[18] Synthesis of Ribulose 1,5-Bisphosphate: Routes from Glucose 6-Phosphate (via 6-Phosphogluconate) and from Adenosine Monophosphate (via Ribose 5-Phosphate)\textsuperscript{1}

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This procedure describes two routes to ribulose 1,5-bisphosphate (RuBP) (Fig. 1).\textsuperscript{2} These routes provide procedures suitable for preparing several hundred grams of RuBP and for the major intermediates lying between starting materials and this metabolite (6-phosphogluconate, 6-PG; ribulose 5-phosphate, Ru-5-P; ribose 5-phosphate, R-5-P). The enzymes required are all used in immobilized form. These immobilized preparations have high stability, and the enzymes can be recovered conveniently in good yields at the end of the reactions and re-used. Both routes require ATP cofactor recycling, and the procedure includes details of an improved synthesis of the acetyl phosphate used as the ultimate phosphate donor for this recycling. The route based on glucose 6-phosphate (G-6-P) also illustrates a convenient procedure for regeneration of NADPH from NADP+ under anaerobic conditions, based on conversion of α-ketoglutarate to glutamate.

The major technical problem in the preparation of RuBP is that of obtaining a product substantially free of xylulose 1,5-bisphosphate (XuBP). This analog is a powerful inhibitor\textsuperscript{3} of ribulosebisphosphate carboxylase (RuBPC) ($K_{m,\text{RuBPC}} = 20 \mu M, K_{m,\text{RuBP}} = 3 \mu M$). The RuBP isomerizes rapidly and spontaneously to XuBP ($t_{1/2} = 48$ hr at pH 8.0, 30°C). Contamination of RuBP by 0.1% of XuBP results in significant inhibition of the enzyme. It is thus important to minimize exposure of the RuBP to isomerizing conditions during and after preparation. In particular,

\begin{enumerate}
  \item Supported by the National Institutes of Health, Grant GM 25643.
  \item S. D. McCurry, J. Pierce, N. E. Tolbert, and W. H. Orme-Johnson, J. Biol. Chem., 256, 6623 (1981). Active RuBPC is a ternary complex (ECM) composed of enzyme (E), CO$_2$ (C), and Mg$^{2+}$ (M). This complex is in equilibrium with an inactive form of the enzyme, and the interconversion of these forms is slow. XuBP combines very tightly with the inactive enzyme and acts as a noncompetitive inhibitor. It also binds less tightly to the ECM form of the enzyme as a competitive inhibitor.
  \item F. Dailey and R. S. Criddle, Arch. Biochem. Biophys. 201, 594 (1980).
  \item J. M. Sue and J. R. Knowles, unpublished observation.
\end{enumerate}

RuBP should not be exposed to pH > 8.\textsuperscript{4} It also isomerizes as its solid barium salt if stored at room temperature; at ~20°C it is stable for months. This procedure includes two procedures for removing small quantities of XuBP from RuBP.

The procedures for the preparation of RuBP summarized in Scheme 1 are the best available when large quantities (>10 g) are required. Their ability to produce large quantities in a practical and economical procedure rests on the availability of good cofactor recycling schemes. The requirement for cofactor recycling necessarily makes these procedures more complex than smaller-scale procedures that do not involve cofactor recycling.\textsuperscript{5} Of the two routes in Scheme 1, that starting from AMP is the more direct and more convenient. The route from G-6-P is, however, not much more complex. Which route is preferable in a particular laboratory depends upon the relative availability of the starting materials and relative desirability of the intermediates generated in each.

\begin{enumerate}
  \item C. Piech, J. Pierce, S. D. McCurry, and N. E. Tolbert, Biochem. Biophys. Res. Commun., 83, 1084 (1978). The rate of degradation of RuBP at pH 8.3 and 30°C was 1.2% per hour. We determined the stability of RuBP (0.5 mM in 0.1 M Tris, pH 8.0, 30°C) in solution by measuring the concentration of RuBP with the coupled enzymic method. The observed half-life for RuBP was 48 hr.
  \item G. D. Kuehn and T. C. Hsu, Biochem. J. 175, 909 (1978).
  \item B. L. Horecker, J. Hurwitz, and A. Weissbach, Biochem. Prep. 6, 83 (1958).
  \item J. Pierce, S. D. McCurry, R. M. Mulligan, and N. E. Tolbert, this volume [9].
\end{enumerate}
(0.02 torr) at room temperature for 24 hr. The THF lost during the drying is trapped using a condenser kept at liquid nitrogen temperature to protect the pump. The white product (60 g, 99%) contains 912 µmol of active ester per gram.

**Diammonium Acetyl Phosphate.** This phosphate is prepared by reaction of acetic anhydride and phosphoric acid using a modification of a published procedure\textsuperscript{11} that was designed to give better control over the concentration of ammonia in the methanol used in workup.

Ethyl acetate (AR grade 2000 ml) and 100% phosphoric acid (150 g, 1.5 mol, prepared by dissolving phosgene pentoxide in 85\% phosphoric acid, and stirring at ambient temperature for 2 hr) are transferred into a 3-liter, three-necked flask, equipped with a strong mechanical stirrer, a dropping funnel (500 ml), and a drying tube (Drierite) for ventilation. The flask is cooled in an ice bath (20 × 20 inches), and when the temperature of the reaction mixture had reached 0° (in about 30 min), the funnel is charged with cold (ca. 0°) acetic anhydride (275 g, 2.7 mol), which is then added dropwise (over 20 min) to the ethyl acetate–phosphoric acid mixture, with vigorous stirring. The resulting solution is stirred further at 0° for a total of 3.75 hr. This interval is critical. Major deviations (± 0.5 hr) result in significant decreases in yields.

A 5-liter, three-necked flask was placed in a cooling bath (25 × 25 inches, containing 2 gal of isopropanol and 1 gal of acetone). Pieces of Dry Ice are added to this bath at a rate that keeps the bath temperature at −35 to −40°. The reaction flask is fitted with a low-temperature thermometer, a drying (KOH) gas outlet tube, and a very strong overhead stirrer. Methanol (AR grade, 2000 ml) is added into the flask and stirred until the temperature reaches about −30°; a rapid stream of gaseous ammonia (Matheson) is bubbled into the well-stirred methanol until its concentration reaches approximately 8 N as determined by titration. (To titrate, 1 ml of the solution is diluted with water to 25 ml and titrated with 1 N HCl to pH 6.0). The addition of ammonia is then stopped, and the gas inlet tube is replaced with a 3-liter funnel. The cold ethyl acetate–acetic anhydride–phosphoric acid mixture is quickly transferred to the funnel, from which it is dropped into the vigorously stirred methanol–ammonia solution. This addition takes approximately 30–35 min, during which time a fine, white suspension appears and the temperature rises to about −10°. The white, precipitated material is quickly separated by filtration on a large Buchner funnel, washed with 1 liter of cold (0°) methanol, 1 liter of cold, anhydrous ether, and finally with 1 liter of cold, dry hexane (a mixture of hexanes). The product is immediately transferred into a large vacuum desiccator.

which is attached to a good vacuum (ca. 2 torr). After about 6 hr of drying, 255 g of a white, fluffy product are obtained. An enzymatic assay showed that this solid contains 89% diammonium acetyl phosphate (1.3 mol) by weight, corresponding to an 87% yield based on phosphoric acid.

Diammonium acetyl phosphate must be stored at 15° in tightly stoppered containers to prevent its decomposition. If properly stored, it will decompose less than 5% per month.

Enzymes

The following enzymes are obtained from Sigma: yeast alcohol dehydrogenase (ADH, EC 1.1.1.1); aldehyde dehydrogenase (ALDH, EC 1.2.1.3); acetate kinase (AcK, EC 2.7.2.1); glucose-6-phosphate dehydrogenase (6-PGDH, EC 1.1.1.49); α-glycerol-3-phosphate dehydrogenase (GDH, EC 1.1.1.8); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12); 3-phosphoglycerate kinase (PGK, EC 2.7.2.3); glutamic dehydrogenase (GDH, EC 1.4.1.3); hexokinase (HK, 2.7.1.1); fructose-1,6-bisphosphate aldolase (FDA, EC 4.1.2.13); ribose-5-phosphate isomerase (PRI, EC 5.3.1.6); phosphoribulokinase (PrK, EC 2.7.1.19); ribulose,1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39); triosephosphate isomerase (TRI, EC 5.3.1.1); transaldolase (TA, EC 2.2.1.2). Ribulose-1,5-bisphosphate carboxylase (RuBPC) is isolated from spinach leaves12,13 instead of using the commercially available source, which might contain PrRuK.

For those enzymes suspended in ammonium sulfate, ammonium sulfate is removed by centrifuging the suspension at 15,000 g for 5 min (3°), and the precipitate is used directly in immobilizations. PrRuK (Sigma, 250 U/313 mg) is dissolved in 10 ml of deoxygenated Tris buffer (50 mM, pH 7.5, containing 3 mM DTT) and dialyzed against 1 liter of the same buffer for 6 hr to remove impurities and the ammonium salts (<50% w/w), which would react with the active ester groups of PAN.

Glucose 6-Phosphate: Hexokinase-Catalyzed Phosphorylation of Glucose

Glucose 6-phosphate is prepared using HK-catalyzed phosphorylation and ATP regeneration.11,15 A 3-liter solution containing glucose (1.4 mol), ATP (10 mmol), MgCl₂ (98 mmol), EDTA (4.8 mmol), and 1.3-

dimercapto-2-propanol (18 mmol) is deoxygenated and maintained under argon. Immobilized heparin kinase (1200 units, 6 ml of gel) and AcK (1200 units, 10 ml of gel) are added to this solution. Diammonium acetyl phosphate (1.4 mol, 90% purity) is added to the stirred reaction solution in 10 portions over 60 hr, and the solution is maintained at pH 7.5 by addition of 4 M KOH solution using a pH controller. The reaction is performed at 25°. After 3 days, 1.2 mol of G-6-P has been formed (0.364 M in 3.3 liters, yield 85%). The solution is separated from the polyclaramide gels by decantation. Barium chloride (0.2 mol) is added to precipitate inorganic phosphate; this material is removed by filtration. Another portion of BaCl₂ (1.3 mol, 271 g) is added, followed by addition of ethanol (1.5 liters). The precipitated solid (677 g) contains 92% Ba G-6-P · 7 H₂O (1.2 mol). The precipitate contains 17% (w/v) of G-6-P and 5% of AcK, 80%.

Enzyme Immobilization

Enzymes are immobilized in PAN gel using the general procedure described previously.16 Each enzyme immobilization is carried out in the presence of substrates or products intended to occupy the active site and protect it against modification during immobilization. The concentration of substrates used to protect the enzymes, and the immobilization yields, are PRI (5-5-P 200 mM and Ru-5-P 2 mM, 36%), PrK (Ru-5-P 15 mM and ATP 15 mM, 30%), AcK (AcP 12.5 mM, and ADP 20 mM, 34%), 6-PGDH (6-PG 2.0 mM and NADP 0.5 mM, 38%), RuBPC (2-ketogluconate 15 mM, NADPH 0.5 mM, and HCl 20 mM, 40%).

A typical example of immobilization procedure of 10 mg of enzyme follows. PAN-1000 (1 g) was placed in a 30-ml beaker containing a stirring bar. HEPES buffer (4 ml, 0.3 M HEPES buffer) containing the concentrations given of active site-protective reagents was added. The mixture was brought into solution within 1 min by vigorous stirring. Aqueous solutions of diethylthreitol (DTT) (50 μl, 0.5 M) and triethylene tetramine (TET, 0.6 ml, 0.5 M) were added; 10 sec later, 1 ml of the enzyme solution in HEPES buffer was added. The mixture gelled within 3 min. The gel was allowed to stand under argon at room temperature for 1 hr to complete the coupling reaction. The gel was ground into fine particles with a pestle in a mortar for 2 min; 25 ml of deoxygenated HEPES buffer (50 mM pH 7.5, containing 3 mM DTT and 50 mM (NH₄)₂SO₄) were added, and grinding was

Yield (%) = units assayed in enzyme-containing gel × 100 units assayed in solution before immobilization
continued for an additional 2 min. The mixture was diluted with the same buffer solution (50 ml), stirred for 15 min to destroy the unreacted active esters, and separated by gentle centrifugation (3000 rpm). The washing procedure was repeated once with the same buffer containing no ammonium salt. The gel particles were then resuspended in the same volume of buffer for assay.

Enzyme Assays

Enzyme assays are carried out spectrophotometrically following the standard procedures of Bergmeyer.17 For the immobilized enzymes, aliquots of the solutions containing the suspended gel particles are taken by unexceptional procedures using volumetric pipettes; the gel particles have a density very similar to that of the solution, and the suspensions can be sampled in the same way as solutions provided that the particles are small and that the suspensions are shaken or stirred well immediately before sampling. During spectrophotometric assays, the cuvettes containing the gel suspensions are stoppered with Parafil and shaken periodically for a few seconds to keep the suspensions well mixed. Almost linear assay responses are obtained if the cuvettes are shaken 15-20 times over the course of assays lasting 1-5 min.

Assays for RuBP

Two methods are employed for RuBP determination. One is a coupled-enzyme method18; the other one is radiometric.19,20 Scheme 2 shows the reactions involved in the coupled enzyme method. As shown in the reactions, one equivalent of RuBP reacts with four equivalents of NADH. Since the assay includes five enzymes, the possibility that impurities in one or several of them might influence the results is relatively large. In particular, we have found that RuBP carboxylase (RuBPC) must be prepared18; the commercially available sources of this enzyme seem to contain PRuK and PRI, both of which contribute to incorrect results.

The radiometric method involves only the first reaction in the coupled-enzyme analysis. The quantity of 14C fixed in 3-phosphoglycerate is counted.

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\text{RuBP} + ^{14}\text{CO}_2 + \text{H}_2\text{O} \xrightarrow{\text{RuBPC}} \text{D-3-PG} + \text{D-glycerate 3-P} \]

18 E. Racker, this series, Vol. 5, p. 266.
19 J. Pierce, S. D. McCurry, R. M. Mulligan, and N. E. Tolbert, this volume [91].

Solutions for the Coupled-Enzyme Assay

1. Tris-HCl buffer, 1.5 M, pH 8.0
2. Sodium bicarbonate, 0.5 M
3. Dithiothreitol (DTT), 0.5 M
4. Adenosine triphosphate, 0.1 M
5. Magnesium dichloride, 0.1 M

Triosephosphate isomerase–glycerol phosphate dehydrogenase (TIM–GDH), each 250 U/0.5 ml in 0.3 M (NH₄)₂SO₄

NADH·Na₂, 4 m/l (7 mg/2 ml of H₂O)

Glyceraldehyde-3-phosphate dehydrogenase–phosphoglycerate kinase (GAPDH–PGK), each 250 units/ml in 0.3 M (NH₄)₂SO₄

RuBP, 10 m/l; dissolve 58.3 mg of Ba₂RuBP in 10 ml of 0.1 N HCl

RuBP carboxylase (70 U/ml) in 0.1 M Bicine buffer, pH 8, containing 10 m/l NaHCO₃, 8 m/l DTT, and 10 m/l MgCl₂ (the buffer solution is deoxygenated with argon first), incubated at 30 for 30 min

The assay system is assembled from these solutions in a 3-ml cuvette: Tris (1.9 ml), NaHCO₃ (0.4 ml), DTT (30 µl), ATP (0.2 ml), MgCl₂ (0.2 ml), TIM–GDH (10 µl), GAPDH–PGK (20 µl), NADH (0.15 ml), and the RuBP sample (10 µl, obtained using a syringe).21 Before adding RuBPC, the solution is mixed and read at 340 nm until no further change in absorbance occurs (Fig. 1, E₁, ca. 5 min). After adding RuBPC (30 units), the decrease of absorbance at 340 nm should be complete within 10 min (E₂). The content of RuBP in the sample (% w/w) is determined from the

21 A 10 µl Hamilton syringe (-0.1 µl) was used instead of a pipette to achieve an accurate volumetric transfer: a 1-µl error in sampling results in a 12% relative error in the result.
difference of absorbance \((\Delta E = E_1 - E_2)\) and calculated according to the equation:

\[
G \text{Bar}_{2} \text{RuBP} = \frac{\Delta E}{4e} \times \frac{V_1}{V_2} \times \frac{V_2 \text{ mw} \times 1000}{1000 \text{ W}}
\]

where \(e = 6.22 \text{ mW cm}^{-1} \text{ cm}^{-1} \) (absorbance coefficient of NADH at 340 nm), \(V_1\) = total volume of the assay solution (2.95 ml), \(V_2\) = the volume of the RuBP sample solution taken (0.01 ml), \(V_3\) = the volume of the original RuBP solution (10 ml), MW = the molecular weight of Ba,\(_{2}\)RuBP (583.3), and \(W\) = the weight of Ba,\(_{2}\)RuBP sample in \(V_3\) (58.3 mg). Figure 1 is a typical curve obtained in this assay.

Radiometric Method. For the radiometric assay, aliquots from the RuBP stock solution are equilibrated in the assay buffer (0.1 M Bicine, pH 8.0) containing Na,\(_{14}\)CO\(_3\) (20 mV, 0.16 Ci/mmol), MgCl\(_2\) (10 mV), and Na,\(_{2}\)-EDTA (0.2 mV). The final concentration of RuBP, determined by the Lowry method,\(^\text{22}\) is 80 \(\mu\)g/ml (2 units/mg). The reaction is initiated by addition of RuBP (the total volume of solution is 7.5 ml) and allowed to run for 1 hr. It is then stopped by addition of acid, and the fixed \(^{14}\)C is counted as described elsewhere.\(^\text{20}\) The details of the procedure may be varied. The important feature is to keep the specific activity of the Na,\(_{14}\)CO\(_3\) constant during the assay and to measure the amount of substrate added accurately. Each sample is assayed in triplicate at the estimated concentrations of 0.2, 0.4, and 0.9 mV. If the substrate is reasonably pure, the concentration as determined by either of the above assay methods should agree very well with determination of the concentration by purely chemical methods, e.g., by the orcinol test\(^\text{23}\) and by organic phosphate determination.\(^\text{24}\)

6-Phosphoglucononate by \(\text{Br}_2\) Oxidation of Glucose-6-Phosphate. To a stirred suspension of Ba,\(_{2}\)Glu-6-P, 7 H\(_2\)O (120 g, 230 mmol) in H\(_2\)O, 912 ml, 24 ml of concentrated HCl are added. Sodium sulfate (34.5 g, 240 mmol) is added, and the resulting solution is stirred for about 5 min. The precipitate is removed by centrifugation, and the supernatant is adjusted to pH 5.4 with 4 N NaOH. Bromine (\(\text{Br}_2\), 30 ml) is added. The solution is stirred for 30 min at 25°, and the pH is controlled at 5.0–6.0. The excess \(\text{Br}_2\) is then removed by passing a stream of argon through the solution for 1 hr, and the resulting solution (containing ca. 200 mmol of 6-PG) is used without further purification.

6-Phosphoglucononate can also be prepared by enzymic oxidation of glucose-6-phosphate.\(^\text{11}\) This procedure is useful as a method for regenerat-

\(^{23}\) B. L. Hornecker, this series, Vol. 3, p. 105.
RuBP in 2 liters of solution using the same quantities of enzymes described above is complete in 20 hr and gives Ba\textsubscript{2}RuBP in 92\% purity (Ba\textsubscript{2}XuBP, 0.2\%\(\)) and 80\% yield (80 mmol) based on 6-PG.

Preparation of Ribose 5-Phosphate from AMP. The R-5-P is easily prepared by acid-catalyzed hydrolysis of AMP\textsuperscript{25} following a slight modified literature procedure to facilitate large-scale preparation. To a suspension of 1 kg of Dowex 50W (H\textsuperscript{+} form, 20–50 mesh), prewashed with 0.2 N HCl (two 2-liter portions) and H\textsubscript{2}O (three 2-liter portions), in H\textsubscript{2}O (with total volume of 2 liters) is added AMP-Na salt (100 g, 288 mmol). The pH of the suspension is 1.5–2.0. The suspension is then brought to a boil on a hot plate with stirring and heating is continued 30 min. The suspension is boiled for 5 min and then cooled immediately by pouring it into 300 g of ice. The mixture is filtered to separate the resin. Enzymic assay\textsuperscript{15} indicates that the filtrate (1.2 liters) contains 250 mmol of R-5-P. The solution is stable at 4 °C for periods of months. When longer stability was required, R-5-P was isolated in the form of the barium salt. The above solution is mixed with BaCl\textsubscript{2} (56.2 g, 270 mmol) followed by addition of ethanol (1.2 liters) and cooling. The precipitated material (78 g) contains 94\% by weight Ba\textsubscript{2} R-5-P (200 mmol).

Preparation of Ribulose 1,5-Bisphosphate from Ribose 5-Phosphate. A representative conversion of R-5-P to RuBP involved stirring R-5-P (0.2 mol, generated from its barium salt by adding 73.1 g (0.2 mol) of BaR-5-P to 1010 ml of 0.2 M H\textsubscript{2}SO\textsubscript{4} with vigorous stirring for 30 min and centrifuging to remove the precipitate) under argon for 40 hr at pH 7.8 in a 3-liter reaction mixture containing PRI (100 units, 2 ml), PruK (800 units, 160 ml), Ack (800 units, 5 ml), each immobilized separately in PAN gel, MgCl\textsubscript{2} (30 mmol), DTT (10 mmol), and ATP (1 mmol). Solid AcP(NH\textsubscript{4})\textsubscript{2} (0.25 mol) was added in 10 equal portions over this period. Isolation of Ba\textsubscript{2}RuBP followed the procedure outlined above: 94 g of solid was obtained, containing 72\% Ba\textsubscript{2} RuBP (116 mmol, 58\% yield based on R-5-P), and 0.34\% Ba\textsubscript{2} XuBP. The recovered enzyme activities were: PRI, 94\%; PruK, 92\%; Ack, 92\%. For further purification, 45 g of this material in 800 ml of H\textsubscript{2}O was mixed with 300 g of Dowex 50W (H\textsuperscript{+} form, 200–400 mesh) and stirred for 30 min at 25 °C, filtered, and washed with 100 ml of H\textsubscript{2}O. The resulting solution was passed through Dowex 1 (800 g, 200–400 mesh, Cl\textsuperscript{−} form) supported in a 2-liter filter, and washed with 5 liters of 40 m\textsuperscript{−} aqueous HCl to remove Ru-5-P and other impurities. RuBP was then desorbed by washing the resin with 2 liters of 0.15 M HCl, 0.1 M NaCl, and precipitated as described previously with 160 mmol of BaCl\textsubscript{2}. The product (32 g) contained 94\% Ba\textsubscript{2} RuBP and 0.16\% Ba\textsubscript{2} XuBP by weight.

Determination of the Degree of Inhibition of Ribulosebisphosphate Carboxylase by Inhibitors Present in the Ribulose 1,5-Bisphosphate Using the Radiometric Assay Method. RuBP is a very unstable compound, particularly in basic solution, and the decomposition products, especially XuBP, are inhibitors of RuBPC.\textsuperscript{26} The binding of XuBP to RuBPC is slow. Because the binding is slow, the presence of small amounts of this potent inhibitor does not decrease the initial rate in an enzyme assay. In order to determine whether XuBP or other similar inhibitors were present, we incubated RuBPC with a solution of RuBP (600 μg of RuBPC and 2.25 μmol of RuBP in 7.5 ml of the solution described previously for the radiometric assay). Aliquots were taken periodically (approximately every 0.25–2 min), and the quantity of labeled phosphoglyceric acid was measured. When the reaction was complete (10 min), an additional 2.25 μmol of RuBP were added and the reaction again was followed. The difference between these rates measures the degree of inhibition of the second reaction by impurities present in the first: the rates will be the same in the two runs if there are no inhibitors present. A sample of RuBP that had been purified only to the extent that it had been precipitated as BaRuBP after the synthesis gave an inhibition in the second stage of 64\%. If this inhibition were caused entirely by XuBP, it would represent a contamination in the range of 0.1%. Treatment of the RuBP sample by passage through a Dowex 1 (Cl\textsuperscript{−} column) to separate mono- and diphosphate resulted in an inhibition of 50\%. The small amount of XuBP suggested by this assay can be removed by the aldolase–glyceraldehyde 3-phosphate dehydrogenase purification described below. It is very difficult, if not impossible, to prevent the re-formation of XuBP. While the rate of epimerization of RuBP to XuBP during the purification under acidic conditions is not known, it is clear that under conditions used for the RuBPC assay (pH 8.0, 30 °C), RuBP undergoes β-elimination and rearrangement at the rate of 1.25% per hour.\textsuperscript{4}

Purification of Ribulose 1,5-Bisphosphate. The level of contamination by XuBP present in these preparations would be unacceptable in many experiments. Two procedures are used for further purification.

In one, the sample (1 g, 72\% purity with 0.4\% XuBP) is treated with Dowex 50 (10 g, H\textsuperscript{+} form, 200–400 mesh), sonicated, filtered to remove resin, and purified on a Dowex 1 (200–400 mesh) column (1.5 × 20 cm) run with 30 m\textsuperscript{−} HCl (100 ml), followed by HCl gradient formed by introducing 1 liter of 0.15 M HCl through a mixing chamber containing 200 ml of 30 m\textsuperscript{−} HCl. Fractions of 20 ml are collected, and pentolose phosphates are detected using the orcinol test\textsuperscript{24} or by enzymic assay for RuBP. The cluted RuBP (fractions 15–25) is isolated as the barium salt (0.6 g). It contains 96\% Ba\textsubscript{2} RuBP and 0.02\% Ba\textsubscript{2} XuBP.

\textsuperscript{25} J. R. Sokatch, Biochem. Prep. 12, 2 (1968).

\textsuperscript{26} S. D. McCurry and N. E. Tolbert, J. Biol. Chem. 252, 8344 (1977).
A second purification is based on the selective enzymic destruction of XuBP aldolase cleaves XuBP 20-30 times more rapidly than RuBP (see Scheme 3). Incubation of a sample of RuBP contaminated with XuBP for a sufficiently long time (~10-20 min) effectively guarantees that all XuBP is digested by aldolase, provided that this reaction is coupled with glycerol-3-phosphate dehydrogenase (GDH) to convert the cleavage product dihydroxyacetone phosphate to glycerol phosphate. An NADH regeneration system based on coupled alcohol and aldehyde dehydrogenase (ADH--AldDH) is used for the GDH-catalyzed reaction. A sample of Ba$_2$RuBP (1 g, 72% purity, 0.4% XuBP) is suspended in 30 ml of H$_2$O at 4°C and treated with 10 g of Dowex 50 as described above. The solution is adjusted to pH 7.0 by slow addition of 1 N KOH at 0°C. To the solution is added 50 units of fructose-1,6-diphosphate aldolase (FDPA), glycerol-3-phosphate dehydrogenase (GDH), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (AldDH), followed by NAD (15 mg) and ethanol (0.1 ml). The mixture is stirred at 25°C for 10 min, then acidified to pH 3.0. The RuBP is purified by anion-exchange chromatography as described previously and isolated as Ba$_2$RuBP. The resulting material (0.5 g, 94% purity) shows no (~0.1%) detectable contamination by XuBP.

Determination of XuLactose 1,5-Bisphosphate (XuBP). The same principle as described above is followed to determine the content of XuBP in a sample of RuBP. In the absence of ADH--AldDH for NADH regeneration, the decrease in the absorbance at 340 nm is measured over a period of 10 min. and the change of absorbance is used to calculate the content of XuBP. Ba$_2$RuBP (20 mg) is dissolved in 0.1 N HCl (0.3 ml). Sodium sulfate (0.2 ml of 0.2 M Na$_2$SO$_4$) is added to precipitate the barium ion as barium sulfate. The supernatant, after centrifugation, is added to 2.5 ml of triethanolamine buffer (0.2 M, pH 7.6) containing 0.2 mM of NADH. Glycerol phosphate dehydrogenase (5 units) and fructose-1,6-diphosphate aldolase (5 units) are added to the mixture. The decrease of absorbance at 340 nm ($\Delta$E) is recorded over a 10-min interval, and the content of XuBP is calculated according to the equation

$$\% \text{Ba}_2\text{XuBP} = \frac{\Delta E}{6.22} \times V \times \frac{\text{MW}}{W} \times 100$$

where V = total volume of assay solution in milliliters, MW = molecular weight of Ba$_2$XuBP (583.3), and W = weight of Ba$_2$RuBP sample in micrograms (ca. 20,000 µg). This method would detect 0.01% (0.005 µmol) of Ba$_2$XuBP in a 20-µg sample based on $\Delta$E = 0.01.

[19] Isomers of $\alpha$-D-Apiofuranosyl 1-Phosphate and $\alpha$-D-Apiose 1,2-Cyclic Phosphate

By J. Mendicino, Raga Hanna, and E. V. Chandrasekaran

The study of the biosynthetic reactions involving intermediates of $\alpha$-apiose has been impeded by the lack of chemically pure phosphate esters of this branched-chain sugar. A more careful characterization of the small amounts of $\alpha$-apiose derivatives formed in tissue extracts is possible if compounds of known structure and purity are available. Four monophosphate esters are formed when $\beta$-$\alpha$-apiose tetraacetate is treated with anhydrous phosphoric acid. Two isomers of $\alpha$-apiose, $\alpha$-$\text{apio-1-furanosyl}$ 1-phosphate and $\alpha$-$\text{apio-1-furanosyl}$ 1-phosphate, are obtained in the highest yield. Almost none of the corresponding $\beta$-$\text{apio-1-furanosyl}$ 1-phosphate and $\beta$-$\text{apio-1-furanosyl}$ 1-phosphate is found. Several cyclic phosphate esters of $\alpha$-apiose are also formed when $\beta$-$\text{apio-1-furanosyl}$ 1-phosphate is treated with crystalline phosphoric acid. The $\alpha$-$\text{1,2-cyclic}$ $\alpha$- and $\beta$-furanosylapiose phosphodiesters are formed from the corresponding $\alpha$-$\text{apio-1-furanosyl}$ 1-phosphate esters when they are treated with alkali to remove the 0-acetyl groups as shown in the following reactions.

$$\alpha$-Apiose $\xrightarrow{\text{pyridine}} \alpha$-Apiose tetraacetate

$$\beta$-Apiose tetraacetate $\xrightarrow{\text{crystalline}}$ mixture of $\alpha$-apiose 1-phosphate esters

Mixture of D-ribose 1-phosphate esters 1,2,4
D-ribose-5-phosphate, 1,3-D-ribose-5-phosphate, 1,5-D-ribose-3-phosphate.

One of the cyclic phosphodiester derivatives has properties that are identical to those of a compound formed during the enzymatic conversion of UDP-D-glucuronic acid to D-ribose derivatives in extracts of parsley and L. minor. 2 This compound arises from the breakdown of UDP-D-ribose, since hydrolysis of some nucleoside diphosphate sugars under alkaline conditions yields cyclic 1,2-phosphodiester derivatives of the sugar moiety. 2 The cyclic 1,2-phosphodiester formed by the migration of the phosphate group of D-ribose-5-phosphate 1-phosphate is identical to the D-ribose cyclic phosphate intermediate formed from UDP-D-glucuronic acid in tissue extracts. 3

Reagents

Anhydrous pyridine dried by distillation over calcium hydride
Acetic anhydride
Dry tetrahydrofuran
Crystalline phosphoric acid dried and stored over magnesium perchlorate
LiOH
LiCl
K₂B₄O₇

Synthesis of D-ribose Tetraacetate

The disosympolidentine derivative 2 is prepared from D-ribose. A thoroughly dried syrup of D-ribose is then prepared from crystalline di-o-isopropylindeneapiose 1,5 3, 58° (ethanol). The syrup (10 g) is dissolved in a mixture of 67 g of dry pyridine and 50 g of acetic anhydride, which has been previously cooled to 0°. After 3 days at 3° the reaction mixture is poured into 100 ml of ice water. The solution is extracted five times with 50-ml portions of dichloromethane, and the combined extracts are neutralized by shaking them with water and solid NaHCO₃ until effervescence ceases. The chloroform extract is washed with water and dried over anhydrous MgSO₄. The solvent is removed, and the dry syrup is stored under vacuum over magnesium perchlorate. The yield of product is 72° based on D-ribose tetraacetate, and the sample has 6° 18.4. A weighed sample is assayed by the Somogyi-Nelson procedure. It contains

46° by weight of D-ribose, which corresponds to the value expected for D-ribose tetraacetate.

Synthesis of the 1-Phosphate Esters of D-Ribose

Dry crystalline phosphoric acid is dissolved in tetrahydrofuran, and the D-ribose tetraacetate is dissolved in chloroform or tetrahydrofuran. The two solutions are mixed, and the solvent is removed under reduced pressure. This procedure ensures complete mixing of very small amounts of the two compounds in the resulting melt, and there is no reaction until the solvent is removed. In this manner, thoroughly dried D-ribose tetraacetate (10 mmol) is treated with 70 mmol of crystalline phosphoric acid in an evacuated reaction flask for 3 hr at 50°. The resulting dark syrup is dissolved in 50 ml of dry tetrahydrofuran, and it is then poured into 50 ml of ice-cold 1 N LiOH. The suspension is adjusted to pH 10.0 with LiOH, and it is kept at room temperature for 12 hr. Insoluble lithium phosphate is removed by filtration, and the precipitate is washed with 200 ml of 0.01 N LiOH. The filtrate and washes are combined and passed through a Dowex 50-NH₄ column (5 x 7 cm), and the column is washed with water until the eluate is free of acid-labile phosphate. The solution is then concentrated to 25 ml under reduced pressure. The yield of D-ribose-1-P, determined by analysis of acid-labile phosphate, is 30%.

The mixture of D-ribose 1-phosphate esters, approximately 2500 µmol, is applied to a Dowex 1 column (4.5 x 8 cm) in the bicarbonate form, and the column is washed with 10 liters of 10 m M K₂B₄O₇, 2.5 m M NH₄OH. The phosphate esters are then eluted successively with solutions containing increasing concentrations of LiCl and decreasing concentrations of borate ion. Four peaks are eluted from the Dowex 1 column. Fraction I is eluted with 10 liters of 1 m M K₂B₄O₇, 2.5 m M NH₄OH. Fraction II is eluted with 5 liters of 0.1 m M K₂B₄O₇, 2.5 m M NH₄OH. Then fraction III is eluted with 10 liters of the same solution. Finally, fraction IV is eluted with 10 liters of 0.5 m M LiCl. The four peaks are collected and concentrated by evaporation under reduced pressure to 25 ml. Precipitated salt is removed by filtration. The samples of each fraction are then applied to Whatman 3 MM papers. Repeated development with a solvent containing 20% ethanol and 80% acetone causes LiCl and other salts to move off the paper, whereas the phosphate esters remain near the origin of the chromatogram. After chromatography, two thin strips one-third of the distance in from the sides of the paper are cut out and developed with the FeCl₃-sulfosalicylic acid reagent 5 to reveal phosphate-containing areas. The developed strips are

TABLE I
Properties of Isomers of d-Apino-1-Phosphate Isolated by Ion-Exchange Chromatography

<table>
<thead>
<tr>
<th>Property</th>
<th>Fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (μmol)</td>
<td></td>
<td>190</td>
<td>80</td>
<td>1250</td>
<td>900</td>
</tr>
<tr>
<td>Assay (mmole of d-apino)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reducing sugar after acid hydrolysis</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fructose-H₂SO₄ test as d-apino</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid-labile phosphate</td>
<td></td>
<td>1.06</td>
<td>1.03</td>
<td>0.97</td>
<td>0.99</td>
</tr>
<tr>
<td>Total phosphate</td>
<td></td>
<td>1.05</td>
<td>1.08</td>
<td>1.06</td>
<td>1.03</td>
</tr>
<tr>
<td>Hydrolysis rate constants (km⁻¹) at 26°C</td>
<td></td>
<td>0.010</td>
<td>0.007</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>H₂SO₄, 0.5 N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂SO₄, 0.25 N</td>
<td></td>
<td>0.004</td>
<td>0.003</td>
<td>0.035</td>
<td>0.083</td>
</tr>
</tbody>
</table>

used as guides to cut out the appropriate areas of the untreated portions of the chromatogram. The phosphate esters are eluted from the paper with water, and the solutions are adjusted to pH 7.0 with NaOH and concentrated to 15 ml under reduced pressure. The yields and properties of each peak are shown in Table I.

The reaction mixture for the hydrolysis of the phosphate esters contains, in 5 ml, 0.5 N H₂SO₄ and 0.25% ammonium molybdate or 0.25 N H₂SO₄ and 0.125% ammonium molybdate, 0.1 ml of reducer, and 0.5 μmol of sugar phosphate. The reaction is carried out at 26°C. The rate constants are calculated from slopes of the straight lines obtained by plotting the logarithm of ester concentration against time and applying the formula \( K = 2.3 \log (\text{ester}_{t_1} / \text{ester}_{t_2}) \).

Most of the d-apino that is phosphorylated is recovered in fractions III and IV (2150 μmol compared to 2500 μmol applied to the column). Only about 10% of the total acid-labile sugar is present in fractions I and II.

The barium and cyclohexylammonium salts of the monophosphate esters in fractions III and IV are prepared from the lithium salt in greater than 95% yield. The compounds in fractions I and II, which do not form insoluble barium salts, may be further purified by paper chromatography in solvent systems A and D shown in Table II.

Taken collectively, the analytical and chromatographic data show that the isolated products are isomeric monophosphate esters of d-apino. The purified fractions from each peak contain no free reducing sugar or P₄, but during acid hydrolysis equivalent amounts of d-apino and P₄ are liberated. Support for the chemical identity of the synthesized phosphate esters is indicated by the stoichiometry between the total phosphate, acid-labile phosphate, and reducing sugar present in each compound. The isolated sugar phosphates have the same molar extinction coefficient and spectrum as d-apino in the fructose-H₂SO₄ test. On paper chromatography in three different solvent systems, the sugar released from each fraction after treatment with 0.1 N HCl at 100° for 15 min was recovered virtually quantitatively in a single well-defined region, with an Rf in each case identical with that of d-apino. Although they did not form furfuraldehyde when treated with strong acid, d-apino and the sugars formed by hydrolysis of the sugar phosphate esters reacted with the benzidine-chloroanisyl acid reagent on paper chromatograms to give a yellow spot with a white fluorescence under ultraviolet light. They gave a yellow-brown spot when the chromatograms were sprayed with p-anisidine hydrochloride. It is advised that correct protective procedures be used when spraying paper chromatograms with benzidine reagent because of its carcinogenicity.