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Note

Preparative enzymic synthesis and isolation of D-*threo*-2-pentulose 5-phosphate (D-xylulose 5-phosphate)

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D-*threo*-2-Pentulose 5-phosphate (D-xylulose 5-phosphate, **5**) is the product of D-*erythro*-2-pentulose 5-phosphate (D-ribulose 5-phosphate, **6**) conversion by D-ribulose 5-phosphate 3-epimerase[1] and of the transketolase reaction[2] starting from D-*altro*-2-heptulose 7-phosphate (D-sedoheptulose 7-phosphate) and D-glyceraldehyde 3-phosphate (**3**). However, these reactions are not applicable for the synthesis of pure preparations of **5**, since this product cannot be easily separated from a mixture of phosphorylated compounds, and particularly other pentose phosphates. Alternatively, D-*threo*-2-pentulose can be directly converted by a specific kinase into the corresponding phosphate ester. Unfortunately, the enzyme is not commercially available and the reported isolation procedure[3] provides only a partially purified product, unsuitable for synthetic purposes.

The first reliable method for the synthesis of **5** was described by Srere *et al.*[4], who used hydroxypyruvic acid (**4**) as a donor for the transketolase reaction and D-*arabino*-2-hexulose 1,6-bisphosphate (D-fructose 1,6-bisphosphate, **1**) as a source of **3**, in the presence of aldolase. However, conditions reported for that procedure gave a very low recovery of **5**. A higher yield of **5** was obtained by Wood[5], who synthesized the pentose phosphate directly from DL-glyceraldehyde 3-phosphate, but the final product was partially contaminated by **6** and contained large proportions of unreacted L-glyceraldehyde 3-phosphate. Compound **5** is also commercially available, but preparations are very expensive and the purity does not exceed 70–85%.

To overcome these inconveniences and because of the need of relatively large amounts of pure substrate suitable for metabolic studies, we have developed a conveniently fast and inexpensive method for the preparation of **5**, and describe herein the optimal conditions for synthesis and isolation and a detailed analysis of the contaminants in the final product.

Compound **5** was obtained by a combination of two enzymic reactions. The first step (*1*) consists in the cleavage of **1** by aldolase to give equimolar concentra-

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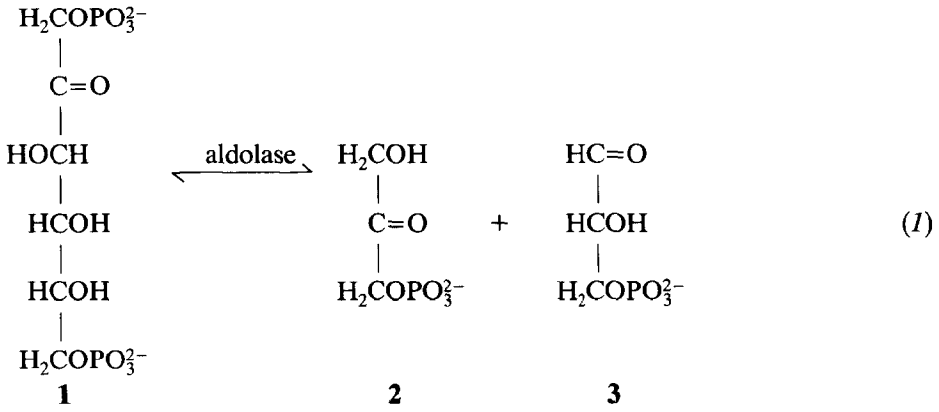
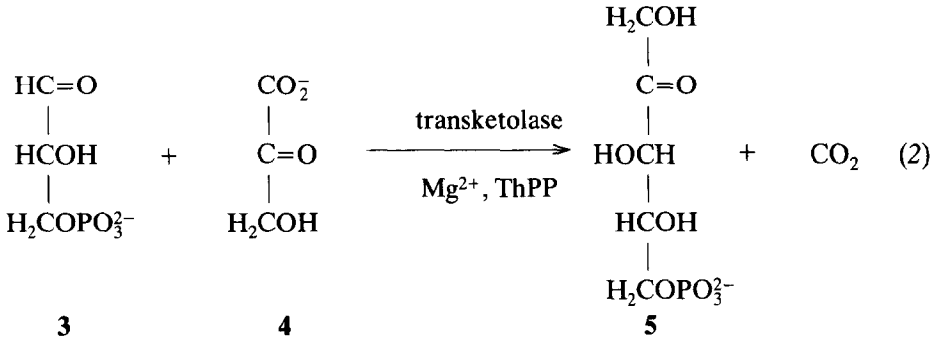
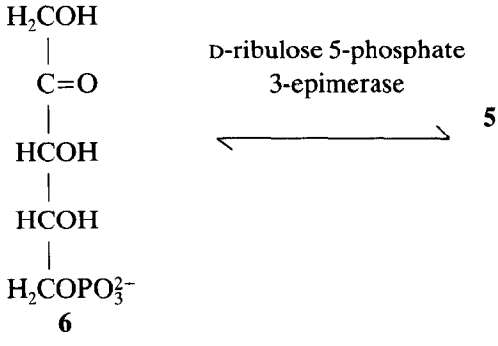
tions of dihydroxyacetone phosphate (**2**) and **3**. The latter intermediate is then condensed (*2*) with the active glycolaldehyde from compound **4** in the presence of yeast transketolase, which requires thiamine diphosphate and Mg^{2+} ions for activity[6]. According to the stoichiometry of the reactions, 1 μmol of **5** is expected from 1 μmol of **1**, as described in reactions *1* and *2*, and since CO_2 is liberated in the medium, overall equilibrium is irreversibly shifted to the right. Optimal rates of transketolase activity could be obtained by use of a five-fold excess of **4** over **1**.







Direct synthesis of **5** by the action of yeast transketolase on hydroxypyruvic acid and D-glyceraldehyde 3-phosphate was not applicable because of the cost and lability of pure triose phosphate preparations; therefore, alternative precursors were employed. Either D-*arabino*-2-hexulose 1,6-bisphosphate (**1**) or DL-glyceraldehyde 3-phosphate can be indifferently used for this purpose, yielding a product that needs to be further purified from **2** or L-glyceraldehyde 3-phosphate, respectively. In view of the chromatographic properties and of the biological importance of **2** over L-glyceraldehyde 3-phosphate, compound **1** was preferred as precursor in the present method. In this respect, we essentially followed the scheme of reactions described by Srere *et al.*[4], but the conditions for synthesis and isolation were modified as follows. The absolute amounts of aldolase and transketolase were greatly reduced in order to lower contaminating activities and to avoid the conversion of **5** into undesirable products. For the same reason, the reaction was not allowed to reach completion, and the incubation was stopped when ~80% of **1** had been transformed. Maximal initial rates of synthesis of **5** were obtained by a preincubation of **1** with aldolase to provide a sufficiently high level of **3**, and by raising the concentration of **4** up to 15mM. A five-fold excess of transketolase over aldolase (relative activity) in the final mixture was effective in driving the reaction towards the synthesis of **5** within a reasonable time.

As shown in the Experimental section, the synthesis was quite reproducible and the yields of final products agree well with the stoichiometry of the reactions.

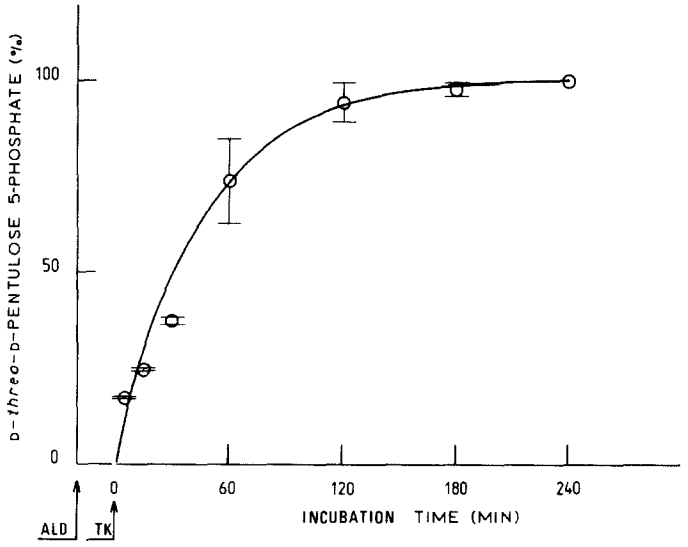


Fig. 1. Kinetics of the synthesis of D-*threo*-2-pentulose 5-phosphate (**5**). For substrate analysis, 50-μL aliquots were taken from the incubation mixture at 5, 15, 30, 60, 120, 180, and 240 min after transketolase addition and mixed with 50mM hydrochloric acid (50 μL) to terminate the reaction. For the determination of **5**, see legend to Table I. Values (○) are expressed as percentage of the maximum at 240 min, and reported as the average ± standard deviation of four separate incubations.

To separate **5** from other components of the incubation mixture, ion-exchange chromatography was found much more reliable than other methods, *e.g.*, barium–ethanol precipitation, and allowed a relatively fast isolation of both **5** and **2**. The final recovery of **5**, after chromatography and evaporation, accounted for ~60% of the initial amount of **1**, and the purity of the preparation reached 95–97%, as estimated by enzymic and organic phosphate analysis. Contamination by **6** did not usually exceed 2% relative to **5**. The kinetics of a typical preparation of **5** are shown in Fig. 1.

EXPERIMENTAL

Materials. — Baker's yeast transketolase (type X) (EC 2.2.1.1), D-ribulose 5-phosphate 3-epimerase (EC 5.1.3.1), D-ribose 5-phosphate isomerase (EC 5.3.1.6), thiamine diphosphate (ThPP), D-fructose 1,6-diphosphate (Na_3 salt), and Li β-hydroxypyruvate were obtained from Sigma Chemical Co. Rabbit muscle aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1) (5000 U/mg), glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) (170 U/mg), and NADH were purchased from Boehringer Mannheim Biochemicals, and AG 1-X8 anion- and AG 50W-X8 cation-exchange resins from Bio-Rad Laboratories. AG 1-X8 resin was converted into the formate form as indicated by the supplier.

Enzymic assays. — All the assays were performed in a Gilford spectrophotometer. D-*threo*-2-Pentulose 5-phosphate (**5**), D-*erythro*-2-pentulose 5-phosphate (**6**), D-ribose 5-phosphate, D-*arabino*-2-hexulose 1,6-bisphosphate (**1**), and triose phosphates were essentially assayed according to Racker[7–9], and Michal and Beutler[10]. The formation of **5** throughout incubation was determined by use of rat liver transketolase, prepared in our own laboratory[11]; the final product was assayed with yeast transketolase.

Acid hydrolysis and organic phosphate determination. — The purified product

TABLE I

CONVERSION OF D-*arabino*-2-HEXULOSE 1,6-BISPHOSPHATE (**1**) INTO **5** AND **2** BY ACTION OF ALDOLASE AND TRANSKETOLASE[a]

Time	*Compounds (μmol)*		
	1	**5**	**2**
Start of reaction	149	0	0
End of reaction observed	32	116.6	94.5
Theoretical		117.0	117.0

[a]The enzymic determination of compounds was performed on 25-μL samples, in the presence of 2mM D-ribose 5-phosphate and 1 μg of triose phosphate isomerase, by sequential addition of α-glycerophosphate dehydrogenase, rat liver transketolase (s.a., 1.5–2 U/mg, see Experimental section) and aldolase, which would determine compounds **2**, **5**, and **1**, respectively (see Experimental section). The values are the average of the assays on two separate samples.

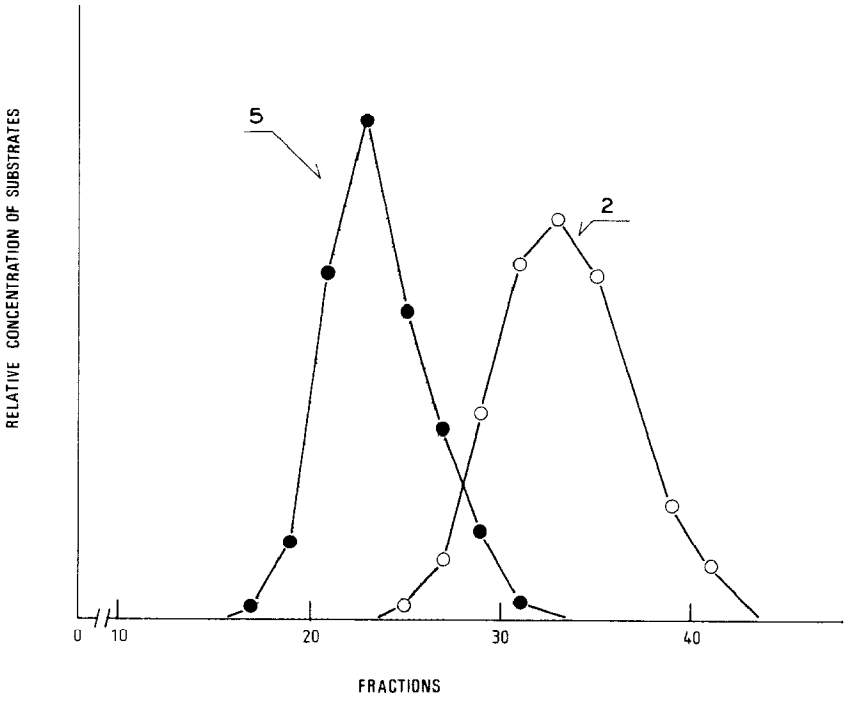


Fig. 2. Ion-exchange chromatography on AG 1-X8 anion-exchange resin. Elution was performed at a flow rate of 2 mL/min and 4.5-mL fractions were collected. Compounds **5** (—●—●—) and **2** (—○—○—) were determined on 20-μL samples, as reported in the legend to Table I, but rat liver transketolase was replaced by yeast transketolase (s.a., 18 U/mg; see Experimental section)

was hydrolyzed with M HCl for 6 h at 100°. Samples at various time intervals were taken and analyzed for inorganic phosphate release, 5 h being sufficient to achieve a complete hydrolysis of phosphoric esters in the mixture. Inorganic phosphate was determined according to Tashima and Yoshimura[12].

Preparation of D-threo-2-*pentulose 5-phosphate* (**5**). — A mixture (50 mL) containing 3mM D-*arabino*-2-hexulose 1,6-bisphosphate (**1**), 15mM Li hydroxypyruvate (**4**), 2.5mM $MgCl_2$, and 0.2mM ThPP, in 25mM Tris-HCl buffer, pH 8, was incubated at 37°. The reaction was started by the addition of 0.5 unit of aldolase (9 U/mg) and 20 min later 2.5 units of yeast transketolase (18 U/mg) were added. The production of **5** was determined in samples withdrawn from the solution at various time intervals after transketolase addition. The incubation was carried out for 4 h and terminated by adding to the mixture wet Dowex AG 50W-X8 cation-exchange resin (H^+, 2 g). As shown in Table I, 116.6 μmol of **5** were produced, as expected from the amount of cleaved compound **1**. The suspension was quickly shaken and filtered through a coarse, sintered-glass funnel. The filtrate having pH ~2 was mixed with water (~1 vol.) to reach a final conductivity of ~2 mSiemens, and then thoroughly degassed under vacuum. This last step was necessary in order to avoid CO_2 bubbling during chromatography. The de-areated solution was passed through

a column (1 × 15 cm) of AG 1-X8 (HCO_2^-) anion-exchange resin which was washed with distilled water (30 mL). Monophosphate sugars were isocratically eluted with 1.3M formic acid (300 mL; conductivity ~7 mSiemens), and D-*threo*-2-pentulose 5-phosphate (**5**) was the first compound displaced from the resin, fairly well resolved from the triose phosphates (Fig. 2). Fractions 16–27 containing the bulk of compound **5** (~50 mL) were pooled and evaporated twice at 37° (water bath) almost to dryness. The concentrated fraction was taken up in water (5 mL) and the pH adjusted to ~6 by dropwise addition of 0.1M NaOH under stirring. The content of **5** in the final solution, as determined in the presence of an excess of D-ribose 5-phosphate and of yeast transketolase, was 86 μmol, which represent 60% of the initial concentration of compound **1**. Enzymic analysis of isolated preparations of **5** showed the presence of the following contaminants: D-*erythro*-2-pentulose 5-phosphate (**6**), 2%; D-ribose 5-phosphate, 1.5%; D-glyceraldehyde 3-phosphate (**3**), 0.5%; and dihydroxyacetone phosphate (**2**), 1.5%, relative to the amount of D-*threo*-2-pentulose 5-phosphate (**5**), which accounted for 95–97% of total inorganic phosphate released by exhaustive HCl hydrolysis.

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