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Preparation of a Mixture of Nucleoside Triphosphates Suitable for Use in Synthesis of Nucleotide Phosphate Sugars from Ribonucleic Acid Using Nuclease P_1, A Mixture of Nucleoside Monophosphokinases and Acetate Kinase

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ABSTRACT

This paper (*1*) describes the enzymatic syntheses of a mixture of adenosine, guanosine, cytidine, and uridine triphosphates (ATP, GTP, CTP, and UTP) from ribonucleic acid (RNA). RNA was hydrolyzed by nuclease P_1 to a mixture of 5′-nucleoside monophosphates. This mixture was converted to the nucleoside triphosphates using a mixture of nucleoside monophosphate kinases and acetate kinase, with acetyl phosphate as the ultimate phosphoryl donor. The nucleoside monophosphokinases were extracted from brewer's yeast in a four-step procedure. The specific activity of the yeast enzyme preparation after gel permeation chromatography was sufficiently high that the yeast kinases could be immobilized in volumes that were practical for laboratory scale syntheses. Conversions from NMP to NTP in a mixture containing 0.34 mol of total nucleoside phosphates were: ATP, 90%; GTP, 90%; CTP, 60%; and UTP, 40%.

Index Entries: ATP; GTP; CTP; UTP; RNA.

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INTRODUCTION

The nucleoside di- and monophosphate sugars are central intermediates in the Leloir pathway for biosynthesis of oligo- and polysaccharides (*2*). These activated sugars are, in turn, derived from the nucleoside triphosphates (NTPs). We have explored potential routes to mixtures of these NTPs, acknowledging that the effort spent in preparing each as a separate pure entity may not be repaid in terms of its utility. This paper describes a two-step conversion of RNA to a mixture of the NTPs (Scheme I). We rely on nuclease P_1 to convert RNA to a mixture of the NMPs and a mixture of nucleoside monophosphate kinases from yeast to effect the difficult (for N=C, G, and U) conversion of NMPs to NDPs (*3*). The conversion of all the NDPs to NTPs is easily accomplished using either acetate kinase (*4*) or pyruvate kinase (*5*).

Nuclease P_1 is used extensively for the production of 5′-GMP from RNA (*6*), and its properties have been studied in detail. It requires Zn(II) and catalyzes efficient hydrolyses of ribonucleic acid and heat-denatured deoxyribonucleic acid to 5′-ribo- and deoxyribonucleoside monophosphates at 65°C in acidic solution (optimal pH ~5.3) (*7,8*). It has been immobilized successfully onto a titanium support (*9*). The immobilized nuclease P_1 had enhanced thermal stability and catalyzed RNA hydrolyses at 60°C for 30 d with retention of two-thirds of its original activity (*10*).

The nucleoside monophosphokinases catalyze the transfer of the γ-phosphoryl group of ATP to a nucleoside monophosphate. Guanylate kinase (E.C. 2.4.7.8), adenylate kinase (E.C. 2.7.4.3), and UMP/CMP kinase (E.C. 2.7.4.4) are each specific for the base of the acceptor nucleotide; their catalytic properties are otherwise quite similar. They require Mg(II); pH optima are between 7 and 8; they possess an oxygen-sensitive, essential sulfhydryl residue (*11,12*). The specific kinases have been isolated from several sources, but the procedures are laborious (*12*). This work has utilized a mixture of the nucleoside monophosphokinase activities from brewer's yeast (*13*) and applied it to the conversion of a mixture of nucleoside monophosphates to nucleoside triphosphates.

RESULTS

Preparation of Nucleoside Monophosphates: Nuclease P_1-Catalyzed Hydrolysis of Ribonucleic Acid

A mixture of nucleoside monophosphates was prepared by a protocol that combined features of the procedures of Leuchs (*14*) and Kuninaka (*15,16*). When a large amount of nuclease P_1 was present (i.e., ~6 U nuclease P_1/mmol substrate), concentrated solutions of RNA (15% RNA, w/v) were hydrolyzed effectively. The use of large quantities was dictated

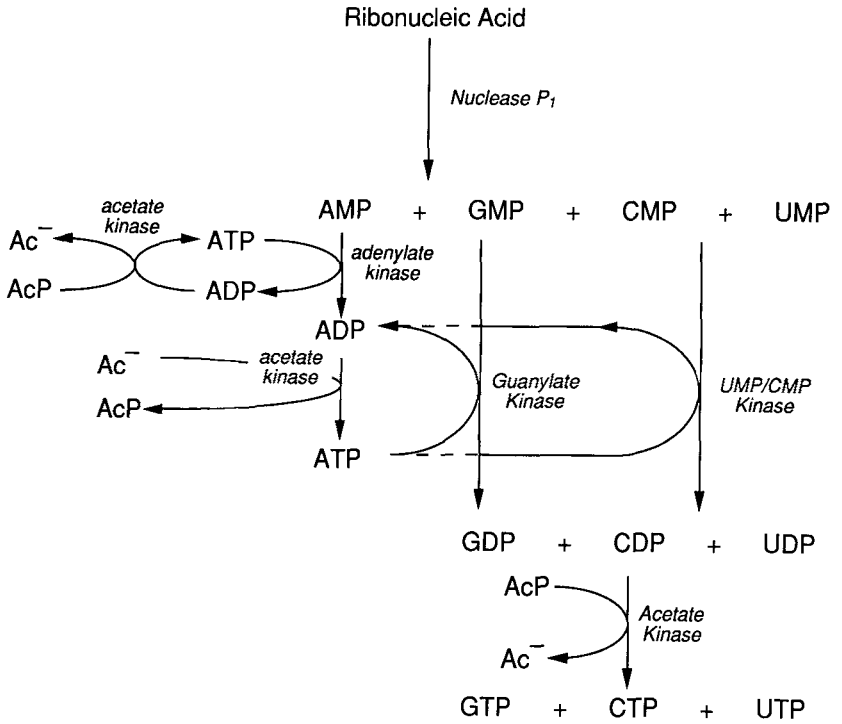

Fig. 1. General scheme for an enzyme-catalyzed synthesis of nucleoside triphosphates from ribonucleic acid.

by the instability of nuclease P_1. Nuclease P_1 deactivated by a first-order process with half-life of ~1 h (at pH 5.6 and 65°C). The deactivation appears to be thermal, rather than owing to oxidation or loss of Zn(II): thiol reducing agents and EDTA accelerated deactivation (perhaps by complexation with the Zn(II); higher concentrations of Zn(II) did not inhibit the deactivation reaction. We are not able to immobilize or stabilize nuclease P_1 in a PAN gel (*17–19*). Soluble nuclease P_1 hydrolyzed a 15% ribonucleic acid solution quantitatively in 1 h in 15 m*M* sodium acetate, 0.1 m*M* $ZnSO_4$, pH 5.6 at 65°C. The completeness of the reaction was corroborated by the presence of roughly equimolar amounts of each mononucleotide; partially-digested ribonucleic acid samples have different product distribution patterns: in particular, an excess of CMP and a deficiency of GMP (*20*).

Purification of Nucleoside Monophosphokinases from Brewer's Yeast

The nucleoside monophosphokinases were purified through the first two steps of Lieberman's procedure using certain modifications (37°C, 1 h autolysis, addition of DTT) (*13,21*). Two separate gel chromatography steps were used. The second on P-100 separated the kinases from a smaller (30–60 kD) protein that inhibited the subsequent UDP-glucose pyrophos-

phorylase-catalyzed condensation of RNA-derived UTP with glucose-1-phosphate. All purification steps in this preparation are summarized in the Experimental section.

Immobilization of Yeast Nucleoside Monophosphokinases

The partially-purified preparation of nucleoside monophosphokinases was immobilized by the standard reaction conditions used with PAN (22). Maximal immobilization yields of 50% were obtained when the reaction was performed in a solution containing 0.5 m*M* AMP, 0.5 m*M* GMP, 0.5 m*M* UMP, 0.5 m*M* CMP, and 1.0 m*M* ATP.

Stability of the Nucleoside Monophosphokinases

The stability of the yeast kinases in solution decreased as the purification progressed. As an ethanol fraction, guanylate and UMP/CMP kinases retained all of their original activity after 40 d storage at 3°C in 10 m*M* β-mercaptoethanol. The fractions purified by gel chromatography were significantly less stable: activity decreased by 10–15% after 1 wk at 3°C. The purified preparations were usually immobilized and used in synthesis immediately. The lifetimes of the kinases were not significantly enhanced by immobilization: after 30 d at 3°C, a mixture of kinases immobilized in PAN retained these percentages of their original activities: guanylate kinase, 16%; UMP/CMP kinase, 33%; and adenylate kinase, 37%.

Synthesis of Nucleoside Triphosphates

Reactions were carried out at pH 7.5 in argon-flushed vessels at ambient temperature. The AMP in the mixture was first converted to ATP using acetate kinase, adenylate kinase, and acetyl phosphate. When the transformation of AMP to ATP was 80–90% complete, the reaction was charged with the immobilized yeast nucleoside monophosphokinases to initiate the synthesis of UTP, GTP, and CTP.

When the total concentration of nucleotides in the reaction mixture was less than 25 m*M*, reaction yields ranging from 80–90% were obtained for each NTP (Table 1). If the solutions of nucleoside monophosphates were more concentrated, the yields of ATP and GTP remained near 90%, but the yields of UTP and CTP dropped to 40 and 60%, respectively. This observation and the characteristic long reaction times can be most easily rationalized by substrate inhibition of the UMP/CMP kinase. The competitive inhibition of the UMP/CMP kinase by its substrates, UMP and CMP, and several adenosine nucleotides has been documented previously (23).

The nucleoside triphosphate-enriched solutions were not purified. The resolution of the products can be accomplished by anion exchange chromatography, but the reported techniques are inconvenient (24).

Table 1
Representative Syntheses of UTP, ATP, GTP, and CTP

Reactants, mmol[a] (m*M*)	I	II	III	IV
AMP	100 (111)	12 (9.2)	1.3 (2.5)	0.037 (3.7)
GMP	41 (45)	3.6 (3.3)	1.1 (2.8)	0.010 (1.0)
CMP	138 (153)	10 (8.7)	2.68 (6.7)	0.025 (2.5)
UMP	78 (87)	10 (8.7)	1.9 (4.8)	0.025 (2.5)
Acetyl Phosphate	524	96	22	0.9
ATP[b]	0.3	0.3	0.0054	0.008
Catalysts, units (% recovery)				
Adenylate Kinase[c]	208 (50)	125	16.5	2.3
Guanylate Kinase	62 (85)	3.7	11.5	0.106
UMP/CMP Kinase	19 (50)	7.5	16.7	0.7
Acetate Kinase	338 (30)	123	37.5	10
Products, mol (% yield)				
ATP	89 (89)	10.8 (90)	1.1 (87)	0.035 (95)
GTP[d]	38 (92)	3.3 (92)	1.0 (90)	0.009 (90)
CTP	83 (60)	8.5 (88)	2.1 (80)	0.022 (87)
UTP[d]	31 (40)	8.5 (85)	1.0 (50)	0.022 (88)
Yield based on acetyl phosphate	90	64	49	20
Reactor volume, L	0.9	1.3	0.4	0.01
Reaction time, da	7	12	5	1.75
pH, T (°C)	7.6, 25	7.5, 25	7.5, 25	7.5, 25

[a]Values were estimated by HPLC. The differing ratios of AMP: GMP: CMP: UMP for these reactions reflects the lack of complete nuclease P_1 hydrolysis in the preparation of the nucleotide samples. These samples were prepared before we amended the hydrolysis protocol.

[b]ATP value = the initial quantity of ATP added to initiate the reaction.

[c]Value for adenylate kinase is the sum of the activity represented in the yeast kinase and the immobilized commercial preparation added prior to the initiation of UTP, GTP, and CTP synthesis.

[d]Since UDP and GDP coelute in the HPLC analyses, and the conversion of GMP to GTP was much more rapid than the conversion of UMP to UTP, any peak that eluted was usually ascribed to UDP. This assumption was checked by comparing the ratios of the total peak areas for the guanidine and uridine moieties throughout the reaction. The ratios were usually constant.

Synthesis of UDP-Glucose

The usefulness of the unpurified mixture of nucleoside triphosphates for preparation of nucleoside phosphate sugars was demonstrated by a synthesis of UDP-glucose (UDP-Glc). UTP that was present as 8–25 mol% of the mixture of nucleoside triphosphates was converted to UDP-Glc using UDP-Glc pyrophosphorylase and inorganic pyrophosphatase (*25*). In a typical reaction, 1.9 mmol UTP in a 300 mL aliquot of a nucleoside triphosphate solution was condensed with 2 mmol glucose-1-phosphate. The course of this condensation reaction, employing the crude UTP prepara-

tion, was indistinguishable from a reaction that used commercially-prepared, pure UTP. The yield of UDP-Glc, based on UTP, was 80%. The mixture was also used successfully without further purification in a synthesis of trehalose that required UDP-glucose regeneration.

DISCUSSION

The preparation of the guanylate and UMP/CMP kinases reported here is laborious, but preferable to purchasing the expensive commercial preparations. It has several attractive features. Brewer's yeast is inexpensive and readily available. The yeast preparation contained UMP/CMP-, adenylate-, and guanylate kinase activities (*11*). The purification sequence involved only four steps and should be easily scaled; the purified enzyme fraction could be obtained in 3–4 d. The major weakness in the yeast nucleoside monophosphokinase preparation was that the activity of UMP/CMP kinase obtained was lower than guanylate and adenylate kinases. This feature contributed to the low yields of UTP and CTP in the larger-scaled syntheses.

We used acetate kinase and acetyl phosphate for regeneration of ATP. Although the synthesis of acetyl phosphate (*26*) is rapid and economical, this regeneration system has two disadvantages. First, acetyl phosphate is unstable in solution. Second, high concentrations of cations (especially ammonium ion) present in the reactions can result in formation of insoluble complex magnesium phosphate salts (*27*). These precipitates remove essential Mg(II) from solution and occlude the enzyme-containing gel particles. An alternative method of immobilization based on membranes, MEEC (*28*), was not explicitly tested here, but should work (*29*). PEP would be superior to AcP as a P_1 donor (*30*).

The strategy of employing a crude mixture of nucleotides has several shortcomings. First, an unidentified minor constituent(s) in the NMP preparation completely inactivated UDP-Glc pyrophosphorylase if the nucleotide mixture was allowed to react with any yeast NMP kinase fraction that preceded the P-100 gel chromatography. Second, the presence of all four nucleotides inhibited the UMP/CMP phosphorylating system in large-scale syntheses using concentrated solutions of substrates. Although these problems were remedied, they highlight potential limitations in the use of partially-purified materials in syntheses: either the reactor efficiency and product yield or the reactant concentration was compromised to offset the problem of competitive inhibition.

In summary, this procedure provides a practical route to mixtures of nucleoside triphosphates and provides an entry into the nucleoside diphosphate sugars. However, it is probably inferior to the fermentation route reported by Tochikura in the synthesis of UDP-Glc (*31*). The efficiency of this fermentation synthesis may reflect the fact that UDP-glucose

is a central and primary metabolite in yeasts, bacteria, and a host of mammalian systems (32). The fermentation route has its own limitations, however: first, although it can easily provide large amounts of the nucleotide-sugars, it is not useful for synthesis of nucleoside triphosphates. Second, the fermentation reactions cannot be used in cofactor-regeneration schemes without coupling multiple separate reactors.

EXPERIMENTAL

General

Reactions were maintained at constant pH with a Chemtrix (Hillsboro, OR) pH controller coupled to a peristaltic pump. Centrifugations for enzyme purification procedures were carried out in a refrigerated Sorvall (Wilmington, DE) Superspeed RC-5. Nucleotide analyses were carried out by HPLC using a Waters (Milford, MA) radial compression C-18 column. Protein fractions were concentrated in an Amicon (Danvers, MA) ultrafiltration unit with 10,000 molecular weight cutoff filters. All biochemicals, enzymes, and cells were obtained from Sigma (St. Louis, MO) unless otherwise stated. PAN-1000 (22) and diammonium acetyl phosphate (26) were prepared as previously described. Gel permeation beads were obtained from Bio-Rad (Richmond, CA). Water was doubly distilled from a Corning (Corning, NY) Model 3B glass still. Welding grade argon was used as an inert atmosphere. Enzymatic activities are reported in units of μmol/min at 25°C. Acetate kinase (33), acetyl phosphate (34), UDP-Glc (35), UDP-Glc-pyrophosphorylase (36), and inorganic pyrophosphatase (37,38) were assayed by literature procedures.

Measurement of AMP, GMP, and (U + C)MP

The concentration of the four nucleotides (39) can be measured in a single determination with adenylate-, guanylate-, and UMP/CMP kinases coupled to a pyruvate kinase (PK)/lactate dehydrogenase (LDH) indicator reaction (Eqs. (1-9).

For AMP =

$$\text{AMP} + \text{ATP} \xrightarrow{\text{adenylate kinase}} 2\ \text{ADP} \quad (1)$$

$$2\ \text{ADP} + 2\ \text{PEP} \xrightarrow{\text{PK}} 2\ \text{ATP} + 2\ \text{pyruvate} \quad (2)$$

$$2\ \text{pyruvate} + 2\ \text{NADH} \xrightarrow{\text{LDH}} 2\ \text{lactate} + 2\ \text{NAD}^+ \quad (3)$$

For GMP:

$$\text{GMP} + \text{ATP} \xrightarrow{\text{guanylate kinase}} \text{GDP} + \text{ADP} \quad (4)$$

$$\text{ADP} + \text{GDP} + 2\ \text{PEP} \xrightarrow{\text{PK}} 2\ \text{pyruvate} + \text{GTP} + \text{ATP} \quad (5)$$

$$2\ \text{pyruvate} + 2\text{NADH} \xrightarrow{\text{LDH}} 2\ \text{lactate} + 2\ \text{NAD}^+ \quad (6)$$

For CMP:

$$\text{CMP} + \text{UMP} + 2\ \text{ATP} \xrightarrow{\text{UMP/CMP kinase}} 2\ \text{ADP} + \text{CDP} + \text{UDP} \quad (7)$$

$$2\ \text{ADP} + 2\ \text{PEP} \xrightarrow{\text{PK}} 2\ \text{ATP} + 2\ \text{pyruvate} \quad (8)$$

$$2\ \text{pyruvate} + 2\ \text{NADH} \xrightarrow{\text{LDH}} 2\ \text{lactate} + 2\ \text{NAD}^+ \quad (9)$$

Pyruvate kinase phosphorylates CDP and UDP at rates only 0.02 times the rate of ADP phosphorylation, so their contribution to the overall PK/LDH reaction is negligible. The following reagents were dissolved in 10 mL of 0.3*M* triethanolamine buffer (pH 7.5) containing 9 m*M* in $MgAc_2$: PEP (14.5 μmol); NADH (4 μmol); PK (70 U); LDH (70 U); and ATP (1.6 μmol). For each sample determination, 0.5 mL of the reagent mixture was combined with 0.2 mL distilled water and 25–50 μL of the sample. The nucleotides were measured in this sequence: AMP, GMP, (U+C)MP. The absorbance of the assay solution was read before the addition of 4 μL of adenylate kinase suspended in an ammonium sulfate solution (~4 U). When the absorbance became constant, 10 μL guanylate kinase in 50% glycerol (~0.25 U) was added to the cuvet. When NADH oxidation ceased, 15 μL pyrimidine nucleoside monophosphokinase, dissolved in distilled water (~0.12 U), was added to the reagent mixture. The change in absorbance upon the addition of each specific kinase was proportional to a concentration of nucleotide (Eqs. 10–12).

$$[\text{AMP}] = \Delta A_{340} \cdot \text{Vol}_{\text{assay}} (6.22 \cdot 2 \cdot \text{Vol}_{\text{sample}})^{-1} \quad (10)$$

$$[\text{GMP}] = \Delta A_{340} \cdot \text{Vol}_{\text{assay}} (6.22 \cdot 2 \cdot \text{Vol}_{\text{sample}})^{-1} \quad (11)$$

$$[(\text{U+C})\text{MP}] = \Delta A_{340} \cdot \text{Vol}_{\text{assay}} (6.22 \cdot \text{Vol}_{\text{sample}})^{-1} \quad (12)$$

Measurement of the Total Mononucleotide Concentration

This assay is based upon the absorbance of nucleic acids at 260 nm (*40*). Polyribonucleic acid was removed from the mononucleotide-containing sample (0.1 mL) by precipitation with 0.2 mL of 0.25% (w/v) uranyl acetate (UO_2·$acetate_2$) in 10% (v/v) perchloric acid. The tube was rapidly mixed by vortexing, then placed in an ice/water bath for 20 min before sedimenting (5000*g*) any insoluble RNA. The supernatant was diluted, and the absorbance of the sample was measured against air at 260 nm. A blank of distilled water was treated in an analogous manner (Eq. 13).

$$[\text{NMP}]_{\text{total}} = (A_{\text{sample}} - A_{\text{blank}}) \cdot \text{Dilution factor} (10.6\ \text{Vol}_{\text{sample}})^{-1} \quad (13)$$

Assay of Nuclease P_1

This assay (*9,41*) involved the incubation of nuclease P_1 with yeast RNA, separation of the mononucleotide products from the polymer by precipitation with uranyl acetate, and measurement of the absorption of the mononucleotides in the supernatant at 260 nm. A 0.10 mL aliquot of a dilute solution of nuclease P_1 (~5 U of enzyme) initiated reaction in a mixture containing: 0.18 mL of 30 m*M* sodium acetate buffer, pH 5.33; 0.20 mL of a solution of yeast RNA (5 mg/mL) in acetate buffer; and 20 μL of 10 m*M* $ZnSO_4$. Distilled water (0.10 mL), instead of enzyme, was added to the blank reaction sample. The test tubes were incubated for 15 min at 37°C. The reaction was quenched by the addition of 1.0 mL of a solution containing 0.25% (w/v) uranyl acetate in 10% (v/v) perchloric acid. The tube was rapidly vortexed, then placed in an ice/water bath for 20 min before sedimenting (50,000*g*) the undigested RNA. The supernatant was diluted by a factor of 10, and the absorbance of the sample was measured against air at 260 nm. Enzymatic activity was calculated using Eq. (14)

$$\text{Units/mL} = (A_{\text{sample}} - A_{\text{blank}}) \cdot 10 \cdot \text{Dilution Factor} \cdot (10.6 \cdot 15 \cdot 0.10) \quad (14)$$

Assay of Adenylate Kinase

All three nucleoside monophosphokinase assays (*42*) used the coupling of the NADH-dependent reduction of pyruvate, formed after phosphorylation of nucleoside diphosphates.

$$\text{XMP} + \text{ATP} \xrightarrow{\text{NMP kinase}} \text{ADP} + \text{XDP} \quad (15)$$

$$\text{ADP} + \text{XTP} + 2\ \text{PEP} \xrightarrow{\text{PK}} 2\ \text{pyruvate} + \text{XTP} + \text{ATP} \quad (16)$$

$$2\ \text{pyruvate} + 2\ \text{NADH} \xrightarrow{\text{LDH}} 2\ \text{lactate} + 2\ \text{NAD}^+ \quad (17)$$

The rate of oxidation of NADH was monitored spectrophotometrically at 340 nm and was directly proportional to 1/2 of the rate of adenylate kinase-catalyzed phosphorylation of AMP. Any ATPase activity or the presence of nonenzymatically-derived pyruvate in the assay sample was accounted for by the measurement of background oxidation of NADH in the absence of the nucleoside monophosphate.

The following reagents were added to a 1.0 cm semimicro quartz cuvet: 700 μL of 0.1*M* triethanolamine, pH 7.6 buffer; 60 μL of 2.0*M* KCl; 20 μL of 0.5*M* $MgCl_2$; 20 μL of 0.1*M* ATP; 20 μL of 50m*M* PEP; 10 μL of 25 m*M* NADH; 10 μL each of LDH and PK suspended in ammonium sulfate solutions (~9 and 4 U, respectively); and 150 μL of the adenylate kinase sample and distilled water. The rate of change of absorbance at 340 nm was measured at 25°C until the rate reached a constant value. The substrate, AMP (30 μL of a 50 m*M* solution), was added. The difference in rates was proportional to the activity of adenylate kinase.

Assay of Guanylate Kinase Activity

The following reagents were mixed in a cuvet and the rate of change of absorbance at 340 nm (*43*) was measured until it reached a constant value: 100 μL of 1.0*M* Tris-chloride, pH 7.6; 40 μL of 0.5 m*M* $MgCl_2$; 50 μL of 0.1*M* ATP; 20 μL of 50 m*M* PEP; 20 μL of 25 m*M* NADH; 10 μL of a LDH solution (~9 U); 10 μL of a PK solution (~4 U); and 570 μL distilled water and enzyme. The guanylate kinase-dependent reaction was initiated by the addition of 60 μL of 50 m*M* GMP. The differences in rates was proportional to the guanylate kinase activity.

Assay of UMP/CMP Kinase Activities

A cuvet containing the following reagents (*44*) was monitored at 340 nm: 70 μL of 1.0*M* Tris-chloride, pH 7.6 buffer; 90 μL of 0.1*M* ATP; 40 μL of 0.5*M* $MgCl_2$; 10 μL of 2.0*M* KCl; 60 μL of 50 m*M* PEP; 20 μL of 25 m*M* NADH; 50 μL of 1.0*M* DTT; 40 μL of a suspension of pyruvate kinase in ammonium sulfate solution (~16 U); 20 μL of a suspension of LDH in ammonium sulfate solution (18 U); and 555 μL distilled water and the sample. When the absorbance change was constant, 45 μL of 200 m*M* CMP was added to initiate the UMP/CMP-dependent reaction. Since the rate of pyruvate kinase-catalyzed phosphorylation of CDP is 0.03 times that of ADP, its contribution to the production of pyruvate—and the subsequent oxidation of NADH—was treated as negligible. UMP/CMP kinase activity was calculated to be equal to the rate of oxidation of NADH.

Preparation of AMP, GMP, CMP, and UMP from Ribonucleic Acid

A 2 L, three-necked, round-bottomed flask, attached to an overhead stirrer, was immersed in a water bath of 65°C. Equal volumes (494 mL) of 30 m*M* sodium acetate, pH 5.3 buffer, and distilled water were added to the reaction vessel and warmed to the bath temperature. Ribonucleic acid (100 g, 85%, Sigma) was slowly added to the rapidly-stirred solution. When the RNA had dissolved, 3 mg of nuclease P_1 (~1500 U) and 10 mL of 20 m*M* $ZnSO_4$ were added to the solution to initiate the hydrolysis. After 3–4 h, 453 mg EDTA was added to the reaction mixture. The solution was cooled to ambient temperature, and the pH was lowered to 2 to precipitate any remaining high molecular weight RNA and protein. The dark-brown solution was filtered (Schleicher and Schuell #595 paper). The filtrate was neutralized with potassium hydroxide to pH 7.0. The filtrate had the expected peaks on HPLC: CMP (2.6 min), UMP (3.2 min), GMP (4.0 min), AMP (6.5 min), and some minor impurities (<1%) that eluted after AMP.

Table 2
Purification of Nucleoside Monophosphokinases from Yeast[a]

Enzyme fraction	Volume, mL	Units	Protein, mg	Specific activity	Yield, %
GMP Kinase					
Autolysate[b]	225	–	–	–	–
Ethanol	250	215	3209	0.067	100
P-200 Chromatography	50	189	53	3.54	88
P-100 Chromatography	30	150	10	15.2	70
UMP/CMP Kinase					
Autolysate[b]	250	–	–	–	–
Ethanol	250	140	3209	0.043	100
P-200 Chromatography	50	86	53	1.72	61
P-100 Chromatography	30	35	10	3.5	25

[a]The preparation began with 150 g of brewer's yeast.
[b]The initial autolysate could not be assayed by the standard assay methods.

Preparation of Nucleoside Monophosphokinases from Brewer's Yeast

Unless otherwise indicated, all manipulations were executed at 2°C (*13*). Yields in each step are summarized in Table 2.

Autolysis

In a capped, 2 L Erlenmeyer flask, 150 g brewer's yeast was suspended in 450 mL of 0.1*M* potassium bicarbonate, containing 0.8 m*M* DTT, and incubated in a heated air chamber for 1 h at 37°C. Yields decreased with longer incubation times (Table 3). The suspension was cooled to 5°C prior to centrifugation (10,000*g*, 10 min, 0°C). Approximately 225 mL of a brown supernatant was obtained.

Ethanol Fractionation

The literature procedure (*13*) was followed, except for the following modifications. When this procedure was modified by performing the second step—ethanol fractionation—under an oxygen-free atmosphere, a NADH-oxidase activity was activated to the extent that it obscured the measurement of the activity of the nucleoside monophosphokinases. As a consequence, no care was taken to minimize exposure of the enzyme preparation to oxygen, and the second purification step was executed, as described in previous efforts (*13,45*). Most of the ethanol-precipitated

Table 3
Yield of NMP Kinase Activity as a Function of Incubation Time during Autolysis

Incubation time, h	Kinase Activity		U/mL UMP/CMP
	AMP	GMP	
1.0	50.5	5.10	2.87
2.0	37.1	2.20	2.17
3.0	27.5	0.10	–[a]
4.0	18.9	0.50	–[a]
5.0	15.8	0.29	0.76
6.0	9.0	0.04	0.71

[a] Activity was not measured.

protein was not soluble in buffered aqueous solutions; however, the NMP kinases dissolved selectively.

Sephadex G-150 beads, swollen in buffer (50 m*M* sodium phosphate, pH 7.6, 10 m*M* β-mercaptoethanol) were packed into a 2.5×95 cm glass column. The gel bed was equilibrated in the same buffer for 2 d prior to the application of the kinase preparation from the ethanol fractionation. The effluent was collected in fractions of 10 mL and each protein-containing fraction was assayed for enzymatic activity. Fractions with greater than 2 U guanylate kinase activity were pooled and concentrated 10-fold by ultrafiltration. β-Mercaptoethanol was added to the enzyme sample to a final concn. of 10 m*M*.

Biogel P-100 gel chromatography (Biogel P-60 gave similar results) was a necessary purification step to separate UDP-glucose pyrophosphorylase-inhibiting protein(s) from the nucleoside monophosphokinases. The P-100 column (1.5×50 cm) was equilibrated in 50 m*M* potassium phosphate; pH 7.6, 10 m*M* β-mercaptoethanol. The P-200 (or G-150) gel fraction (~10 mL) was applied to the column and collected in fractions of 6 mL vol. Fractions with greater than 0.5 U guanylate or UMP/CMP kinase activity were pooled. The volume was reduced to 5–10 mL by ultrafiltration.

Immobilization of the Mixture of Yeast Nucleoside Monophosphokinases

Ten grams of PAN-1000 was rapidly dissolved in 32 mL of 0.3 m*M* Hepes, 15 m*M* $MgCl_2$, pH 7.6, 0.4 mL of 50 m*M* GMP, 0.4 mL of 50 m*M* CMP, 0.4 mL of 50 m*M* UMP, and 0.4 mL of 100 m*M* ATP. The P-100 enzyme preparation (8 mL), 0.4 mL of 1.0*M* DTT, and 6.8 mL of 0.5*M* triethylenetetramine (TET) were quickly added to the rapidly stirred mixture. The gel set within 15 s; it was allowed to stand under a moderate stream of argon for 60 min. The gel containing immobilized enzyme was ground to fine particles and washed: 1×50 m*M* Hepes, 50 m*M* $(NH_4)_2SO_4$,

10 m*M* $MgCl_2$, pH 7.5 buffer with 10 m*M* β-mercaptoethanol; 2×0.3*M* Hepes, 10 m*M* $MgCl_2$, pH 7.5, 10 m*M* β-mercaptoethanol. The yield on immobilization for each kinase activity was approximately 50%.

Immobilization of Acetate Kinase

Acetate kinase was immobilized as described in previous publications (*22,46*).

Immobilization of Inorganic Pyrophosphatase

Lyophilized enzyme, which contained citrate and Tris salts, was dissolved in 600 μL distilled water ([protein] = 1.67 mg/mL). In a 20 mL beaker equipped with a stirring bar, 4.0 mL of 0.3 *M* Hepes, 15 m*M* $MgCl_2$, pH 7.6 buffer, and 100 μL of 45 m*M* sodium pyrophosphate were mixed. To this rapidly stirred solution, 1.0 g of PAN-1000, 850 μL of 0.5 *M* TET, and the enzyme-containing solution were added. The gel was ground and washed according to standard procedures (*22*). Reducing agents (i.e., dithiothreitol) can irreversibly inactivate the disulfide-containing inorganic pyrophosphatase; therefore, DTT was omitted from the immobilization reaction.

Immobilization of UDP-Glc Pyrophosphorylase

The enzyme was dissolved in 30 m*M* dithiothreitol to a protein concn. of 4.6 mg/mL. This solution (165 μL, 0.76 mg protein) was incubated with 10 μL of 50 m*M* UDP-Glc. The enzyme–substrate mixture was added immediately after the following reagents were thoroughly mixed: 1.0 g PAN-1000; 4.0 mL of 0.3*M* Hepes, 15 m*M* $MgCl_2$, pH 7.6 buffer; 50 μL of 0.5*M* DTT; and 850 μL of 0.5*M* TET. The gel set within 30 s and stood at ambient temperature for 1 h before it was ground and washed. The suspension was diluted to 50 mL and stored in 0.3*M* Hepes, 15 m*M* $MgCl_2$, pH 7.6, 10 m*M* DTT buffer. Average yields on immobilization were ~30%.

Stability of Nuclease P_1 at 65 °C

Nuclease P_1 (0.5–2.0 U) was dissolved in 5 mL vol. of 15 mL sodium acetate, pH 5.3, and 0.1 m*M* $ZnSO_4$. The flasks were immersed in a 65 °C water bath, and the solutions were stirred. Aliquots (0.10 mL) were removed and measured for nuclease P_1 activity by the uranyl acetate method (*9,43*).

Conversion of Hydrolyzed RNA into Nucleoside Triphosphate

The larger-scale reactions were performed in an argon-flushed, 2 L, round-bottomed flask. The reaction vessel was coupled to a pH controller, and the reaction solution was maintained at pH 7.5 with 2.0*N*

KOH throughout the synthesis. Conversion of AMP into ATP typically preceded the ATP-requiring syntheses of GTP, CTP, and UTP. ATP synthesis was initiated by the addition of immobilized commercial preparations of adenylate kinase (60 U) and acetate kinase (330 U) to a reaction mixture containing the following (in mmol): AMP (100), GMP (41), CMP (138), UMP (102), ATP (0.24), KCl (9), $MgCl_2$ (45), and dithiothreitol (4.5). The initial reaction volume was 0.6 L. Diammonium acetyl phosphate was added to the stirred reaction mixture as a solid (~ 30 mmol$(10h)^{-1}$). The relative concentrations of AMP and ATP were measured by HPLC and enzymatic assays. After 2 d, or when ATP represented 80–90% of the total adenosine nucleotide pool, the reaction was charged with 300 mL immoblized nucleoside monophosphokinases (UMP/CMP kinase, 19 U; guanylate kinase, 62 U; and adenylate kinase, 148 U). Lower amounts of acetyl phosphate were added to reflect the lower activities of guanylate and UMP/CMP kinases. Magnesium chloride (~ 5 mmol/d) was added throughout the course of the reaction to restore the soluble magnesium levels that were depleted owing to precipitation of complex magnesium phosphates. After 7 d, the products were decanted from the insoluble gel and inorganic salts following sedimentation. The total nucleotide composition, as determined by HPLC and enzymatic assays, was: NMP, 27%; NDP, 10%; and NTP, 63%. The nucleoside triphosphate-enriched solutions were stored at $-20°C$ until use in a UTP-dependent reaction.

Conversion of UTP into UDP-Glc

A 300 mL aliquot of an enzymatically-generated nucleoside triphosphate solution was thoroughly deoxygenated with argon and added to a 500 mL, three-necked, round-bottomed flask equipped with a pH controller. The nucleotide solution, which contained 1.9–2.0 mmol each of UTP, CTP, GTP, and ATP, was treated with glucose-1-phosphate (2.1 mmol), immobilized UDP-Glc pyrophosphorylase (5.5 U, 10 mL), immobilized UDP-Glc pyrophosphatase (16 U, 5 mL), and $MgCl_2$ (3.2 mmol). The mixture, which was maintained at pH 7.5, was rapidly stirred at ambient temperature. After 24 h, HPLC indicated that the reaction was complete: a peak corresponding to UDP-Glc had appeared, and the peak corresponding to UTP was not present. The supernatant obtained after centrifugation was used in sucrose and trehalose syntheses without further purification or characterization. No detectable degradation of UDP-Glc had occurred after the solution was stored for 5 mo at $-15°C$.

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