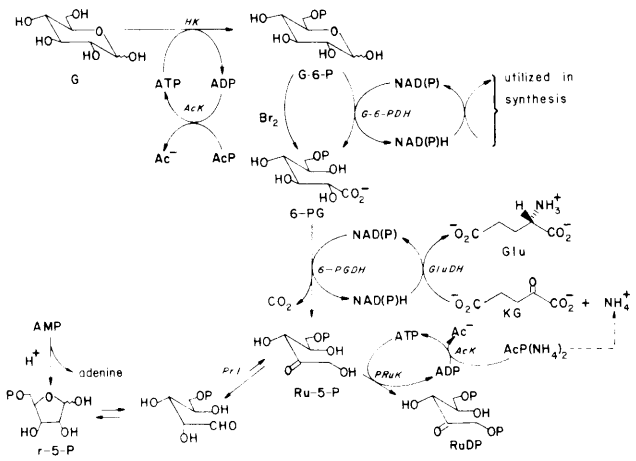
Practical Enzymatic Syntheses of Ribulose 1,5-Bisphosphate and Ribose 5-Phosphate[1]

Sir:

Ribulose-bisphosphate carboxylase (EC 4.1.1.39) is the central enzyme in fixation of CO_2 by plants and as such is an object of current interest in enzymology.[2] The normal substrate for this enzyme, ribulose 1,5-bisphosphate (RuDP), is expensive and has been difficult to prepare in quantity.[3] Here we report two enzymatic syntheses easily capable of generating RuDP in quantities greater than 0.1 mol. These syntheses also provide a new route to ribose 5-phosphate and illustrate the practicality of an anaerobic system for regeneration of NAD(P) from NAD(P)H based on glutamate dehydrogenase. The relevant reactions are summarized in Scheme I.

The first step in conversion of glucose 6-phosphate[4] to RuDP was oxidation to 6-phosphogluconate (6-PG) either with Br_2[5] or enzymatically, using glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and NAD(P).[6] The two-step conversion of 6-PG to RuDP was carried out in one vessel. A 3-L solution containing 0.2 mol of 6-PG, 0.2 mol of α-ketoglutarate, 1 mmol of ATP, 10 mmol of dithiothreitol (DTT), and 30 mmol of $MgCl_2$ was degassed with argon. Acetate kinase (AcK, EC 2.7.2.1, 800 U), 6-phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44, 800 U), glutamate dehydrogenase (GluDH, EC 1.4.1.2, 800 U), and phosphoribulokinase (PRuK, EC 2.7.1.19, 800 U), each separately immobilized in PAN gel,[7] were added, and the solution was blanketed with argon.[8] Diammonium acetyl phosphate[9] (48 g of 90% pure material, 0.25 mol) was added as a solid in 10 equal portions over 40 h and the pH controlled at 7.9.[10,11] The solution was decanted from the gel; the enzymatic activities recovered in the gel were: 6-PGDH, 92%; AcK, 92%; GluDH, 94%; PRuK, 92%. Acid-washed activated charcoal (30 g) was added to the decanted solution to remove colored impurities. The pH was adjusted to 4.5, and $BaCl_2$ (0.5 mol) was added. Most of the phosphate present in solution precipitated as barium phosphate, while the RuDP remained in solution. The pH was adjusted to 6.5, and ethanol (the same volume as the aqueous phase) was added to precipitate Ba_2RuDP. The product (98 g) showed 78% purity when assayed enzymatically[12] (131 mmol, 66% based on 6-PG).

Scheme I[a]



[a] Enzyme-catalyzed syntheses of ribulose 1,5-bisphosphate and ribose 5-phosphate. Abbreviations (*italics* = enzymes): *HK* = hexokinase; *G-6-PDH* = glucose-6-phosphate dehydrogenase; *PrI* = phosphoribose isomerase; *GluDH* = glutamate dehydrogenase; *PRuK* = phosphoribulokinase; *6-PGDH* = 6-phosphogluconate dehydrogenase; *AcK* = acetate kinase; G = glucose; G 6-P = glucose-6-phosphate; 6-PG = 6-phosphogluconate; Glu = glutamate; Ru-5-P = ribulose-5-phosphate; RuDP = ribulose-1,5-bisphosphate; R-5-P = ribose 5-phosphate; AcP = acetyl phosphate; KG = 2-ketoglutarate. Enzymatic conversion of G-6-P to 6-PG was ordinarily accomplished by using a procedure in which the NADH and NADPH produced was consumed in some other coupled, enzymatic synthesis step.

Ribulose 1,5-bisphosphate was also prepared in a second procedure from ribose 5-phosphate (R-5-P). This latter compound was prepared either by acid-catalyzed hydrolysis of AMP[13] or by an enzymatic route from 6-PG. In the enzymatic procedure, a 2-L solution containing 0.2 mol of 6-PG, 0.22 mol of α-ketoglutarate, 0.25 mol of NH_4Cl, 10 mmol of DTT, and 0.2 mmol of NADP was degassed with argon. To this solution was added 6-PGDH (800 U), GluDH (800 U), and phosphoribose isomerase (EC 5.3.1.6, 800 U), separately immobilized in PAN gel.[7] The reaction mixture was stirred under argon and the pH controlled at 7.8. After 40 h the solution was decanted from the gel and treated with $BaCl_2$ (0.25 mol). Ethanol (the same volume as the aqueous phase) was added at 0 °C. The resulting precipitate was collected by filtration and washed with ethanol. After being dried, the solid (58 g) contained ribose 5-phosphate (52 g of BaR-5-P, 72% yield based on 6-PG, 90% purity).[8] The recovered enzymatic activities in the gel were 6-PGDH, 92%; GluDH, 94%; PrI, 92%.

A representative conversion of R-5-P to RuDP involved stirring R-5-P (0.2 mol) under argon for 40 h at pH 7.8 in a 3-L reaction

(1) Supported by the National Institutes of Health, Grant GM 26543.

(2) Jensen, R. G.; Bahr, J. T. *Annu. Rev. Plant Physiol.* **1977**, *28*, 379-400. McFadden, B. A. *Bacteriol. Rev.* **1973**, *37*, 286-319. Siegelman, H. W., Hind, G., Eds., "Photosynthetic Carbon Assimilation"; Plenum Press: New York, 1978.

(3) For small-scale (0.5 g) preparations of RuDP without cofactor regeneration, see: Kuehn, G. D.; Hsu, T. C. *Biochem. J.* **1978**, *175*, 909-912. Horecker, B. L.; Hurwitz, J.; Weissbach, A. *Biochem. Prepn.* **1958**, *6*, 83.

(4) Pollak, A.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 2366-2367.

(5) Horecker, B. L. *Methods Enzymol.* **1957**, *3*, 172-174.

(6) Wong, C.-H., unpublished. The G-6-PDH from *Leuconostoc mesenteroides* accepts both NAD and NADP as cofactors: DeMoss, R. D.; Gunsalus, I. C.; Bard, R. C. *J. Bacteriol.* **1953**, *66*, 10-16.

(7) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324-36. Activities (μmol min^{-1}) refer to immobilized enzyme and are approximate (±10%); immobilization yields ranged from 30 to 40%.

(8) Enzymatic assays: Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Verlag Chemie: New York, 1974. Enzymes were obtained from Sigma or Boehringer-Mannheim.

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

(10) The ammonium ion introduced with the acetyl phosphate was sufficient for glutamate synthesis.

(11) Because 6-PGDH contains an essential and easily autoxidized SH group, NAD(P) regeneration schemes based on dioxygen are unsatisfactory for the 6-PGDH-catalyzed synthesis of Ru-5-P (cf., for example; Irwin, A. J.; Jones, J. B. *J. Am. Chem. Soc.* **1977**, *99*, 556-561). NAD(P) regeneration based on GluDH can be carried out anaerobically and is therefore compatible with 6-PGDH. GluDH accepts both NAD and NADP: Olson, J. A.; Anfinsen, C. B. *J. Biol. Chem.* **1953**, *302*, 841-856.

(12) Racker, E. *Methods Enzymol.* **1957**, *5*, 266-275. Commercial ribulose bisphosphate carboxylase (RuDPC) (Sigma) has low purity, and contaminating activities (possibly PrK) make it unsuitable for use in this assay. The RuDPC was obtained from spinach leaves, by using the procedure of Ryan et al. (Ryan, F. J.; Barker, R.; Tolbert, N. E. *Biochem. Biophys. Res. Commun.* **1975**, *65*, 39-46), and had specific activity of 2.5 U mg^{-1}. A solution of RuDP is unstable at room temperature ($\tau_{1/2}$ = 48 h at pH 8.0, 30 °C) and should be stored in the absence of water at low temperature: Paech, C.; Pierce, J.; McCurry, S. D.; Tolbert, N. E. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 1084-1092. No solution should be allowed to become more basic than pH 8 once RuDP is present, to avoid formation of xylulose diphosphate (a strong inibitor of ribulose-bisphosphate carboxylase). To determine the level of inhibitors present in the preparations of RuDP, a sample of RuDP is allowed to react to completion under assay conditions. The inhibitors react only slowly under these conditions, and remain at the conclusion of the reaction. Additional RuDP is then added, and the initial rate measured. The difference between the initial rates of these two reactions provides an estimate of the concentrations of inhibitors. Contamination by xylulose diphosphate (<1% of RuDP) would give up to 50% inhibition.

(13) The method of Sokatch (Sokatch, J. R. *Biochem. Prep.* **1968**, *12*, 2) was used with modifications making it applicable to 200-g quantities of AMP. AMP was obtained from Kyowa Hakko Kogyo.

mixture containing PrI (1000 U), PRuK (800 U), AcK (800 U) (each immobilized separately in PAN gel[7]), $MgCl_2$ (30 mmol), DTT (10 mmol), and ATP (1 mmol); solid AcP (0.25 mmol) was added in 10 equal portions over 40 h. Isolation of Ba_2RuDP followed the procedure outlined above: 94 g of solid was obtained, containing 116 mmol of RuDP (72% purity, 58% yield based on R-5-P). The recovered enzymatic activities were PrI, 94%; PRuK, 92%; AcK, 92%.

Both of these preparations of RuDP use commercial PRuK and yield product of moderate purity. To obtain purer RuDP, either this product may be purified or the conditions of synthesis altered to give RuDP of higher initial purity. In order to accomplish the latter, it is necessary to use more PRuK to shorten the reaction time and minimize hydrolysis of RuDP in solution. Commerical PRuK (Sigma, 250 U/313 mg) was passed through a column of Bio-Gel P-150 (2 × 80 cm) in 50 mM Tris–3 mM DTT, concentrated by ultrafiltration, and immobilized, giving gel having activity of 6 U mL^{-1}. Conversion of R-5-P (100 mmol) to RuDP by using 800 U of PRuK in 2 L of solution was complete in 20 h and gave Ba_2RuDP in 92% purity and 80% yield (80 mmol) based on R-5-P.

A purification of lower purity RuDP was accomplished by treating a suspension of 45 g of Ba_2RuDP (72% purity) with 300 g of Dowex 50 (H^+ form, 200–400 mesh) in 800 mL of H_2O to remove Ba^{2+}. The resulting solution was passed through Dowex 1 (800 g, 200–400 mesh, chloride form), supported in a 2-L glass filter, and washed with 5 L of 40 mM aqueous HCl, to remove Ru-5-P and other impurities. RuDP was then desorbed by washing the resin with 2 L of 0.15 M HCl–0.1 M NaCl and precipitated as described previously. The product (32 g) was 94% Ba_2RuDP.

Both ribose 5-phosphate and ribulose 1,5-bisphosphate were prepared by using two different routes. Which route is superior depends on circumstances. The acid-catalyzed hydrolysis of AMP is the shorter route to R-5-P. Since it requires no enzymes, it is preferable when only a modest quantity of R-5-P (a few hundred grams) is required. This procedure has the further advantage that it generates a product uncontaminated by Ru-5-P.[14] The route from glucose to R-5-P is more complex, but it has two advantages: first, it can be used to regenerate NAD(P)H from NAD(P); second, it is potentially much less expensive as a route to large quantities of R-5-P than that starting with AMP. Thus, this procedure is more applicable to large-scale processes and integrated schemes for biotransformations in which the reduced nicotinamide cofactors can be utilized for other purposes. Similar considerations apply to RuDP. We have available in our laboratory large quantities of 6-PG as a byproduct of nicotinamide coenzyme regeneration using G-6-P.[6] The effort required to convert this material to RuDP is comparable to that required when starting with R-5-P.

In summary, for a synthetic effort whose sole purpose is the synthesis of RuDP, the procedure starting from AMP is simpler. When 6-PG is available for other reasons, or when the oxidation of 6-PG to Ru-5-P can be usefully coupled to another reduction via NAD(P)H, the route starting with G-6-P or 6-PG is superior.

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(14) An equilibrium mixture of Ru-5-P and R-5-P contains 77% R-5-P at pH 7.6; Dobrogrosz, W. J.; Demoss, R. D. *Biochim. Biophys. Acta* **1963**, *77*, 629–638.

Chi-Huey Wong, Stephen D. McCurry
George M. Whitesides*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

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