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D-XYLULOSE-1-PHOSPHATE: ENZYMATIC ASSAY AND PRODUCTION IN ISOLATED RAT HEPATOCYTES

Debra A. Barngrover, Hope C. Stevens and William L. Dills, Jr.

Division of Nutritional Sciences and Section of Biochemistry, Cell and Molecular Biology, Division of Biological Sciences, Cornell University, Ithaca, NY 14853

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SUMMARY

A specific enzymatic assay for D-xylulose-1-phosphate (D-threopentulose-1-phosphate) was developed, based on the measurement of glycolaldehyde, which is formed by aldolase cleavage of the D-xylulose-1-phosphate. This assay was used to confirm the identity of the product of fructokinase phosphorylation of D-xylulose and the production of glycolaldehyde and D-xylulose-1-phosphate in D-xylulose-treated isolated rat hepatocytes. An alternative pathway of xylitol metabolism in the liver, through D-xylulose-1-phosphate to glycolaldehyde, is proposed. Because glycolaldehyde is a known oxalate precursor, this pathway may explain the synthesis of oxalate from xylitol.

The known metabolism of xylitol involves oxidation by a polyol dehydrogenase to D-xylulose (D-threopentulose)[1] which is then phosphorylated by xylulokinase (EC 2.7.1.17) to xylulose-5-phosphate (1). In vitro, it has been shown that xylulose is also a good substrate for liver fructokinase (EC 2.7.1.3) (2, 3). The presumed product from this reaction is xylulose-1-phosphate, which is a substrate for aldolase (EC 4.1.2.13) yielding dihydroxyacetone phosphate and glycolaldehyde (5). In light of these enzymatic studies and the fact that glycolaldehyde is a known precursor of oxalate (6, 7) we propose that the metabolism of xylitol through an alternative pathway involving xylulose-1-phosphate (Fig. 1), could account for the formation of [^{14}C] oxalate from [^{14}C] xylitol noted by Rofe, et al. (8).

To study this proposed alternative pathway, a specific enzymatic assay for xylulose-1-phosphate was developed. This assay was used to confirm the identity

1. All sugars and sugar esters mentioned in this paper are of the D-configuration, where appropriate.

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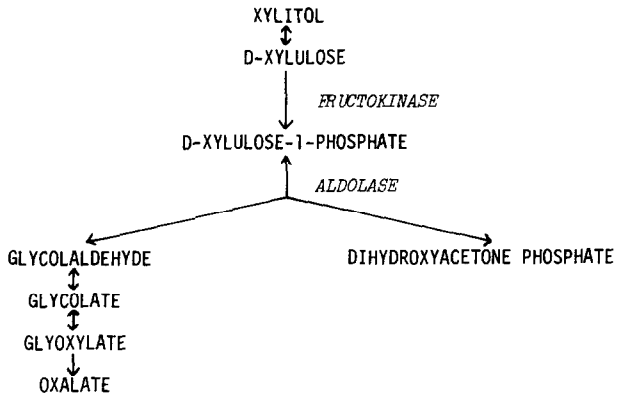

Fig. 1 Alternative pathway of xylitol metabolism. This simplified diagram is not meant to indicate that oxalate is the sole product of glycolaldehyde metabolism. For further details of oxalate metabolism see Hodgkinson (7).

of the product of fructokinase phosphorylation of xylulose and to identify xylulose-1-phosphate as a product of xylulose metabolism in isolated rat hepatocytes. A preliminary report on this assay has been presented (9).

MATERIALS AND METHODS

DEAE-Sephacel was obtained from Pharmacia Fine Chemicals. The sugars, cofactors, enzymes, and phosphocellulose used were obtained from Sigma Chemical Co. All other chemicals used were reagent grade. Absorbances were measured using a Gilford 250 spectrophotometer and an attached Gilford 6051 recorder.

Bovine liver fructokinase was purified by a variation (4) of published methods (3). The preparation was free from xylulokinase, hexokinase and L-iditol dehydrogenase activities. Fructokinase activity was measured with a lactate dehydrogenase-pyruvate kinase coupled system (10). The kinetic data was graphed on a double reciprocal plot and analyzed using linear regression (11). Rat liver aldolase was isolated according to the method of Leuthardt and Wolf (12), as modified by Eggleston (13). Xylulose-5-phosphate was analyzed using the method of Racker (14). Phosphate was analyzed using the Ames method (15).

Synthesis of Xylulose-1-Phosphate:

The method of Byrne and Lardy was used (5). The final reaction mixture contained 0.1 M Piperazine-N-N'-bis (2-ethane-sulfonic acid) buffer, pH 6.5, 0.5 mmol dihydroxyacetone phosphate (Li salt), 1.0 mmol glycolaldehyde and 0.9 mg muscle aldolase (specific activity = 12 U/mg) in a final volume of 5 ml. After 3 hours at 32° C, the xylulose-1-phosphate was separated from the other components on a DEAE-Sephacel column (2.5 x 90 cm) in the acetate form (16). The compounds were eluted with a 1500 ml linear gradient of 0.05-0.8 M sodium acetate, pH 4.5. Based on phosphate analysis, approximately 60% of the added dihydroxyacetone phosphate had been converted to xylulose-1-phosphate. The sugar phosphate prepared was keto-pentose positive (cysteine-carbazole assay, λmax = 540 nm (17)), and reacted with both rabbit muscle aldolase and rat liver aldolase, but not transketolase.

Xylulose-1-Phosphate Assay:

This assay is based on the glycolaldehyde assay of Goedde and Langenbeck (18), coupled with muscle aldolase cleavage of xylulose-1-phosphate and can be used to measure both metabolites sequentially in the same cuvette. Since alcohol dehydrogenase has a low affinity for glycolaldehyde, large amounts of enzyme must be used for stochiometric conversion. The initial assay mixture consisted of 0.5 ml Tris buffer (0.1 M, pH 7.4), 0.02 ml β-NADH-Na_2 (10 mg/ml) and 0.45 ml sample, containing between 4 and 100 nmol of glycolaldehyde or xylulose-1-phosphate. A control cuvette containing water instead of sample must be included to correct for extinction changes upon addition of enzymes and the slow background rate of NADH oxidation, which should be less than 0.0003 μmol/min. After mixing the cuvettes, the initial extinction (E_1) was read at 340 nm. Glycolaldehyde was measured by adding 200-240 U yeast alcohol dehydrogenase and recording the extinction (E_2) every 5 min for 15-30 min, until the change in extinction equals the background rate. The use of a recorder attached to the spectrophotometer makes the recording of the intermediate values unnecessary. The change in absorbance ($E_1 - E_2$) minus the absorbance change in the control cuvette was used to calculate the concentration of glycolaldehyde in the added sample as follows (19):

$$c\ (\mu mole/ml) = \frac{(\Delta E_{sample} - \Delta E_{control}) \times V}{\varepsilon \times v \times d}$$

Where: V = Total volume in cuvette (ml)
v = Volume of added sample (ml)
ε = Extinction coefficient for NADH ($cm^2/\mu mol$)
d = Light path (cm)

Xylulose-1-phosphate was then measured by adding 1.5 U of rabbit muscle aldolase and following the same procedure.

The enzymes in the assay system used must be free of other dehydrogenases, particularly lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and L-iditol dehydrogenase. If necessary, the muscle aldolase can be purified by fructose bisphosphate elution from a phosphocellulose column (20).

Isolation and Incubation of Hepatocytes:

Male Sprague-Dawley rats (Blue Spruce, Altamont, NY), 200-400 gm were maintained on a 12-hour light-dark schedule in stainless steel cages and were fed stock diet (Charles River Rat, Mouse and Hamster Formula, Syracuse, NY) and water ad libitum. Hepatocytes were isolated from fed rats, two hours into the light cycle, according to the method of Seglen (21) with modifications (22). The wet weight of the cells and trypan blue exclusion were determined according to the methods of Seglen (21). Trypan blue was excluded by > 90% of the cells.

The hepatocytes were incubated in 25 ml polycarbonate Erlenmeyer flasks at 37° C and 90 oscillations per min in a shaking water bath. The cells (70-80 mg/flask), plus substrate and suspension buffer for a total volume of 2.5 ml, were gassed with 95% O_2 : 5% CO_2 and incubated for 5 min. The suspensions were then deproteinized with 0.5 ml ice cold 3 N perchloric acid and centrifuged in a clinical centrifuge. The supernatants were immediately neutralized with 1 N potassium hydroxide and frozen until assayed.

RESULTS

Specificity of Xylulose-1-phosphate Assay:

The assay was linear with samples containing from 4 to 100 nmol xylulose-1-phosphate. For a sample with a mean concentration of 0.17 μmol/ml the standard deviation was 0.013 μmol/ml with a coefficient of variation of 8%. At cuvette concentrations up to 15 μmol/ml, the following compounds did not react significantly: fructose-1-phosphate, fructose-1,6-bisphosphate, fructose-6-phosphate, xylulose-5-phosphate, xylulose, xylitol, and acetoacetate. Acetaldehyde was the major potential interfering substrate. Its presence in the sample was easily detected by a very fast rate of NADH oxidation upon addition of alcohol dehydrogenase. Glycolaldehyde can still be measured in its presence by first adding a small amount of alcohol dehydrogenase (5-10 U) and then, when the reaction is complete, adding the larger amount of alcohol dehydrogenase necessary to measure the glycoaldehyde. Since acetone is frequently contaminated with acetaldehyde, methanol instead of acetone is recommended for rinsing cuvettes. Other aldehydes which react with alcohol dehydrogenase include glyceraldehyde, butyraldehyde, propionaldehyde, valeraldehyde, and isobutyraldehyde (18).

Fructokinase and Xylulose:

Xylulose was a good substrate for fructokinase with a K_m of 0.45 ± 0.07 mM (relative Vmax = 65.0 ± 0.05% of fructose). The product of fructokinase phosphorylation of xylulose was confirmed to be the 1-phosphate by use of the two specific xylulose phosphate assays (xylulose-1-phosphate was produced but not xylulose-5-phosphate).

The recovery of xylulose-1-phosphate added to hepatocytes (0.6 - 3.7 μmol/g wet weight) ranged between 93 and 106%. This confirmed that the ester was stable to the deproteinization, neutralization and storage procedures used. The levels of glycolaldehyde, xylulose-1-phosphate and xylulose-5-phosphate in hepatocytes incubated with 0-20 mM xylulose are presented in Figure 2. Significant amounts of both xylulose phosphates and glycolaldehyde were found at all levels of xylulose tested. During the short incubations used, less than 5% of the added xylulose was

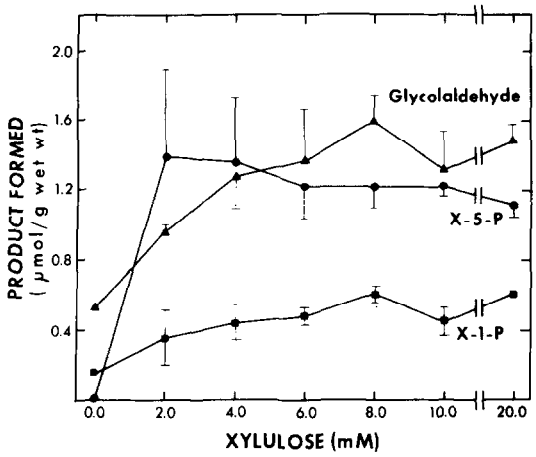

Fig. 2 Production of xylulose-1-phosphate (■), glycolaldehyde (▲) and xylulose-5-phosphate (●) in isolated rat hepatocytes incubated with the indicated levels of xylulose for 5 min. For details of hepatocyte preparation and incubation see text. Data is given as means ± SD for duplicate incubations.

metabolized. There were no significant amounts of xylulose-1-phosphate or glycolaldehyde recovered from hepatocytes incubated with 10 mM glucose, fructose, or sorbitol.

DISCUSSION

Earlier papers which reported the phosphorylation of xylulose with fructokinase (2, 3) do not identify the product formed. The use of the xylulose-1-phosphate specific assay reported here confirmed the identity of the product and also demonstrated the production of xylulose-1-phosphate from xylulose in isolated hepatocytes. This reaction forms the basis for the proposed pathway (Fig. 1) by which oxalate precursors can be formed from xylitol. Calcium oxalate is a common constituent of kidney and bladder stones. Known oxalate precursors include ascorbic acid, glycolaldehyde, glycine, tryptophan, glycolate, ethylene glycol, and glyoxylate (27). Xylitol is implicated as a precursor to oxalate both in human cases (23, 24, 25) and animal studies (8, 26), though no direct pathway for xylitol conversion to oxalate has previously been identified. Further studies are underway to detail the conditions under which xylitol and xylulose can be converted to xylulose-1-phosphate, glycolaldehyde, and other metabolites in the liver.

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