PENTOSE PHOSPHATES FORMED BY MUSCLE ALDOLASE*

by

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Although the ribose of nucleotides may be synthesized directly by oxidation of hexoses[1,2], there is considerable evidence which indicates that other pathways may also be involved[3,4,5].

Pentose derivatives have been synthesized from glycolaldehyde and FDP[6] by extracts of muscle[8] and bacteria[9]. RACKER[10] stated that the product so synthesized by crystallized rabbit muscle aldolase was not ribose-5-phosphate, and FORREST, HOUGH AND JONES[11] concluded that pea aldolase yielded "optically active keto-pentose containing largely D-xyloketose". (*Cf.* also [12,13]).

In view of the lack of specificity exhibited by crystalline muscle aldolase toward ketohexose phosphates[7], the ketopentose phosphates which it yields have been investigated by procedures which do not include exposure to basic conditions. Chromatography on Dowex-1-chloride was used to separate the components of the reaction mixtures. This technique as well as paper chromatography indicated that only one pentose derivative—D-xylulose-1-phosphate—is formed from glycolaldehyde and dihydroxyacetone phosphate. Glycolaldehyde-phosphate similarly yields a pentose diphosphate which on enzymic dephosphorylation yields xylulose.

MATERIALS AND METHODS

Materials

Fructosediphosphate. Commercial FDP was purified by chromatography on Dowex-1-chloride with dilute HCl as the eluting agent essentially as described below. The purified material, in selected fractions of the column eluate, was isolated as the Ba salt at pH 6.8 and contained only a trace of inorganic P.

Glycolaldehyde-2-phosphate and Glycolaldehyde were prepared as described[7]. The latter, which contained a trace of pyridine, melted at 81–83° and the once-melted material, after solidification, melted at 92–95° (reported m.p. 96°[14]). Its phenylhydrazone (once recrystallized) melted at 157–59° (reported m.p., 162°[14]).

D-*Ribulose* was prepared from D-ribulose-*o*-nitrophenylhydrazone which was a gift of Dr. SEYMOUR COHEN[15].

Aldolase was crystallized from rabbit muscle extract as needles at pH 7.5 and was recrystallized twice at pH 6.0 as bipyramids using the procedure of TAYLOR *et al.*[16]. One electrophoresis run in phosphate buffer, pH 7.4, ionic strength 0.1, showed a single component. It contained an enzymically detectable trace of triosephosphate isomerase.

Potato acid phosphatase was prepared according to a procedure kindly furnished by B. L. HORECKER. The activity of the material used was 400 μM of phosphorus liberated per hour mg protein under the test conditions[17].

* This investigation was supported in part by a research grant (A-531) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

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Methods

Ion exchange chromatography. COHN[18] used Dowex-1 with dilute HCl as an eluting agent to separate ribonucleotides. Satisfactory resolution of phosphorylated sugars has been obtained using the same resin and dilute HCl (0.025–0.10 *N*) for elution. The exact column dimensions and the concentration of HCl used for elution are described with each experiment. Dowex-1, 200–400 mesh, 10% crosslinkage (The Dow Chemical Company), was washed by suspension and decantation to remove about 20% of the finest material and also the small amount (1%) of dark, rapidly settling particles. It was broken in with 1 *N* HCl and 1 *N* NH_4OH. Before each chromatogram it was washed with an excess of 1 *N* HCl and then water.

As suggested by COHN[18], the sample was placed on the column in a solution of low eluting power (anion concentration = 0.01 *M*) at pH 6.5. The column was then washed with water before introducing the eluting agent. Small amounts of aldolase were bound by the resin but were eluted at or ahead of the breakthrough of even the weakest eluting agent. The sample, water wash, and initial portion of the eluting agent were introduced by pipette. Elution was carried out at an average flow rate of 0.8 ml/cm²/min which required a net air pressure of about 10 cm of mercury with the columns used. The main portion of the eluting agent was introduced from a reservoir connected directly in the air pressure line. The top of the resin bed was protected by a close fitting alundum plate. The eluate was separated into fractions using a volume-controlled automatic fraction collector. Fraction zero was the breakthrough of the eluting agent. If enough water wash is used to rinse out the acid released by the sample, the breakthrough is readily detected by a drop in pH.

Combined fractions selected to contain a single compound may be concentrated for further study by precipitation as the barium salt or by lyophilization. Lyophilization avoids exposure to barium ion and alkaline conditions and was used in all cases for the unknown pentose phosphates.

Analytical determinations were carried out as described[7].

RESULTS

Pentosemonophosphate

Synthesis and separation. By systematic variation of length and diameter of the resin bed and the concentration of HCl used for elution, an ion exchange procedure capable of separating inorganic phosphate, triose phosphate, pentose monophosphate and FDP was developed (Fig. 1). A large scale synthesis designed to furnish a quantity

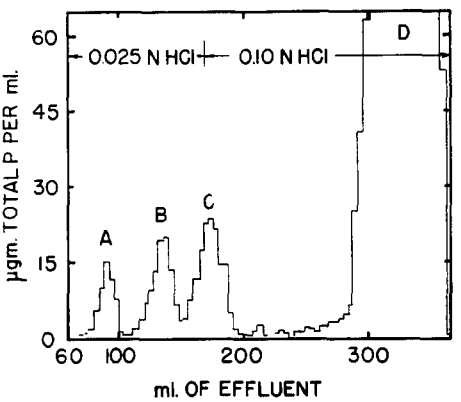

Fig. 1. Ion exchange separation of sugar phosphates. A, 6.8 µmoles orthophosphate; B, 12.6 µmoles triose phosphate; C, 19 µmoles pentose phosphate; D, 152 µmoles HDP. The sample (21 ml) resulted from the incubation of 183 µmoles HDP, 420 µmoles glycolaldehyde, and 8 mg of aldolase at p.H 7.0 for 30 min at about 20°. The aldolase *in this experiment only* had been partially inactivated by dialysis against distilled water at pH 4.1. The Dowex-1-chloride resin bed was 1.2 cm × 44 cm.

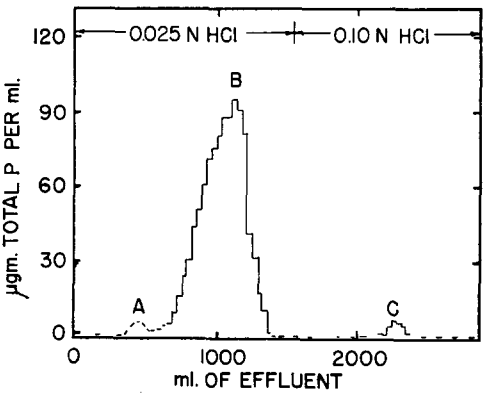

Fig. 2. Ion exchange separation of pentose-phosphate. A, Orthophosphate; B, 1.23 m moles Pentose phosphate; C, 0.03 m moles HDP. The sample (149 ml) resulted from the incubation of 0.745 m moles HDP, 3 m moles glycolaldehyde and 74 mg aldolase at pH 6.5 for 2.5 h at 30°. The aldolase had been dialyzed against 0.003 *M* $KHCO_3$. The resin bed was 4 cm × 33 cm.

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of the unknown pentose phosphate adequate for identification is described in Fig. 2. The resulting equilibrium under the conditions used gave a pentose yield of 97% calculated from the initial quantity of FDP.

Homogeneity. The conclusion that the condensation with glycolaldehyde resulted in a single pentosemonophosphate was based on the following evidence. Selected fractions of the pentose peak had a constant ratio of apparent pentose to organic phosphorus, and specific analyses for inorganic phosphate, and for the alkali-labile phosphate of triosephosphate were negative. The pentose phosphate synthesized is alkali stable[7]. A solution of the ketopentosemonophosphate as the free acid was optically active; $a_D^{23} = -16.8°$ ($c = 11.9$ in 4 N HCl). After the rotation had been measured, analysis showed less than 5% of the organic phosphorus had been hydrolyzed. The specific rotation of the two derivatives made (see below) confirmed the presence of only

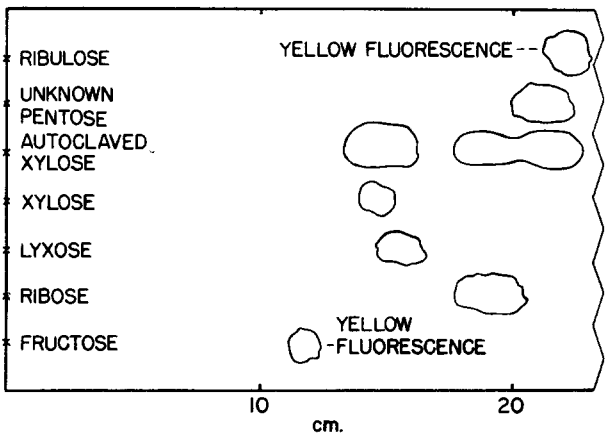

Fig. 3. Identification of pentose from the monophosphoric ester. The tracing is of a descending chromatogram carried out according to the method of JERMYN AND ISHERWOOD (*J. Biochem.*, 44 (1949) 402) using ethyl acetate-acetic acid-water (2.9-1.0-2.9) as solvent on Whatman No. 1 paper. The paper was dried and the sugars detected (S. CHERNICK, I. L. CHAIKOFF AND S. ABRAHAM, *J. Biol. Chem.*, 193 (1951) 793) by spraying with 0.2% diphenylamine-0.1 *M* oxalic acid in 50% alcohol and then heating in an oven at 90° C for five to ten minutes. The most critical examination is possible under ultraviolet light. The numerous minor components of the autoclaved xylose which are obvious only under ultraviolet light were not indicated in the tracing.

a single optical isomer. The free sugar was prepared from the unknown pentose phosphate by incubating 80 μmoles of the latter with 80 μmoles $MgCl_2$, 400 μmoles acetate buffer and 250 units of potato phosphatase in a total volume of 4 ml at pH 5.0 for 15 hours at 37°. Analysis at 14 hours showed that 92% of the esterified phosphate had been hydrolyzed. The cooled incubation mixture was deproteinized with 5% trichloroacetic acid, passed through freshly regenerated IR-120 and IR-4B resins to remove all cations and anions including any remaining pentose phosphate, and concentrated to 1.5 ml. Paper chromatography of the free pentose, Fig. 3, showed a single spot.

Identification. Pentose phenylosazones were prepared[19] from D-ribose, and D-xylose and directly[20] from the unknown pentose phosphate. Their observed melting points and observed mutarotations are given in Table I[21]. Although the melting points of pentose phenylosazones (except for that of DL-xylose)[22] are of no value for the identification of a specific pentose, the mutarotations are characteristic. LEVENE AND LAFORGE[22] used

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this technique to identify the phenylosazone of the ketopentose, L-xylulose, found in human, nondietary pentosuria. The course of mutarotation during the periods reported by LEVENE AND LA FORGE (in agreement with the data in Table I) is a consistent change in one direction as opposed to the biphasic mutarotation shown by some free sugars. The observed mutarotation of the unknown pentose phenylosazone indicated that it was D-*threo* pentose phenylosazone.

TABLE I

IDENTIFICATION OF PENTOSE PHENYLOSAZONE BY ITS MUTAROTATION

Compound	*Melting point**	$[\alpha]_D$ *in ethanol***		
D-*threo*-Pentose phenylosazone	165–167°	1 h, −26	13 h, −44	48 h, −48
Unknown Pentose phenylosazone	161–163°	1 h, −25 ± 5	72 h, −37 ± 3	96 h, −42 ± 8
D-*erythro*-Pentose phenylosazone	164–166°	1 h, −20		144 h, −7

* Uncorrected.

** Rotations were measured at room temperature (20–23°) in a 2 dm tube; the concentrations, in the order listed, were: 0.52, 0.2, and 0.84 %.

The phenylosotriazole of the unknown pentose was prepared[23] from the osazone and crystallized from ether at −10°. Its melting point and specific rotation were similar to those of D-*threo* pentose phenylosatriazole (Table II). Furthermore, admixture of the latter with the unknown produced no depression of melting point.

The two derivatives (phenylosazone and phenylosotriazole) prepared do not differentiate between xylulose and xylose. Paper chromatography (Fig. 3) of the free sugar showed that the unknown pentose was not xylose and that it was different from all sugars tested, including D-ribulose, D-ribose, D-xylose, and D-lyxose, with the exception of one component of autoclaved xylose[25].

TABLE II

PHENYLOSOTRIAZOLE DERIVATIVES OF PENTOSES

Compound	*Melting point*	*Specific rotation*
D-*threo* Pentose phenylosotriazole	88.0–90.0° C	$[\alpha]_D^{20}$ −32.3°
Unknown Pentose phenylosotriazole	87.5–88.5° C	$[\alpha]_D^{23}$ −30.9° ± 3
D-*erythro* Pentose phenylosotriazole	80.0–81.0° C	$[\alpha]_D^{20}$ + 23.1°

The known specificity of aldolase leads to the prediction that the D-xylulose carries the phosphate on carbon atom No. 1. The lability of the phosphate grouping to 1 *N* HCl[26] and to phenylhydrazine acetate[20] is likewise in agreement with the ketopentose-1-phosphate structure. We therefore conclude that the product synthesized from glycolaldehyde and triose phosphate by muscle aldolase is a single pentose monophosphate — D-xylulose-1-phosphate.

Pentose diphosphate

Synthesis and isolation. TUNG *et al.*[7] incubated glycolaldehydephosphate (alkali stable phosphate) and synthetic dihydroxyacetonephosphate (alkali-labile phosphate)

with 5 times crystallized aldolase free of triose phosphate isomerase and observed an increase in the apparent pentose concentration (orcinol reaction) but no disappearance of alkali-labile phosphorus. It was concluded that a pentosediphosphate was formed but that one of the phosphate ester linkages was alkali-labile.

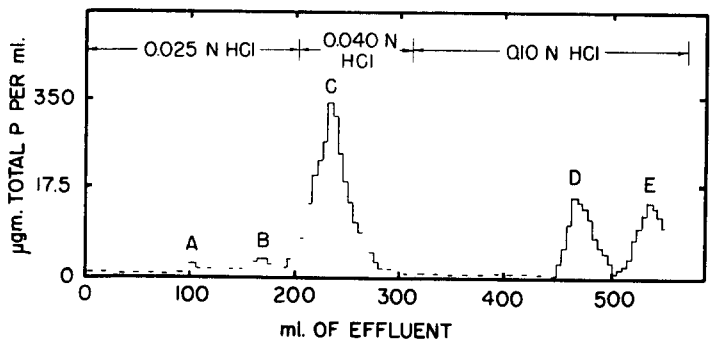

Fig. 4. An ion exchange chromatogram of a small-scale synthesis of pentose diphosphate. A, 1.16 μmoles orthophosphate; B, 2.06 μmoles triose phosphate; C, 42 μmoles glycolaldehyde phosphate; D, 7.2 μmoles HDP; E, 9.1 μmoles pentose diphosphate. The sample (2.65 ml) resulted from the incubation of 13.2 μmoles HDP, 53 μmoles glycolaldehyde phosphate, and 2.65 mg aldolase at pH 6.5 for 2 hours at 37°. The dimensions of the Dowex-1-chloride resin bed were 1.2 cm × 50 cm.

For identification, the unknown pentose diphosphate was synthesized from FDP and glycolaldehyde phosphate. Fig. 4 shows an ion exchange chromatogram of a small scale synthesis. The components of the reaction mixture were distinctly separated. In 5 other experiments, the yield of pentose diphosphate, calculated on the basis of the original diose phosphate concentrations, varied from 30 to 48%. Larger scale syntheses were carried out and the unknown pentose diphosphate fractions were combined and concentrated by lyophilization. This method of concentration resulted in hydrolysis of 6.5% of the organic phosphate. The specific rotation of the pentose-diphosphoric acid, a_D^{23}, was approximately + 10° (c = 1.3).

Identification. The pentose diphosphoric acid is labile to both acid and alkali. Exposure to 1 N NaOH for 15 minutes at room temperature hydrolyzed 44% of the

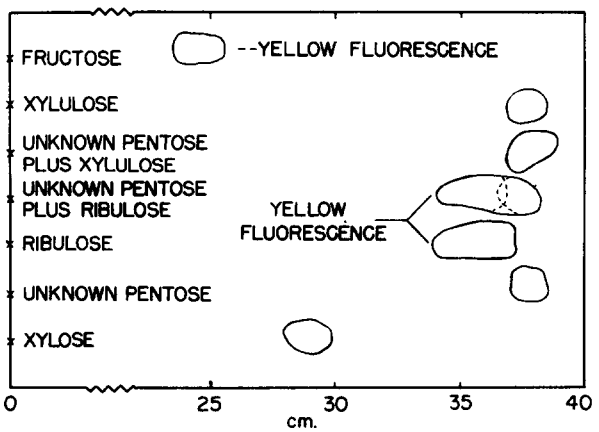

Fig. 5. Identification of pentose from the diphosphoric ester. The tracing is of a descending chromatogram using ethyl acetate-pyridine-water (2.0–1.0–2.0); other conditions as described in Fig. 3.

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organic phosphate. Lability of the 1-phosphate group is to be expected in a ketopentose-1,5-diphosphate since combination of the 5-hydroxyl with phosphate prevents the formation of a furanose ring. Treatment with 0.01 *M* phenylhydrazine acetate at pH 5.0 and 100° liberated 46% of the organic phosphate after 10 minutes.

The free pentose was liberated enzymically from the diphosphate and isolated as described for the pentosemonophosphate above. It was positively identified as xylulose by paper chromatography (Fig. 5). The reference sample of xylulose was that characterized above as originating from D-xylulose-1-phosphate.

These several properties indicate the pentose diphosphate is a derivative of xylulose, and most likely D-xylulose-1,5-diphosphate. A quantity sufficient for preparing derivatives, even on a small scale, was not available.

DISCUSSION

The stereospecificity of the aldolase catalyzed condensation of diose or diose phosphate with dihydroxyacetone phosphate is in keeping with the original findings of MEYERHOF *et al.*[27]. The newly formed hydroxyl groups in the resulting ketopentose phosphates are *trans* to one another and in the same relative configuration as the 3 and 4 positions of fructose. Although this aldol condensation of $C_3 + C_2$ compounds does not give rise to ribose phosphates directly, it may do so indirectly. The above condensation, followed by conversion of xylulose phosphate to ribulose phosphate[28] and finally by the pentosephosphate isomerase[17] reaction may yield ribose phosphate for nucleotide synthesis. Since aldolase can cleave sugar phosphates between carbon atoms bearing *cis* hydroxyl groups[7], it may also synthesize them in small amounts. Continued conversion of ribulose phosphates, so formed, to ribose phosphates would provide a more direct pathway for the synthesis of the latter. The equilibrium concentration of ribulose phosphates formed by aldolase may have been too low to permit detection by the methods employed here.

The synthesis and isolation of xylulose-1-phosphate described here was accomplished in good yield from readily available starting materials and is potentially a practical source of this compound.

SUMMARY

1. The product of the condensation of glycolaldehyde and dihydroxyacetonephosphate, catalyzed by rabbit muscle aldolase, is D-xylulose-1-phosphate.

2. The product of the condensation of glycolaldehyde-phosphate and dihydroxyacetonephosphate is a diphosphoric ester of xylulose, presumably D-xylulose-1,5-diphosphate.

3. Conditions are described for the separation of orthophosphate, diose phosphate, triose phosphate, xylulose-1-phosphate, fructose diphosphate and xylulose diphosphate on Dowex-1-chloride.

RÉSUMÉ

1. L'aldolase du muscle de lapin catalyse la condensation du glycolaldéhyde et du dihydroxyacétone phosphate en D-xylulose-1-phosphate.

2. Le produit de condensation du glycolaldéhyde-phosphate et du dihydroxyacétonephosphate est un ester diphosphorique du xylulose, probablement le D-xylulose-1,5-diphosphate.

3. Une méthode de séparation de l'orthophosphate, du diosephosphate, du triosephosphate, du xylulose-1-phosphate, du fructosediphosphate et du xylulosediphosphate sur Dowex-1-(forme Cl^-) est décrite.

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ZUSAMMENFASSUNG

1. Dass durch Kaninchenmuskelaldolase katalysierte Kondensationsprodukt von Glykolaldehyd und Dihydroxyacetonphosphat ist D-Xylulose-1-phosphat.

2. Das Kondensationsprodukt von Glykolaldehydphosphat und Dihydroxyacetonphosphat ist ein Diphosphorsäure-Ester der Xylulose, wahrscheinlich einer D-Xylulose-1,5-diphosphat.

3. Es werden die Bedingungen beschrieben, unter denen Orthophosphat, Diosephosphat, Triosephosphat, Xylulose-1-phosphat, Fruktosediphosphat und Xylulosediphosphat mit Dowex-1-Chlorid getrennt werden können.

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Received March 31st, 1954