THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 237, No. 3, March 1962
Printed in U.S.A.

A Simple Method for the Preparation of D-Ribulose 5-Phosphate*

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(Received for publication, October 19, 1961)

Since the discovery of D-ribulose 5-phosphate as the product of the oxidation of D-gluconate 6-phosphate (1), this compound has been found to be an intermediate in a number of enzymatic pathways in pentose metabolism in animal tissues (2), microorganisms (3), and plants (4).

It became of interest, therefore, to develop a procedure for the preparation of this substrate, especially in view of the fact that the methods presently available are cumbersome and yield products that are contaminated with variable amount of aldopentose phosphate, usually D-ribose 5-phosphate. In this communication, we describe a simple procedure for the preparation of D-ribulose 5-phosphate by the enzymatic oxidation of D-gluconate 6-phosphate with pyruvate and catalytic amounts of triphosphopyridine nucleotide, essentially as described by Horecker and Smyrniotis (1). Chromatography was unnecessary since no D-ribose 5-phosphate was formed. D-Ribulose 5-phosphate was isolated as the barium and the lithium salt. These products have been characterized both chemically and enzymatically and shown to be completely free of other esters.

EXPERIMENTAL PROCEDURE

Materials—Crystalline D-gluconate 6-phosphate dehydrogenase was prepared as previously described (5). D-Ribose 5-phosphate isomerase was prepared according to Hurwitz *et al.* (6). Crystalline D-lactic dehydrogenase and sodium pyruvate were purchased from Boehringer and Soehne, Germany. TPN, DPNH, and D-gluconate 6-phosphate (sodium salt) were obtained from the Sigma Chemical Company. Both D-gluconate 6-phosphate dehydrogenase and D-lactic dehydrogenase were centrifuged and dissolved in water, before use, to avoid the introduction of excessive amounts of ammonium sulfate. Activated charcoal was purchased from Ditta Faravelli, Milano, Italy. It was prepared by boiling for 10 minutes in 1.0 N HCl, filtered with suction, and washed with deionized water until the washings were neutral. The washed charcoal was dried in air.

Determinations—Pentose was measured by the method of Mejbaum (7), with D-ribose as a standard and a 40-minute heating period. For ketopentose phosphate, a correction was employed, since this yields less color than aldopentose. We used the value reported by Dickens and Williamson (8), who found that pentulose 5-phosphate yields only 70% as much absorption as an equivalent amount of aldopentose. The values obtained by this assay were in agreement with those obtained by the Dische and Borenfreund cysteine-carbazole reaction (9), as modified by Ashwell and Hickman (10). For enzymatic assay of D-ribulose 5-phosphate, we measured the disappearance of ketopentose phosphate by the cysteine-carbazole test, after incubation with D-ribose 5-phosphate isomerase. D-Gluconate 6-phosphate was determined with D-gluconate 6-phosphate dehydrogenase. Pyruvate was determined with DPNH and lactic dehydrogenase. Phosphate was determined by the method of Fiske and SubbaRow (11).

* Supported by a grant from the United States Public Health Service.

RESULTS

Preparation of Barium and Lithium Salts—For the preparation of D-ribulose 5-phosphate from D-gluconate 6-phosphate, the reaction mixture (8.0 ml) contained: sodium D-gluconate 6-phosphate, 240 μmoles; sodium pyruvate, 460 μmoles; TPN, 20 μmoles; D-lactic dehydrogenase, 1.2 mg; glycylglycine buffer, pH 7.6, 300 μmoles; and crystalline D-gluconate 6-phosphate dehydrogenase (specific activity, 190 units per mg of protein), 0.6 mg. The reaction was begun by the addition of the enzyme at 30°. Aliquots were assayed at intervals for D-gluconate 6-phosphate. After 45 minutes, when less then 1% remained, the reaction mixture containing 250 μmoles of pentose phosphate (including TPN) was cooled and treated with 0.78 ml of 45% trichloroacetic acid and 100 mg of activated charcoal (acid-washed), shaken, and left for 10 minutes in ice. The suspension was filtered through a sintered glass filter, and the residue was washed with 2.5 ml of water. To the combined filtrate and washing (11 ml), 1 ml of 1 M barium acetate was added. After 20 minutes in ice, the turbid suspension was centrifuged, the precipitate was washed with 1 ml of water, and the supernatant solution and washing combined (12.2 ml). This solution was treated with 3.6 ml of saturated $Ba(OH)_2$ adjusted to pH 6.6 with saturated KOH, and precipitated with 4 volumes of cold absolute ethanol. After 30 minutes, the precipitate was collected by centrifugation, washed with 80% ethanol, and dried under reduced pressure. The yield of dried barium salt was 104.5 mg (first barium salt precipitate). An aliquot of the Ba salt (55 mg) was dissolved in 5.0 ml of 0.1 M CH_3COOH, and the solution was adjusted to pH 6.6 with 0.01 ml of saturated KOH and 0.5 ml of saturated $(BaOH)_2$, and precipitated with 4 volumes of cold absolute ethanol. After 45 minutes, the precipitate was collected by centrifugation, washed with 80% ethanol, and dried under reduced pressure. The yield of dried barium salt was 44 mg (second barium salt precipitate).

To obtain the lithium salt, 49.5 mg of the first barium salt precipitate were dissolved in 4 ml of 0.1 M CH_3COOH, an aliquot (0.5 ml) was removed for analyses, and the remaining solution was passed through a Dowex 50-H^+ column to remove Ba^{++}. The effluent and washings (4.1 ml) were combined and brought to

TABLE I

Pentose and phosphate analyses of barium and lithium salts

Sample	Dry weight	Total pentose phosphate (orcinol)	Total ketopentose phosphate (cysteine-carbazole)	Inorganic phosphate	Total organic phosphate
	mg	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Ba salt, first precipitate..	104.5	212	211	0	212
Ba salt, second precipitate.....	44	86.2	85.5	0	86
Li salt.....	19	64	62	0	63

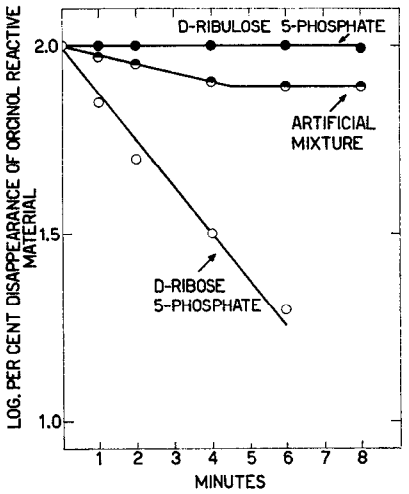

FIG. 1. Bromine oxidation of pentose phosphates. The reaction vessels (8 ml) contained 1.8×10^{-4} M D-ribulose 5-phosphate alone or 1.8×10^{-4} M D-ribose 5-phosphate alone or a mixture of 1.53×10^{-4} M D-ribulose 5-phosphate plus 0.27×10^{-4} M D-ribose 5-phosphate; 0.2 M phosphate buffer, pH 6, and 0.125 volume per volume of bromine. Samples of 0.5 ml were collected at different time intervals and analyzed for total pentose with the orcinol reaction.

pH 6.3 with 4.5 ml of 0.1 N LiOH. The lithium salt was precipitated, at 0°, with 100 ml of 10% methanol in acetone. After 30 minutes, the precipitate was collected by centrifugation, washed with 10 ml of the acetone-methanol mixture, and dried in a vacuum. The yield of dried lithium salt was 19 mg.

Characterization of Product—The barium and the lithium salts together accounted for 150 μmoles of pentose phosphate. This represents a yield of 80% of the phosphogluconate added after suitable correction for the aliquots removed for assay (Table I). In each case, the preparations were completely free of inorganic phosphate, and the ratio of pentose phosphate (orcinol) to pentose phosphate (cysteine-carbazole) to total organic phosphorus was 1.0:0.99:1.0 for the barium salt, and 1.0:0.97:0.98 for the lithium salt. All the phosphate is therefore present as ketopentose phosphate. Further evidence for the absence of aldopentose was obtained by oxidation with bromine. As shown in Fig. 1, D-ribose 5-phosphate is oxidized by bromine at 28° and pH 6.0 with a half-life of $2\frac{1}{2}$ minutes. In 6 minutes, 80% of the aldopentose has been oxidized. During this time, there was no appreciable disappearance of pentose phosphate in the D-ribulose 5-phosphate sample. With a mixture of 15% of D-ribose 5-phosphate and 85% of D-ribulose 5-phosphate, the analysis showed exactly 15% of the pentose to be oxidizable by bromine. Although this procedure is not highly sensitive, it would certainly have detected quantities of aldopentose approaching 5 to 10%.

The ultimate characterization of a phosphate ester depends on its enzymatic activity. Our product was found to be fully active with D-ribose 5-phosphate isomerase (Table II). At equilibrium the reaction mixture contained 30% of ketopentose phosphate and 70% of aldopentose phosphate, in agreement with the equilibrium values reported by Hurwitz *et al.* (6) and Axelrod and Jang (12). Since this enzyme does not catalyze the isomerization of D-xylulose 5-phosphate, this substance cannot have been present in significant amount.

The D-ribulose 5-phosphate preparation was also tested qualitatively for its ability to react with TPNH in the presence of CO_2 and D-gluconate 6-phosphate dehydrogenase. As shown in Fig. 2 in the presence of D-ribulose 5-phosphate and bicarbonate-CO_2 buffer, TPNH was rapidly oxidized until an equilibrium level was reached. If at this point the concentration of

TABLE II

Isomerization of D-ribulose 5-phosphate and D-ribose 5-phosphate in presence of D-ribose 5-phosphate isomerase

The reaction mixture (1.0 ml) contained: 1×10^{-3} M D-ribulose 5-phosphate (Ba or Li salt) or D-ribose 5-phosphate; 4×10^{-2} M Tris buffer, pH 7.1; and 6 μg of D-ribose 5-phosphate isomerase (specific activity, 56 units per mg of protein). The reaction was started by the addition of the enzyme. The temperature was 37°. After 5 and 10 minutes, samples of 0.1 ml were taken and analyzed with the cysteine-carbazole reaction.

Time	Starting substrate		
	D-Ribulose 5-phosphate		D-Ribose 5-phosphate
	Ba salt	Li salt	
min	*μmoles*		*μmoles*
0	0.97	0.98	0.0
5	0.30	0.27	0.29
10	0.29	0.28	0.28

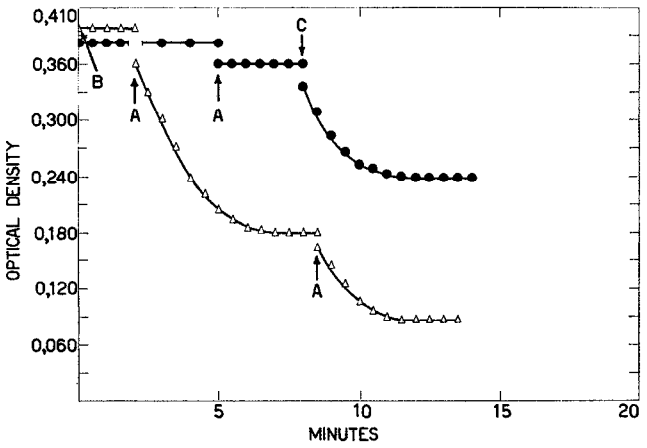

FIG. 2. Oxidation of TPNH with D-ribulose 5-phosphate and CO_2. The reaction mixture (1.0 ml) contained 6×10^{-5} M TPNH; 0.083 M glycylglycine buffer, pH 7.6, and 52 μg of D-gluconate 6-phosphate dehydrogenase (specific activity, 190 units per mg of protein). At $B(\triangle)$ and $C(\bullet)$, we added 1 μmole and 0.3 μmole of D-ribulose 5-phosphate, respectively, and at A, we added 100 μmoles of $NaHCO_3$ saturated with CO_2. The temperature was 30°.

bicarbonate was increased 2-fold, there was further oxidation of TPNH nearly to completion. No TPNH oxidation occurred in addition of bicarbonate CO_2 buffer, in the absence of D-ribulose 5-phosphate, nor with D-ribulose 5-phosphate alone.

Stability of Barium and Lithium Salts—The procedures described above, including bromine oxidation and reaction with D-ribose 5-phosphate isomerase, have all been repeated with the barium and lithium salts after storage for 1 month at 0–2° in a vacuum desiccator. There was no significant change in the analytical data, indicating that the products are relatively stable under these conditions. The lithium salt is a white nonhygroscopic powder, and readily soluble, thus providing a convenient form of the ester since decomposition of the salt is unnecessary.

DISCUSSION

Several procedures have been reported for the preparation of D-ribulose 5-phosphate. These are based on the enzymatic oxidation of D-gluconate 6-phosphate to D-ribulose 5-phosphate (1), the isomerization of D-ribose 5-phosphate by treatment with D-ribose 5-phosphate isomerase (13), or on the phosphorylation of D-ribulose with ATP and ribulokinase (14). All of these procedures have required separation of the phosphorylated sugar by ion exchange chromatography, owing to the presence of other phosphate esters or inorganic phosphate in the reaction mixture.

The product has been obtained in low yield and purity and contaminated with other esters.

The present method requires only two readily available enzymes, D-lactic dehydrogenase and D-gluconate 6-phosphate dehydrogenase, and yields only D-ribulose 5-phosphate since the latter enzyme is free of D-ribose 5-phosphate isomerase. The reaction goes to completion and the product is readily separated from the reaction mixture by precipitation with barium after removal of TPN with charcoal. The yield approaches 80% of theoretical, and chromatography is unnecessary. The product has been shown to be free of detectable quantities of aldopentose phosphate and to contain ketopentose equivalent to organic phosphorus. All of the ketopentose appears to be D-ribulose 5-phosphate on the basis of its reaction with D-ribose 5-phosphate isomerase. The presence of D-xylulose 5-phosphate is further excluded by the results of the cysteine-carbazole test, since, under the conditions we have used, this ester yields only one-third as much color as does D-ribulose 5-phosphate. Both the lithium and the barium salts seem to be relatively stable.

SUMMARY

With the availability of crystalline D-gluconate 6-phosphate dehydrogenase, free of D-ribose 5-phosphate isomerase and D-xylulose 5-phosphate 3-epimerase, it has become possible to prepare D-ribulose 5-phosphate free of D-ribose 5-phosphate and D-xylulose 5-phosphate. The product is isolated directly by precipitation of the barium and lithium salts, both of which are relatively stable. The over-all yield based on D-gluconate 6-phosphate is about 80%. The product has been identified by chemical and enzymatic methods.

Acknowledgment—We thank Professor B. L. Horecker for his kind assistance and valuable discussions.

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