Preparation of a Mixture of Nucleoside Triphosphates from Yeast RNA: Use in Enzymatic Synthesis Requiring Nucleoside Triphosphate Regeneration and Conversion to Nucleoside Diphosphate Sugars[1]

Chi-Huey Wong, Sharon L. Haynie,[2] and George M. Whitesides*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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This paper describes a practical procedure for converting yeast RNA into a mixture of ATP, UTP, GTP, and CTP (Scheme I). This mixture can be used as a source of nucleoside triphosphates for the synthesis of nucleoside diphosphate sugars. These latter substances are required in most enzyme-catalyzed syntheses of oligo- and polysaccharides.[3] In addition, since many ATP-utilizing enzymes (especially phosphotransferases) will also accept GTP, UTP, and CTP,[4] the mixture of nucleoside triphosphates serves

* Inquiries should be addressed to G.M.W. at the Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, MA 02138.

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(3) Nikaido, H.; Hassid, W. Z. *Adv. Carbohydr. Chem. Biochem.* **1971**, *26*, 351–483. Rosevear, P. R.; Numez, H. A.; Barker, R. *Biochemistry* **1982**, *21*, 1421–31.

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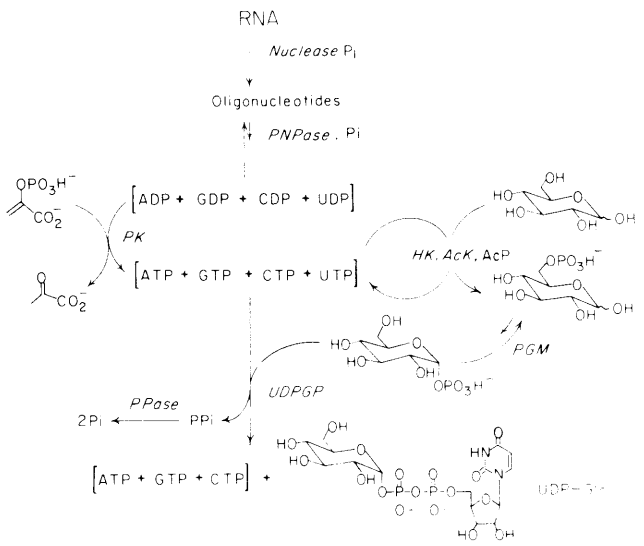
Scheme I[a]



[a] PNPase, polynucleotide phosphorylase; PK, pyruvate kinase; HK, hexokinase; AcK, acetate kinase; PGM, phosphoglucomutase; UDPGP, UDP-glucose pyrophosphorylase; PPase, inorganic pyrophosphatase; AcP, acetyl phosphate.

as a convenient source of phosphate equivalents in other enzyme-catalyzed organic syntheses.[5]

Conversion of RNA to the mixture of nucleoside triphosphates involved two steps. In the first, nuclease P_1 (E.C. 3.1.4.-, in solution) was used to hydrolyze high molecular weight yeast RNA to a mixture of lower molecular weight oligonucleotides.[6] In the second, polynucleotide phosphorylase (PNPase, E.C. 2.7.7.8, immobilized in PAN gel[7]) converted these oligonucleotides into nucleoside diphosphates by reaction with phosphate. This equilibrium conversion strongly favors oligonucleotides.[8] The nucleoside diphosphates were converted to nucleoside triphosphates by phosphorylation in situ by using phosphoenol pyruvate (PEP)[9] and pyruvate kinase (PK, E.C. 2.7.1.40) to drive the reaction.[10] The most expensive enzyme in this synthesis is PNPase ($4.00/unit, Sigma). The initial treatment by nuclease P_1 permits use of PNPase immobilized in PAN gel, in which form the enzyme is both relatively stable and very easily recovered for reuse.

In a representative procedure, yeast RNA (15 g, 95% pure, from Boehringer Mannheim) and 50 units of nuclease P_1 in 20 mL of water (0.1 mM in $MnCl_2$, pH 6.0) was stirred at 50 °C for 1 h. The mixture was adjusted immediately to pH 8.2 by adding cold 5 N NaOH solution and diluted to a total volume of 1 L in a solution that contained potassium phosphate (0.2 M), $MgCl_2$ (2mM), PEP (monopotassium salt, 80 mM), and PAN-immobilized PNPase (8 units in 20 mL of gel)[11] and PK (50 units in 1 mL of gel). The reaction mixture was stirred at room temperature under argon for 4 days, and the pH was controlled between 8.2 and 8.5. Enzymatic analysis[12] of the reaction mixture indicated that it contained 30 mmol of nucleoside triphosphates (68% yield based on RNA); no further increase in the yield of these substances was observed[13] with longer reaction times. The gel particles were removed, the solution was adjusted to pH 3.0 at 4 °C by adding cold concentrated HCl with stirring, and a precipitate was removed by filtration. The filtrate was adjusted to pH 8.0 (5 N NaOH) and concentrated under reduced pressure at 35 °C to a volume of 50 mL. This solution contained a mixture of nucleoside triphosphates (28 mmol, 62% yield based on RNA) in these relative quantities:[14] ATP, 24%; UTP, 28%; GTP, 30%; CTP, 18%. The enzymatic activities recovered after these transformations were PNPase, 75%, and PK, 92%.

The mixture of nucleoside triphosphates obtained in this procedure is contaminated with a number of other components. These other materials do not, however, seem to interfere with the use of the nucleoside triphosphates in further enzymatic processes. In particular, the conversion of the UTP present in the mixture to UDP-glucose and the use of the components of the mixture to act as cofactors in the hexokinase-catalyzed phosphorylation of glucose[15] were both uneventful.

For the synthesis of UDP-glucose (UDP-Glc), 40 mL of the mixture of cofactor (6.2 mmol of UTP) was diluted to 200 mL (pH 8.0). PAN-immobilized UDP-Glc pyrophosphorylase (UDPGP, E.C. 2.7.7.9, 50 units in 1 mL of gel), inorganic pyrophosphatase (PPase, E.C. 3.6.1.1, 60 units in 0.5 mL of gel), phosphoglucomutase (PGM, E.C. 2.7.5.1, 52 units in 1 mL of gel), and glucose 6-phosphate (G-6-P, generated from 6.2 mmol of barium salt by treating with Dowex 50 to remove barium ion)[15] were added. The reaction was conducted under argon for 20 h with pH controlled at 7.5. Enzymatic analysis[16] of the solution indicated it contained 6 mmol of UDP-Glc and 14 mmol of a mixture of other nucleoside triphosphates. After separation of the enzyme-containing gel, the solution could be used directly for UDP-Glc—requiring disaccharide synthesis,[17] or isolated by chromatography using Bio-Rad P-2 (H_2O solvent).[18]

The procedure summarized in Scheme I provides the best method presently available for the preparation of GTP, UTP, and CTP, provided that the *mixture* of nucleoside triphosphates is acceptable. We have explored an alternative scheme for generation of this mixture based on nuclease P_1-catalyzed hydrolysis of RNA to a mixture of nucleoside *mono*phosphates, followed by phosphorylation of these species to triphosphates.[19] We have not found the nucleoside monophosphate kinases required for this scheme to be either easily prepared or easily handled.

The major weakness of the scheme described here is the present expense of commercial PNPase. We note, however, that this enzyme is readily available from *E. Coli* B[20] and could in any event

(4) Bergmeyer, H. U. In "Methods of Enzymatic Analysis"; Academic Press: New York, 1974; p 2081. Chung, A. E. *J. Biol. Chem.* **1967**, *242*, 182–6. Plowman, K. M.; Krall, A. R. *Biochemistry* **1965**, *4*, 2809–25. These enzymes include hexokinase, phosphoglycerate kinase, acetate kinase, pyruvate kinase, phosphofructokinase, NAD kinase, glycerokinase, and glucose 1-phosphate kinase.

(5) Pollak, A.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 2366–7. Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5828–9.

(6) Commercial yeast RNA contains a high proportion of polynucleotides with M_n 10^5–10^6. This material was not a substrate for PAN-immobilized PNPase and was a poor substrate for this enzyme in soluble form. After digestion with nuclease P_1, it became a good substrate for immobilized PNPase.

(7) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–36.

(8) Godefroy-Colburn, T.; Grumberg-Manago, M. In "The Enzymes"; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. 7, pp 533–74. At 20 °C, 80% of ADP present initially is polymerized, corresponding to an equilibrium constant $K = 16$ for the reaction ADP + ApA $\rightleftharpoons$ ApApA + Pi ($\Delta G^{\circ\prime} = -RT \ln K \simeq -1.7$ kcal/mol).

(9) Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765–6.

(10) If the reaction ApApA + Pi $\rightleftharpoons$ ApA + ADP ($\Delta G^{\circ\prime} = +1.7$ kcal/mol) is coupled with the reaction PEP + ADP $\rightleftharpoons$ ATP + pyruvate ($\Delta G^{\circ\prime} = -7.5$ kcal/mol), the free energy ($\Delta G^{\circ\prime}$) for the overall reaction ApApA + Pi + PEP $\rightleftharpoons$ ApA + ATP is −5.8 kcal/mol. Saber, H. A. "Handbook of Biochemistry"; **1970**, The Chemical Rubber Co.: Cleveland, OH, 1970; pp J180–5.

(11) The enzyme PNPase from *M. Lysodeikticus* was immobilized according to the standard procedure.[7]

(12) Determined by PEP and PK coupled with lactic dehydrogenase.

(13) Mononucleotides, dinucleotides, polynucleotides with therminal 3′-phosphate groups, and double-stranded polynucleotides are not substrates of PNPase: Godefroy, T. *Eur. J. Biochem.* **1979**, *14*, 222–31. Godefroy, T.; Cohn, M.; Grunberg-Manago, M. *Ibid.* **1970**, *12*, 236–49. Chou, J. Y.; Singer, M. F. *J. Biol. Chem.* **1970**, *245*, 995–1004.

(14) HPLC was performed by using a Waters Radial-PAK C_{18} column (5-mm i.d.) with 5 mM tetrabutylammonium phosphate in aqueous acetonitrile (18% v/v, H 7.6) as the mobile phase.

(15) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890.

(16) Determined enzymatically with UDP-glucose dehydrogenase and NAD; see Bergmeyer.[4]

(17) Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 5416–5418.

(18) Previous preparations of UDP-glucose: Moffat, J. G. *Methods Enzymol.* **1968**, *8*, 136–42. Kawaguchi, K.; Kawai, H.; Tochikura, T. *Methods Carbohydr. Chem.* **1980**, *8*, 261–9.

(19) Leuchs, H. J.; Lewis, J. M.; Rios-Mercadillo, V. M.; Whitesides, G. M. *J. Am. Chem. Soc.* **1979**, *101*, 5829–30.

probably be made in quantity by recombinant DNA methods.

Registry No. ATP, 56-65-5; UTP, 63-39-8; GTP, 86-01-1; CTP, 65-47-4; nuclease P_1, 54576-84-0; PNPase, 9014-12-4; PEP, 138-08-9; PK, 9001-59-6; UDP-Glc, 133-89-1; glucose, 50-99-7; G-6-P, 56-73-5.

Supplementary Material Available: Procedures for preparation of glucose-6-phosphate by using the XTP's prepared here and for immobilization of PNPase (1 page). Ordering information is given on any current masthead page.

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(20) Kimhi, Y.; Littauer, U. Z. *Methods Enzymol.* **1968**, *12B*, 513–9. Starting from 540 g of the frozen cells, 880 units of PNPase were detected. After several steps of purification, 150 units of the enzyme were isolated with specific activity 6 unit/mg (1 unit will generate 1 μmol of ATP/min from poly(A) in a coupled reaction).