. Then, cold 95% ethanol (1000 mL) was introduced slowly with stirring over 40 min and the solution was allowed to stir for 4 h at 4°C. The resulting white precipitate was collected by centrifugation at 15,000 rpm, washed twice with 300-mL portions of 40% EtOH, once with 300 mL of 95% EtOH, and finally once with 300 mL of acetone. The solids were dried for 10 h at room temperature at a pressure of 0.05 torr. The solids weighed 158 g and contained 125 mmol (82% yield) of ATP having 67% purity based on Ba<sub>2</sub>ATP·4H<sub>2</sub>O. The recovery of enzymes through three runs was 78, 127, and 37% each for AcK, AdK, and PPase.

### Test of Two-Enzyme Systems for Conversion of RMP to RTP

Adenylate kinase was tested with a solution (pH 7.6, 1.5 mL) containing 10 mM RMP, 7 mM ATP, 20 mM MgCl<sub>2</sub>, and 20 U of AdK. The solution was placed in a 10-mm NMR tube, and a 5-mm NMR tube containing D<sub>2</sub>O was inserted into the tube. The tube was allowed to stand at room temperature for 2 h. Then a <sup>31</sup>P NMR spectrum was recorded. A reference spectrum was recorded with a solution containing everything except AdK. Pyruvate kinase was tested with a solution (pH 7.6, 1.5 mL) containing 10 mM RMP, 25 mM PEP, 1 mM ATP, 20 mM MgCl<sub>2</sub>, 20 U of AdK, and 20 U of PK. Acetate kinase was tested with a solution (pH 7.6, 1.5 mL) containing 10 mM RMP, 30 mM AcP, 1 mM ATP, 20 mM MgCl<sub>2</sub>, 20 U of AdK, and 20 U of AcK.

### Synthesis of RTP

A solution (30 mL) containing 1.7 mmol of RMP, 0.02 mmol of ATP, 3.5 mmol of PEP, 1.7 mmol of MgCl<sub>2</sub>, and 0.06 mmol of mercaptoethanol was transferred to a 100-mL three-necked flask equipped with a magnetic stirring bar, three septa, a pH electrode connected to a Chemtrix pH controller, and a nitrogen supply and bubbler. The pH of solution was adjusted to 7.4 and nitrogen was bubbled through the solution after the flask was tightly capped with septa. The reaction was initiated by the addition of 50 U of AdK and 50 U of PK. The solution was maintained under nitrogen and the pH was kept at 7.4–7.6 by the controlled addition of 1 N HCl solution. The progress of the reaction was followed by periodic <sup>31</sup>P NMR spectroscopy. The additional amount of enzymes (100 U of AdK and 70 U of PK) was added three times (at 72, 96, and 120 h). The reaction was stopped when conversion of RMP to RTP was 98% (140 h). RTP was iso-

lated as its barium salt as follows. To the cold solution (35 mL) containing RTP was added slowly an aq BaBr<sub>2</sub> solution (1.2 g BaBr<sub>2</sub>·2H<sub>2</sub>O in 5 mL of water) with stirring, followed by the slow addition of 30 mL of cold ethanol. The heterogeneous mixture was allowed to stand at 4°C overnight. The white precipitate was filtered, washed with two 30-mL portions of cold 40% EtOH, with two 30-mL portions of cold 95% ethanol, and finally with 30 mL of acetone. The resulting solid was dried *in vacuo* in the presence of anhyd P<sub>2</sub>O<sub>5</sub> and weighed 1.34 g. The enzymatic assay indicated that it contained 1.58 mmol of RTP (93% yield, 97% purity based on Ba<sub>2</sub>RTP·4H<sub>2</sub>O).

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