Generation of Cytidine 5'-Triphosphate using Adenylate Kinase

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Summary. A membrane-enclosed enzyme reactor containing adenylate kinase (E.C. 2.7.4.3, rabbit muscle) and pyruvate kinase (E.C. 2.7.1.40) converts cytidine 5'-monophosphate (CMP) and phosphoenolpyruvate (PEP) to cytidine 5'-triphosphate (CTP) and pyruvate on a gram scale.

Cytidine 5'-triphosphate (CTP) is important in the synthesis of complex biological molecules. CTP is a precursor for (inter alia) ribonucleic acids, cytidine 5'-diphosphocholine (CDP-choline), and activated C-8 and C-9 monosaccharides such as cytidine 5'-monophospho-3-deoxy-D-manno-2-octulosonate (CMP-KDO) and cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NANA). Although CTP is commercially available, its high cost (~ $104/mol) limits the scope of its use in enzyme-catalyzed organic synthesis. CTP has been made by fermentation and by chemical syntheses; fermentation methods are, however, less convenient on a laboratory scale than is the method presented here and chemical routes cannot be used when CTP is to be regenerated in the reaction mixture. In this letter we report that cytidine nucleotides are accepted as substrates with useful rates by the commercially available adenylate kinase from rabbit muscle (E.C. 2.7.4.3) and that adenylate kinase can be used in conjunction with pyruvate kinase (E.C. 2.7.1.40) to produce gram quantities of CTP from CMP and phosphoenolpyruvate (PEP).

Scheme 1. Synthesis of CTP from CMP.
Adenylate kinase catalyzes the phosphate transfer reactions that equilibrate two molecules of ADP with one each of AMP and ATP (K ~ 1).\textsuperscript{10} The literature reports that adenylate kinase is highly specific for adenosine nucleotides.\textsuperscript{11} We have confirmed, however, that adenylate kinase will also catalyze the equilibration of CDP, CMP and CTP.\textsuperscript{12} Pyruvate kinase, in turn, catalyzes the highly favored formation of CTP and pyruvate from CDP and PEP (K ~ 10\textsuperscript{3} with adenosine nucleotides).\textsuperscript{13} By coupling these two reactions together (Scheme I), we are able to drive the formation of CTP from CMP and a catalytic amount of CTP (to initiate the adenylate kinase reaction). Although large quantities of adenylate kinase are required to achieve useful rates, the enzyme has a high specific activity for reaction with adenosine nucleotides and an acceptable cost even when used with cytidine nucleotides.\textsuperscript{14} In addition, immobilization\textsuperscript{12} or use of the MEEC technique\textsuperscript{15} allows reuse of the enzymes. Nucleoside phosphate kinases\textsuperscript{16} could also be used to produce CTP, but we found the system involving adenylate kinase to be more practical and efficient.

Here we detail a procedure that generates CTP on a gram scale. In other work, we have used this method to generate CTP on a 5-g scale, and used this material without isolation to synthesize CMP-NANA.\textsuperscript{7} Isolation and purification of CTP prepared by this procedure is straightforward: addition of a solution containing ammonium and magnesium ions\textsuperscript{17} precipitates inorganic phosphate from the reaction mixture, and the addition of EtOH (1:1, v:v) precipitates CTP. Additional material can be recovered using ion exchange chromatography. Experimental procedures for the production and isolation of CTP on a gram scale appear below.

**Experimental**

**Conversion of CMP to CTP.** PEP-K\textsuperscript{+18} (0.56 g, 88% by enzymatic assay, 2.4 mmol) was dissolved in 10 mL of water and placed in a 50-mL plastic centrifuge tube. The pH was adjusted to pH 8 with 10 N NaOH. CMP (1 g, 98%, 2.4 mmol; US Biochemical) and MgCl\textsubscript{2}·6H\textsubscript{2}O (250 mg, 1.2 mmol) were added followed by 1 mL of a basic solution\textsuperscript{17} of MgCl\textsubscript{2}·6H\textsubscript{2}O (1 M) and NH\textsubscript{4}Cl (3.8 M) to precipitate any residual inorganic phosphate. The reaction mixture was centrifuged for 5 min (IEC centrifuge, setting 6, 2700 rpm), the supernatant was decanted into a clean tube, the volume was adjusted to 20 mL and 20 mg of CTP (0.02 mmol, Sigma) was added. The solution was degassed for 30 min with nitrogen, 5 μL of 2-mercaptoethanol was added, and the pH was adjusted to pH 7.8. A section of dialysis tubing (16 mm diameter) containing 0.5 mL of a solution of adenylate kinase (approximately 2,500 U of a suspension in 3.2 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, Sigma) and 0.5 mL of a solution containing pyruvate kinase (approximately 1,000 U of a suspension in 2.1 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, Sigma) in 2 mL of the reaction mixture was added. Addition of 1 N HCl via a peristaltic pump driven by a pH controller maintained the pH near pH 8; \textsuperscript{1}H NMR and \textsuperscript{31}P NMR spectroscopy monitored the progress of the reaction. A white precipitate, presumably the ammonium-magnesium salt of P\textsubscript{i}, formed during the course of the reaction. Additional neutralized PEP-K\textsuperscript{+} (0.45 g, 1.9 mmol) was
added after 2 days. After 4 days $^1$H NMR and $^{31}$P NMR spectroscopy indicated that the ratio of CTP to CMP was 95:5; CDP was not detected.

**Isolation of CTP.** The enzyme-containing bag was removed and dialyzed against 15 mL of H$_2$O three times. To the combined volume (~70 mL) was added 1 mL of a basic solution$^{17}$ of MgCl$_2$·6H$_2$O (1.3 M) and NH$_4$Cl (3.8 M) as above, and the reaction mixture was centrifuged to remove precipitated inorganic phosphate. This step was repeated followed by the addition of 70 mL of EtOH to precipitate CTP. The solution was centrifuged, the pellet was redissolved in water (25 mL) and an additional 70 mL of EtOH was added. Following centrifugation, the precipitate was dissolved in water and the pH of the solution was adjusted to pH 9 with 1 N NaOH. Concentration in vacuo yielded 0.875 g of a white solid whose spectral data matched that of a commercial sample (95 mg). This material was converted to the ammonium salt (714 mg) by stirring it with ion-exchange resin (Dowex 50W-X8, NH$_4^+$ form). Ion exchange chromatography (AG-1X, HCO$_3^-$, eluant: HCO$_3^-$·NH$_4^+$) of the EtOH-containing supernatant followed by neutralization (with Dowex 50W-X8, H$^+$ form) of the fractions containing CTP yielded an additional 243 mg of CTP as the ammonium salt. Total recovery 0.958 g (1.8 mmol, 74%).

**References**

1. Supported by NIH Grant GM 30367 (to GMW). NMR instrumentation was supported in part by NSF Grant CHE-84-10774.
3. American Cancer Society Postdoctoral Fellow, Grant No. PF-2762, 1987-88.
8. Yuan, H.-M. *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Chin Chan* 1979, 20, 75 (CA 93: 68645j). The patent literature also describes fermentation methods. For example, see: CA 92: 4722).
12. Ribavarin mono- and triphosphates are also substrates for adenylate kinase: Kim, M.-J.; Whitesides, G. M.; *Appl. Biochem. Biotech.* in press.

14. $V_{rel}(CTP) \sim 10^{-4}V_{rel}(ATP)$ under the conditions used in this synthesis.


19. The product was characterized by $^1$H NMR and $^{31}$P NMR spectroscopy. Purity > 95%; PEP and $P_i$ (trace) are also present. $^1$H NMR (D$_2$O, DSS reference, 500 MHz) $\delta$ 7.82 (d, 1 H, J=7.6 Hz), 6.02 (d, 1 H, J=7.6 Hz), 5.88 (d, 1 H, J=4.4 Hz), 4.29-4.08 (m, 5 H) ppm; $^{31}$P NMR (D$_2$O, internal P$_i$ reference, 121.5 MHz) $\delta$ -6.1 (1 P, d, J=15.1 Hz), -10.9 (1 P, d, J=13.3 Hz), -19.6 (1 P, br s) ppm.

(Received in USA 16 November 1987)