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Enzymatic Synthesis and Isolation of D-Glucosamine-6-Phosphate Barium Salt

„Enzymatyczna synteza i wyodrębnianie D-glukozamino-6-fosforanu baru

Энзиматический синтез и изолирование бариевой соли глюкозамино-6-фосфорной кислоты

Glucosamine-6-phosphate is the key intermediate of glucosamine metabolism. It has been obtainable by chemical synthesis or enzymatically using yeast hexokinase and adenosine triphosphate (1).

In this paper we report a new method for the enzymatic synthesis of glucosamine-6-phosphate on a preparative scale using polyphosphate: D-glucose (and D-glucosamine) 6-phosphotransferase (EC 2.7.1.63) as an enzyme and inorganic polyphosphate as a substrate. The ester in gram quantities was isolated as barium salt by precipitation with ethanol.

EXPERIMENTAL

Chemicals. D(+)-Glucosamine hydrochloride anhydrous (GlcN) was from Sigma Chem. Co. (St. Louis, Mo., USA). Inorganic polyphosphate (poly-P) of a particularly high molecular weight (unmeasurable by the potentiometric method) was "potassium metaphosphate" from Hopkin and Williams Ltd (Essex, England). Where indicated in the text, potassium polyphosphate of a lower mol. wt ($n=300$) from B.D.H. (Poole, Dorset, England) was employed. Both were freed of orthophosphate (P_i) by suspending either substance in water and centrifugation, then dried with acetone. DEAE-cellulose DE 52 was from Whatman Biochemicals Ltd (Maidstone, Kent, England). β-Mercaptoethanol purum and Tris purum were from Fluka AG (Buchs S. G., Switzerland). The latter was

recrystallized from ethanol before use. All other chemicals employed were analytical grade products from POCh (Gliwice, Poland). Deionized water was used throughout.

Analytical methods. Protein content and phosphotransferase activity were determined as described previously (2). One unit of activity was defined as the amount of enzyme which catalyzes the utilization of 1 μmol P per min at 30°C with the use of B.D.H. polyphosphate and glucose.

Preparation of polyphosphate-glucose (glucosamine) phosphotransferase. The enzyme was isolated in a partially purified state from *Mycobacterium tuberculosis* $H_{37}Ra$. The starting material was a calcium phosphate gel eluate (step 3) obtained as reported earlier (2). A 45 ml volume of the eluate (135 units and 79 mg protein) was dialysed for 20 h against three changes of cold 0.01 M Tris--maleic acid buffer (*pH* 6.2) containing 50 mM glucose, 5 mM β-mercaptoethanol, 1 mM $MgCl_2$ and 0.5 mM EDTA. Some insoluble material was removed by centrifugation and the supernatant (50 ml, 130 units, 51 mg protein) applied to a DEAE-cellulose column which had been equilibrated with glucose-free buffer (*pH* 6.2) containing β-mercaptoethanol, $MgCl_2$ and EDTA as before. After adsorption, the column was washed with 65 ml of the same buffer and the enzyme eluted with 50 ml of 0.1 M NaCl (Fig. 1). The most active fractions were pooled and the enzyme immediately stabilized by an addition of 120 μmol P of poly-P ($n=300$). The eluate (24 ml) contained 89 activity units and 5.6 mg of protein which indicates an 80-fold purification with 66% recovery. The activity toward glucosamine was lower by 40 and 65% with B.D.H.- and Hopkin and Williams polyphosphate, respectively.

Synthesis of glucosamine-6-phosphate. At the start of incubation, the reaction mixture contained in a total volume of 120 ml: 10 mmol Tris-HCl buffer (*pH* 8), 20 mmol NaCl, 1.6 mmol $MgCl_2$, 89 units of enzyme, 450 mg of poly-P (3.9 mmol P) and 500 mg of glucosamine hydrochloride (2.2 mmol). The reaction was conducted with stirring in a water bath at 28—30°C for 6 h. At suitable time intervals, 0.02—0.05 ml aliquots of the reaction mixture were withdrawn and the level of acid-labile phosphate determined. When its value fell down to 50% or more, a new portion of solid poly-P was added. Solid glucosamine and some Tris in 0.4 M solution to keep the *pH* near 8 were also added.

The time-course of glucosamine phosphorylation is shown in Fig. 2. As one can see, with Hopkin and Williams poly-P, the reaction proceeded at an average rate of 33 μmol/min for about 3.5 h, then slowed down due to both decreasing concentration of the phosphate donor (no more additions) and increasing concentration of the product. The incubation

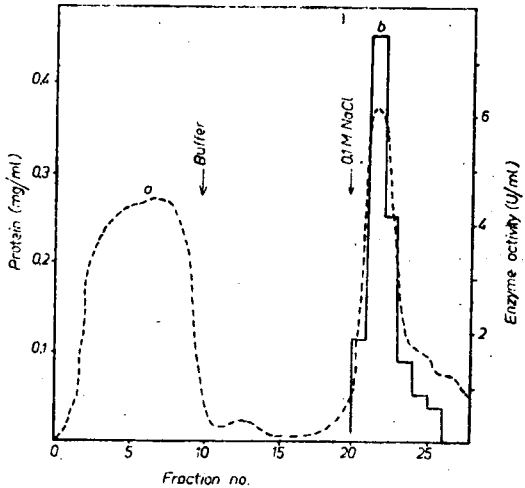


Fig. 1. DEAE-cellulose chromatography of dialyzed calcium phosphate gel eluate. The enzyme preparation in a volume of 50 ml was applied to a DEAE-cellulose column (1×10 cm) equilibrated with Tris-maleic acid β-mercaptoethanol — $MgCl_2$ — EDTA buffer (*pH* 6.2) and after washing, eluted with 0.1 M NaCl. Fractions of 6 ml were collected at a flow rate of 20 ml/h at 4°C and both protein (a) content and enzyme activity (b) determined

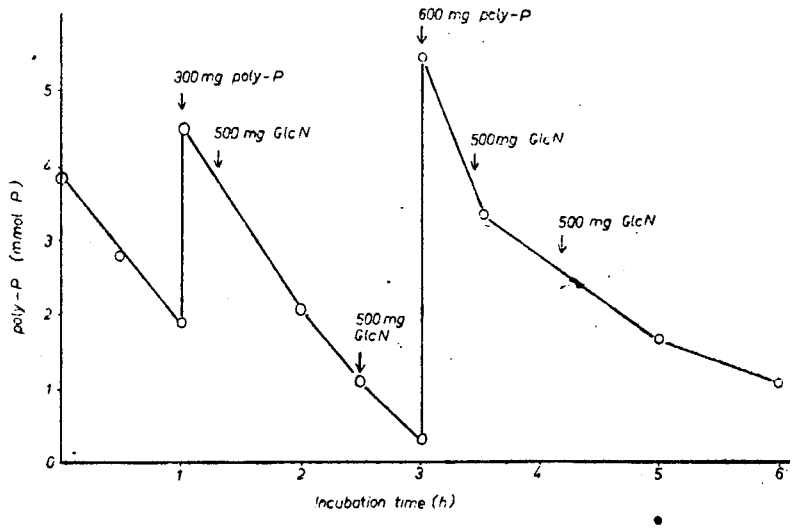
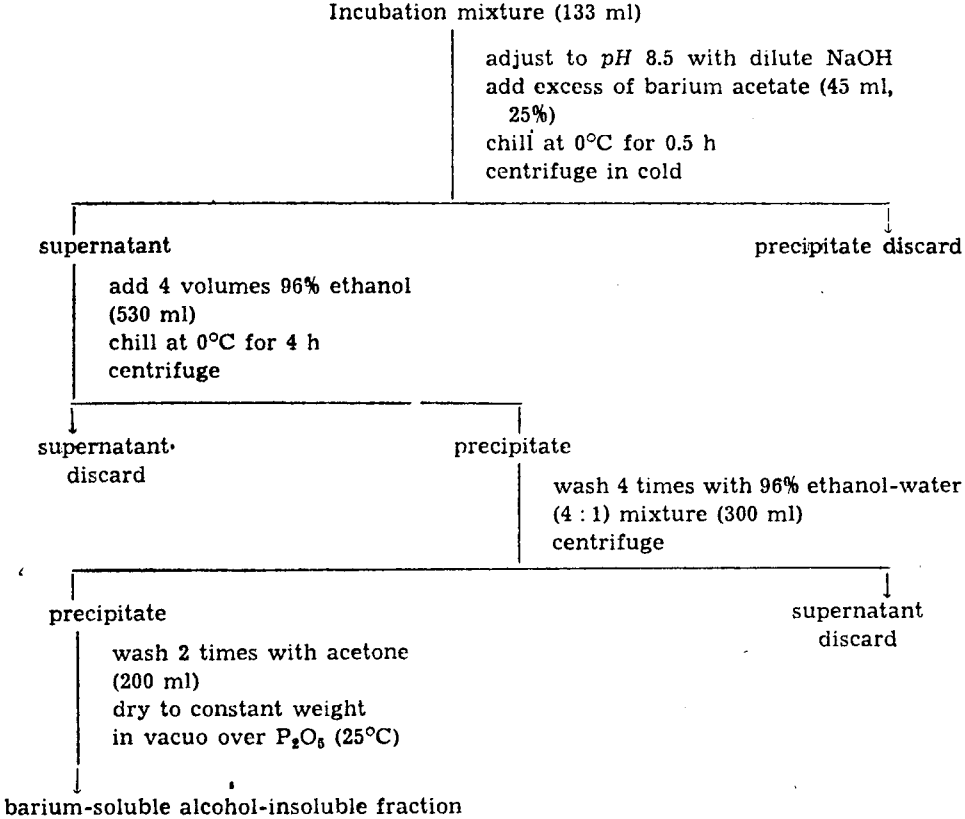


Fig. 2. Time-course of the enzymic synthesis of glucosamine-6-phosphate. The composition of the reaction mixture is given in the text. Arrows indicate additions of polyphosphate and glucosamine. *pH* was maintained near 8 by additions of 0.4 M Tris (not shown)

was terminated after 6 h with about 1.1 mmol P left unutilized. In total, 1.35 g of the polyphosphate (11.4 mmol P), 2.5 g of glucosamine hydrochloride (11.6 mmol) and 15 ml of 0.4 M Tris were added.

Isolation of glucosamine-6-phosphate, barium salt. The isolation procedure of the phosphorylated product as barium salt was based on the method of le Page (4) and that described by Brown (1) with some modifications. The procedure is outlined in the following scheme 1.

SCHEME 1



The isolated compound (white powder) weighing 3.58 g was stored in a dessicator at 4°C. For analytical purposes, if necessary, a suitable portion of the preparation was dissolved in 0.1 N HCl, barium ions removed with Na_2SO_4, the supernatant neutralized and made to a definite volume.

The results obtained for $C_6H_{12}O_8NPBa$ (394.5):

calcd: 18.26% C, 3.07% H, 3.55% N, 7.85% P, 34.81% Ba;
found: 16.6% C, 3.1% H, 2.8% N, 7.0% P, 30.2% Ba.

A comparison of the data indicates that the compound is of 79—89% purity. The rest may be accounted for by water and some contaminating substances; in particular, determinations revealed the presence of 0.07% P_i, 0.13% acid-labile P and 0.2% fructose-6-phosphate. The yield of the compound amounted to at best 80% of the value expected on the basis of poly-P utilization.

DISCUSSION

The method described above makes use of the enzyme phosphorylating glucosamine at the expense of inorganic polyphosphates:

$$(P)_n + \text{glucosamine} = (P)_{n-1} + \text{glucosamine-6-P}$$

At alkaline *pH*'s (*pH* 7.5—8.5), the reaction proved to proceed far to the right due to its exergonic character and a low inhibition of the enzyme by the products: glucosamine-6-P and tripolyphosphate. The latter is formed in a quantity depending on the condensation degree of poly-P used as a substrate (3). In the case of the Hopkin and Williams poly-P consisting of hundreds of P residues per molecule, the amount of $(P)_3$ formed as the end product of an exhaustive phosphorylation is supposed to be negligible (<1%). However, contrary to glucose or glucose-6-phosphate, both glucosamine and glucosamine-6-P tend to decompose at alkaline *pH*'s; therefore, the incubation time was set as brief as possible and *pH* was kept below 8 rather than above that value. In consequence, the incubation was interrupted after 6 h with 10% of the phosphate donor left unutilized. It was removed from the incubation mixture as barium-insoluble fraction prior to the precipitation of barium-soluble ester with ethanol.

In this work, 3.58 g of glucosamine-6-phosphate barium salt of about 85% purity with 80% yield has been prepared. Some attempts to get anhydrous compound by heating at 78°C under diminished pressure ended in failure in that the substance turned yellowish.

Of course, better results could be obtained by using pure enzyme and a more rapidly utilizable polyphosphate (e.g., <100 P residues per molecule), eventually with an improved way of washing barium-soluble alcohol-insoluble fraction. It may be of interest to note that our enzymatic method has given particularly good results when using glucose as the phosphate group acceptor. Under the conditions to be described elsewhere

(Szymona et al.), glucose-6-phosphate barium salt heptahydrate of 96% purity was prepared.

The main advantage of using polyphosphates for preparative scale enzymic synthesis of glucosamine-or glucose-6-P lies in that they are much cheaper than ATP and readily obtainable in large amounts.

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STRESZCZENIE

Stosując polifosforanową gluko(glukozamino)fosfotransferazę jako enzym (EC 2.7.1.63) oraz nieorganiczny polifosforan i glukozaminę jako substraty otrzymano gramowe ilości barowej soli kwasu glukozamino-6-fosforowego z wydajnością 80%. Analiza elementarna końcowego preparatu wykazała ok. 85% czystości związku o wzorze $C_6H_{12}O_8NPBa$.

РЕЗЮМЕ

Применяя полифосфатную глюко(глюкозамино)фосфотрансферазу как фермент (ЕС 2.7.1.63), неорганический полифосфат и глюкозамину как субстраты, получили грамовые количества бариевой соли глюкозамино-6-фосфорной кислоты с выходом 80%. Препарат по элементарному анализу показывает 80% чистоты соединения $C_6H_{12}O_8NPBa$.