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Synthesis of Phosphoenolpyruvate and Its Use in Adenosine Triphosphate Cofactor Regeneration[1]

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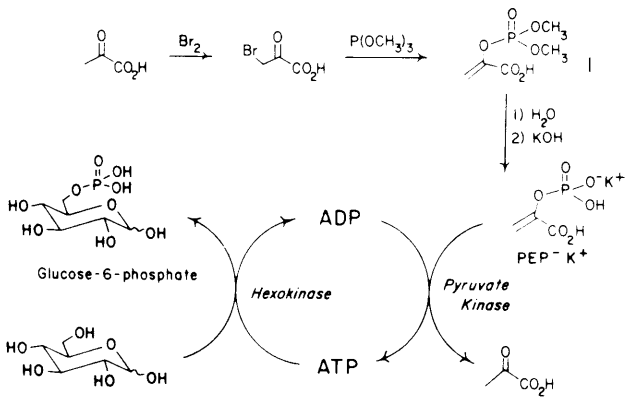
Efficient regeneration of adenosine triphosphate (ATP) in situ from adenosine diphosphate (ADP) is required for many enzyme-catalyzed syntheses. The use of phosphoenolpyruvic acid (PEP) as the ultimate phosphorylating agent for ATP regeneration is an attractive alternative to a procedure reported previously using acetyl phosphate (AcP).[3] Unfortunately, the high cost of PEP has limited its use to analytical-scale reactions.

We report here a convenient synthesis, based on a procedure developed by Clark and Kirby, which can be used to generate PEP in several-mole quantities (Scheme I).[4]

Scheme I. Synthesis of PEP^-K^+ and Use in ATP-Requiring Organic Synthesis



This synthesis has several practical advantages over the previous synthesis. In particular, it accepts crude pyruvic acid as starting material, and it produces PEP directly, in high purity, as an easily handled, stable, crystalline monopotassium salt (PEP^-K^+).[5] We have established the suitability of PEP^-K^+ prepared by this procedure for ATP regeneration using several enzyme-catalyzed syntheses. The preparation of glucose 6-phosphate described here is representative (Scheme I).

ATP regeneration based on PEP^-K^+ and pyruvate kinase has both advantages and disadvantages relative to the scheme based on AcP and acetate kinase.[3] The synthesis and physical properties of PEP^-K^+ are considerably more convenient for laboratory-scale work (several moles) than those of $AcP^{2-}(NH_4^+)_2$,[6] but the cost of the starting materials is higher. PEP is a stronger phosphorylating agent than AcP[7] and can be used more satisfactorily to drive thermodynamically unfavorable reactions.[8] The stability of PEP in solution is much higher than that of AcP.[9] The former can be added in one portion at the beginning of the reaction; the latter must be added continuously. Potassium ion is innocuous as a component of most enzymatic systems (and, in fact, is required for activity of pyruvate kinase[10]); the presence of ammonium ion (from $AcP^{2-}(NH_4^+)_2$) in solution complicates control of the concentration of Mg^{2+}, because $Mg(NH_4)PO_4$ has low solubility in water. Because the K_m of ADP for pyruvate kinase is lower than that for acetate kinase, it is possible to use lower concentrations of A(T,D)P and achieve higher turnover numbers with pyruvate kinase than with acetate kinase.[11]

(1) Supported by grants from the National Institutes of Health (GM 26543 and GM 30367) and from Firmenich SA.

(2) NCI predoctoral fellow, CA 09112 CT.

(3) Pollak, A.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 2366–2367. Rios-Mercadillo, V. M.; Whitesides, G. M. *Ibid.* **1979**, *101*, 5828–5829.

(4) Clark, V. M.; Kirby, A. J. *Biochem. Prep.* **1966**, *11*, 101–104.

(5) The critical element which renders this preparation suitable for large scale work is the discovery that PEP^-K^+ can be precipitated directly from the crude reaction mixture and used without further manipulation in ATP regeneration.

(6) Lewis, J. M.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1979**, *44*, 864–865.

(7) The free energies of hydrolysis in neutral aqueous solution ($\Delta G'_{H_2O}$, kcal/mol) of organic phosphates are representative of their thermodynamic driving force for ATP regeneration. Pertinent values are as follows: PEP, −14.8; creatine phosphate, −10.3; AcP, −10.1; ATP, −7.3; G-6-P, −3.3. Lehninger, A. L. "Biochemistry", 2nd ed., Worth Publishers: New York, 1975; p 398.

(8) We have used PEP^-K^+ to drive phosphorylation of creatine to creatine phosphate in a procedure considerably more straightforward than that described previously with AcP: Shih, Y.-S.; Whitesides, G. M. *J. Org. Chem.* **1977**, *42*, 4165–4166.

(9) The half-lives of PEP and AcP in aqueous solution at 30.5 °C are 98 days (at pH 7.0) and 0.34 days (at pH 7.2), respectively. Benkovic, S. J.; Schray, K. J. *Biochemistry* **1968**, *7*, 4090–4096. DiSabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400–4405.

(10) Lardy, H. A.; Ziegler, J. A. *J. Biol. Chem.* **1945**, *159*, 343–351.

(11) K_m (ADP, pyruvate kinase) = 0.3 mM (McQuate, J. T.; Utter, M. F. *J. Biol. Chem.* **1959**, *234*, 2151–2157). K_m (ADP, acetate kinase) = 1.5 mM (Rose, J. A.; Grumberg-Manago, M.; Korey, S. R.; Ochoa, S. *J. Biol. Chem.* **1954**, *211*, 737–756). The starting concentration of ATP should be ca. 2 K_m.

The costs of the quantities of pyruvate kinase and acetate kinase required to achieve equal rates of ATP regeneration are comparable.[12]

The balance of these factors, in our experience, is that PEP^-K^+/pyruvate kinase is a more convenient and useful system for regeneration of ATP than $AcP^{2-}(NH_4^+)_2$/acetate kinase for syntheses generating products in quantities up to several moles. For larger preparations, AcP/acetate kinase may have economic advantages.

Experimental Section

All reagents and solvents (except for water, which was twice distilled) were obtained commercially and used without further purification.

Bromopyruvic Acid. Pyruvic acid (480 g of 95% pure material, 5.17 mol), 20 drops of concentrated H_2SO_4, and 450 mL of CH_2Cl_2 were added to a 3-L, three-necked flask equipped with an overhead stirrer, an addition funnel, and a reflux condenser connected to a bubbler. Bromine (265 mL, 5.17 mol) was added dropwise over a 3.5-h period to the stirred solution. A white precipitate formed when the addition of Br_2 was nearly complete. The suspension was stirred for an additional hour and diluted with 40 mL of cyclohexene and 200 mL of ligroin (bp 35–60 °C). The reaction mixture was cooled in an ice bath. The bromopyruvic acid was collected by filtration, washed with 300 mL of ligroin, and dried at 0.1 torr for 12 h. Its yield was 804 g [mp 64–67 °C (lit.[4] mp 70 °C), 97% pure,[13] 4.65 mol, 90% yield based on pyruvic acid].

Potassium Phosphoenolpyruvate (PEP^-K^+). Bromopyruvic acid was converted to the dimethyl ester of PEP, 1 (2-hydroxyacrylic acid dimethyl phosphate), in a 12-L, three-necked flask equipped with a reflux condenser connected to a bubbler, an addition funnel, and a magnetic stirrer. A solution of 752 g (4.37 mol) of bromopyruvic acid (97% pure, used without further purification) in 1.25 L of dry ether was added dropwise at a rate sufficient to maintain the ether at reflux (3.5 h) to a stirred solution of 557 mL (4.72 mol) of trimethyl phosphite in 3.85 L of dry ether. The reaction mixture was stirred for an additional hour at ambient temperature, and the ether was removed by rotary evaporation, yielding 1002 g of crude **1** as a brown viscous oil. This oil was dissolved in 1.67 L of water, and the solution was stirred at 20 °C for 15 h. The spontaneous hydrolysis reaction had proceeded to completion in this time and had produced 2.64 mol (60%) of PEP and 0.2 mol (5%) of pyruvate.[14] The solution was cooled in an ice bath, and 267 g of solid KOH (85% pure, 4.0 mol) was added (to produce a solution with pH 2.8) followed by 2.7 L of absolute ethanol. The white precipitate which formed was collected by filtration, washed with 800 mL of cold absolute ethanol, and dried at 0.1 torr, yielding 531 g of PEP^-K^+ (95% pure,[5,14,15] 2.45 mol, 50% yield based on crude pyruvic acid).

Glucose 6-Phosphate. The synthesis was carried out under argon in a 2-L, three-necked flask equipped with a pH electrode and a magnetic stirrer. A solution of 0.800 mol of glucose, 0.800 mol of PEP^-K^+, 35 mmol of $MgCl_2$, and 10 mmol of 2-mercaptoethanol in ca. 800 mL of doubly distilled water was adjusted to pH 7.6 with solid KOH, transferred to the reaction flask, and degassed with a stream of argon. ATP (1.20 mmol) and an aqueous suspension (0.78 L) of 1260 U of pyruvate kinase and 863 U of hexokinase (each separately immobilized in PAN gel[16]) were added. The reaction mixture (1.6 L) was stirred at 20 °C and maintained at pH 7.5–7.6 by occasional addition of a few drops of 12 M HCl. After 8.5 days the reaction mixture contained 0.77 mol of G-6-P (96%), 0.42 mol of pyruvate (53%), and no PEP.[14] The immobilized enzymes were separated by centrifugation, washed with 300 mL of doubly distilled H_2O, and again separated by centrifugation. The turnover numbers (and residual activities) of the components of this system were as follows: hexokinase, 2×10^7 (100%); pyruvate kinase, 4×10^7 (70%); ATP, 587 (not recovered). To the combined supernatant reaction mixture and wash solution was added 244 g of $BaCl_2 \cdot 2H_2O$ (1.00 mol), and the solution was adjusted to pH 7.0 with solid KOH and stored at 0 °C overnight. The white precipitate which formed was collected by filtration, washed in succession with 300 mL of cold H_2O, 400 mL of cold 50% aqueous ethanol, and 200 mL of cold 95% aqueous ethanol, and then dried at 0.1 torr. The yield of BaG-6-P·$7H_2O$ was 367 g (100% pure,[14,15] 0.704 mol, 88% yield).

Registry No. 1, 4185-81-3; ATP, 56-65-5; ADP, 58-64-0; $PEP-K^+$, 4265-07-0; pyruvic acid, 127-17-3; bromopyruvic acid, 1113-59-3; glucose, 50-99-7; glucose 6-phosphate, 56-73-5; pyruvate kinase, 9001-59-6; hexokinase, 9001-51-8.

(12) Pyruvate kinase is less expensive and more stable than acetate kinase. The pronounced inhibition of pyruvate kinase by pyruvate (K_I = 1 mM, Reynard, A. M.; Hass, L. F.; Jacobsen, D. D.; Boyer, P. D. *J. Biol. Chem.* **1966**, *236*, 2277–2283) requires that a 10-fold larger number of units of this enzyme be used than that predicted on the basis of noninhibited assay to achieve satisfactory regeneration rates at high pyruvate concentrations.

(13) The purity of bromopyruvic acid (a substrate analogue of pyruvic acid for L-lactic dehydrogenase) was determined by enzymatic assay[14] and from its ^1H NMR spectrum.

(14) Assays of biological materials were performed following procedures in Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Verlag Chemie Weinheim/Bergstr., Germany: 1974: PEP, pyruvic acid and bromopyruvic acid, p 1456; glucose 6-phosphate, p 1283; pyruvate kinase, p 774; hexokinase, p 473. Assays of immobilized enzymes were performed as described in ref 16.

(15) The ^{31}P NMR spectrum of PEP^-K^+ (0.5 M, H_2O) consisted of a single peak at −4.51 ppm (85% H_3PO_4 external reference); that of BaG-6-P (0.1 M HCl) was a single peak at 0.47 ppm. The ^1H NMR spectrum of PEP^-K^+ (in D_2O) consisted of a multiplet at 5.88 ppm (1 H) and a multiplet at 5.54 ppm (1 H) from an internal DSS reference.

(16) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–6336.