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Preparation of Phosphoenolpyruvate from D-(−)-3-Phosphoglyceric Acid for Use in Regeneration of ATP[1]

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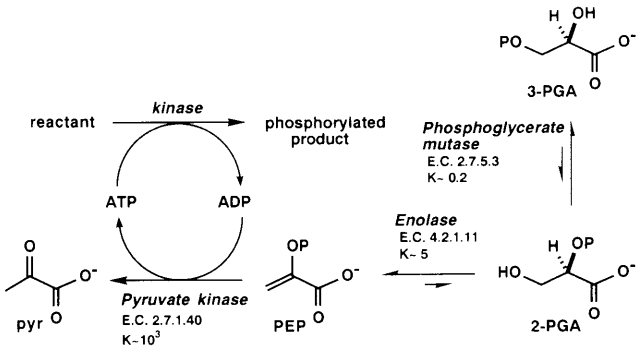
Adenosine 5′-triphosphate (ATP) is the most useful phosphoryl donor in enzyme-catalyzed synthesis.[4] The most convenient procedure for in situ regeneration of ATP from ADP uses phosphoenolpyruvate (PEP) and pyruvate kinase (Scheme I). Commercial PEP is expensive and must usually be synthesized chemically for use in large-scale synthesis. Here we describe a practical procedure (the PGA method) that uses two inexpensive, commercially available enzymes to generate PEP in situ from the relatively inexpensive D-(−)-3-phosphoglyceric acid (3-PGA) (Scheme I).[6]

Scheme I. Regeneration of ATP Using PEP[a]



[a] PEP can be synthesized in a separate chemical step or generated as shown from 3-PGA. P = phosphate; 3-PGA = D-(−)-3-phosphoglyceric acid; 2-PGA = D-(+)-2-phosphoglyceric acid; PEP = phosphoenolpyruvate; pyr = pyruvate.

Phosphoglycerate mutase[7] converts 3-PGA to D-(+)-2-phosphoglyceric acid (2-PGA); enolase[7] then forms PEP. Consumption of PEP by conversion of ADP to ATP, catalyzed by pyruvate kinase,[7] drives the overall process.[8,9]

We have tested the PGA method by using the reactions shown in Scheme II. CTP, GTP, and UTP are the nucleoside triphosphates needed for the syntheses of the most common nucleoside phosphate sugars used in Leloir pathway biosyntheses (reactions 1–4).[10] Dihydroxyacetone phosphate (reaction 5) is used in reactions catalyzed by aldolases,[11] and arabinose 5-phosphate (reaction 6) is a precursor to 3-deoxy-D-*manno*-2-octulosonic acid 8-phosphate (KDO-8-P).[12] In all cases, procedures based on 3-PGA were as effective as and more convenient than procedures based on chemically synthesized PEP.[5]

The synthesis of CTP from CMP illustrates the PGA method.[13] A suspension of 173 g of 3-PGA (barium salt, dihydrate, ~95%, 461 mmol) in 500 mL of water was stirred vigorously with ~600 mL of ion-exchange resin (Dowex 50W-X8, H^+ form, 20–50 mesh) for 30 min at room temperature. The resin was removed by filtration and washed with four 100-mL portions of water.[14] The combined, clear, pale-yellow filtrates were neutralized with solid KOH and used directly in the next step.

CMP (free acid, 71 g, 220 mmol), ATP·Na_2·$3H_2O$ (1.33 g, 2.20 mmol), $MgCl_2$·$6H_2O$ (51 g, 250 mmol), and triethanolamine (1.9 g, 10 mmol) were added to the solution of 3-PGA, and the pH was adjusted to pH 7.6 with 5 N KOH. The solution (total volume of 1 L) was degassed for 30 min with N_2; 2-mercaptoethanol (0.25 mL, 3 mmol) was added. The enzymes[15] were then added, and

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(2) Du Pont Fellow 1986–1987.
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(4) Chenault, H. K.; Simon, E. S.; Whitesides, G. M. In *Biotechnology & Genetic Engineering Reviews*; Russell, G. E., Ed.; Intercept: Wimborne, Dorset, 1988; Vol. 6, Chapter 6.
(5) Hirschbein, B. L.; Mazenod, F. P.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 3765.
(6) From US Biochemical Corp.: PEP = $4800/mol; 3-PGA = $250/mol.
(7) Phosphoglycerate mutase: Ray, W. J., Jr.; Peck, E. J., Jr. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic: New York, 1972; Vol. VI, Chapter 12. Most commercial preparations of 3-PGA contain 2,3-diphosphoglyceric acid, an activator of phosphoglycerate mutase. Enolase: Wold, F. *Ibid.* Vol. V, Chapter 18. Pyruvate kinase: Kayne, F. J. *Ibid.* Vol. VIII A, Chapter 11.
(8) The isolation of PEP in 15–20% yield from 3-PGA using enzyme preparations from yeast has been reported: Ganti, T.; Csoka, A. *Magy. Kem. Foly.* **1975**, *81*, 335–6; *Chem. Abstr.* **1975**, *83*, 162155v. Csoka, A.; Ganti, T. Hung. Teljes 8,931, 1974; *Chem. Abstr.* **1975**, *82*, 96491c.
(9) The three enzymes required are stable under the conditions used[7] and are inexpensive ($/1000 units): pyruvate kinase (0.3, Biozyme); enolase (4.75, Sigma); phosphoglycerate mutase (4.00, Sigma); 700 units of enzyme will convert 1 mol of reactants to products per day under assay conditions.
(10) Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5635.
(11) Bednarski, M. D.; Simon, E. S.; Bischofberger, N.; Fessner, W.-D.; Kim, M.-J.; Lees, W.; Saito, T.; Waldmann, H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627. Durrwachter, J. R.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 4175.
(12) Bednarski, M. D.; Crans, D. C.; Dicosimo, R.; Simon, E. S.; Stein, P. D.; Whitesides, G. M.; Schneider, M. *Tetrahedron Lett.* **1988**, *29*, 427.
(13) CMP is available from Miwon Foods Co., Ltd., Seoul, Korea ($200/kg).
(14) The resin was regenerated with HCl by following the procedure recommended by the manufacturer.

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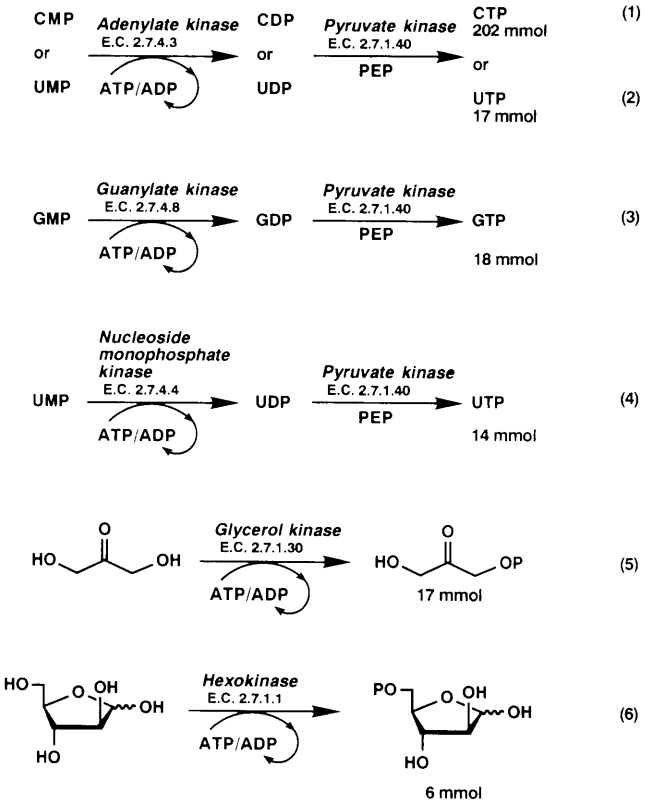
Scheme II. Reactions Tested by Using the 3-PGA Method To Regenerate ATP According to Scheme I[a]



[a] Amount refers to quantity isolated. P = phosphate; CTP = cytidine 5′-triphosphate; UTP = uridine 5′-triphosphate; GTP = guanosine 5′-triphosphate.

the solution was stirred at 22–24 °C under a positive pressure of N_2. Addition of 3 M HCl using a pH stat controlled the pH at 7.5–7.8. After 48 h, consumption of HCl had ceased but ^{1}H NMR spectroscopy indicated that the reaction was not complete. An additional 32 g of 3-PGA (barium salt, 84 mmol) was converted to the K^+ salt as described and added to the reaction mixture. After an additional 24 h, ^{1}H NMR spectroscopy indicated that the reaction was complete. A total of 130 mL of 3 N HCl had been consumed.

To isolate CTP, ~150-mL portions of the clear, pale-yellow reaction mixture were transferred into 12 500-mL polypropylene centrifuge tubes and 150 mL of absolute ethanol was added to each tube. A white precipitate formed immediately. The tubes were cooled in an ice bath for 15 min and then centrifuged (4 °C, 10000*g*, 10 min). The combined, sticky pellets were dissolved in 600 mL of water, the resulting solution was divided among six 250-mL centrifuge tubes, and 100 mL of absolute ethanol was added to each tube. The tubes were again cooled and centrifuged. Lyophilization of the combined pellets provided 145 g of an off-white powder containing 202 mmol of CTP according to enzymatic analysis[16] (92% yield; 90% purity for CTP·K_3).[17]

The primary advantage of the PGA method as a route to PEP is that it requires less time, produces less organic waste, and is more convenient than the chemical synthesis. Its starting materials are commercially available. It is, however, more expensive. The convenience outweighs the difference in cost for reactions carried out on a scale <1 mol. The convenience of the PGA method, along with its reliance on commercially available reagents, recommends it as a general technique for the in situ regeneration of ATP.

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(15) All enzymes were from Sigma: adenylate kinase (10000 units, lyophilized powder from chicken muscle), pyruvate kinase (5000 units, lyophilized powder from rabbit muscle), enolase (4000 units, lyophilized powder from yeast), and phosphoglycerate mutase (5000 units, suspension in ammonium sulfate from rabbit muscle). In this example, we used an excess of the enzymes because their cost is low compared with the value of the product.

(16) Keppler, D. In *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Bergmeyer, J., Grassl, M., Eds.; VCH: Weinheim, 1985; Vol. VII, p 432.

(17) The water content (7.14%) was determined by the Karl Fischer method. Thin-layer chromatography [poly(ethylenimine)–cellulose, eluant: 2.0 N HCOOH/2.0 N LiCl, 1:1, v/v] indicated that ATP was present in addition to CTP; neither CMP nor CDP was detected. Comparison of the ^{1}H and ^{31}P NMR spectra with those of authentic CTP from Sigma indicated the presence of ~1% each of ATP, dipyruvate, 3-PGA, inorganic phosphate, and ethanol.