THE PHOSPHORYLATION OF D-(+)-GLUCOSAMINE BY CRYSTALLINE YEAST HEXOKINASE

by

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HARPUR AND QUASTEL[1] have recently reported that D-(+)-glucosamine is phosphorylated at the expense of adenosinetriphosphate (ATP) in the presence of aqueous extracts of an acetone powder of beef brain. It was further shown that glucose, fructose, and glucosamine compete as substrates for the phosphorylating enzyme involved, presumably brain hexokinase.

In this paper the rapid phosphorylation of D-(+)-glucosamine by ATP in the presence of crystalline yeast hexokinase is reported. It is shown that the number of moles of glucosamine phosphorylated equals the number of moles of "acid-labile" phosphate transferred from ATP. The product of the reaction has been isolated as the barium salt of an "acid-stable", reducing phosphate ester of glucosamine. The extent of oxidation of the ester by sodium periodate together with its elemental composition establish this substance as D-glucosamine-6-phosphate.

EXPERIMENTAL

Preparation of Enzyme

Crystalline yeast hexokinase was isolated from autolysates of baker's yeast by a modification of the methods of BERGER, SLEIN, COLOWICK, AND CORI[2], and KUNITZ AND MCDONALD[3]. This procedure will be described in a later paper. The enzyme was recrystallized three times before use and was stabilized with insulin[2] against loss of activity on dilution.

D-(+)-*glucosamine and* ATP

D-(+)-glucosamine hydrochloride was obtained from HOFFMAN-LA ROCHE. The specific rotation of its aqueous solution was $[\alpha]_D^{20°} = +71.5°$ (*c*, 1.50). IRVINE AND EARL[4] report $[\alpha]_D^{20°} = +72.5°$ (*c*, 1.35, water) as the equilibrium rotation of this salt.

A commercial sample of ATP obtained from Rohm and Haas as the tetrasodium salt was used without purification. It assayed 72% in the hexokinase-glucose test system.

Rate of Glucosamine Phosphorylation and its Correspondence with ATP *Utilization*

This was determined by incubating at 30° solutions of the composition given in Fig. 1 for various times, and then adding to each of them (volume, 3.2 ml) 3.0 ml of *ca.* 0.15 *M* $Ba(OH)_2$ to stop the enzymatic reaction. Then 3.0 ml of $ZnSO_4$ solution (adjusted in concentration so that 10.0 ml of $ZnSO_4$ were acidimetrically equivalent to

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9.8 ml of the $Ba(OH)_2$) was added to each (method of SOMOGYI) and the mixture shaken vigorously for one minute. The amount of glucosamine present in the filtrate was determined by the method of NELSON[5], using reagent 50 of SHAFFER AND SOMOGYI[6] and a D-glucosamine hydrochloride standard. Control experiments showed that substantially all of the phosphorylated glucosamine was removed by the barium-zinc precipitation.

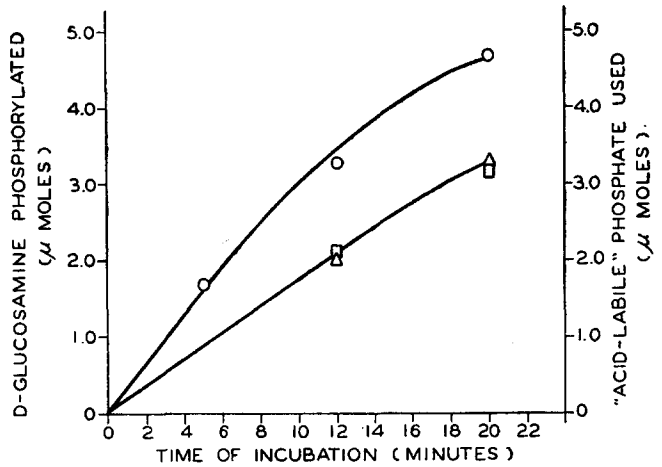

Fig. 1. Rate of phosphorylation of D-glucosamine by ATP in the presence of crystalline yeast hexokinase and $MgCl_2$. The reaction mixture was incubated at p_H 7.8 and 30° and contained, initially: D-glucosamine·HCl, $3.1 \cdot 10^{-3}$ *M*; ATP, $6.7 \cdot 10^{-3}$ *M*; $MgCl_2$, $1.1 \cdot 10^{-2}$ *M*; amorphous insulin, 47 μg/ml; tris-(hydroxymethyl)-amino methane buffer, $6.0 \cdot 10^{-2}$ *M*.
○, 0.84 μg hexokinase/ml; △, 0.46 μg hexokinase/m. □, "acid-labile" phosphate used.

The amount of "acid-labile phosphorus" in the reaction mixtures was determined before and after incubation by analyzing suitable aliquots of a trichloracetic acid filtrate of the solutions by the method of FISKE AND SUBBAROW[7]. In each case, the amount of inorganic phosphorus (present in the ATP solution used) was subtracted from the total orthophosphate found in the solutions after heating them for 10 minutes in 1 *N* H_2SO_4 at 100°.

N-acetyl-D-glucosamine is not phosphorylated in a system similar to that which phosphorylates D-glucosamine rapidly.

Enzymatic Preparation and Subsequent Isolation of a Phosphorylated Derivative of D-glucosamine

A solution was prepared containing the following substances at the indicated initial concentrations: D-glucosamine hydrochloride, $1.87 \cdot 10^{-2}$ *M*; ATP, $6.35 \cdot 10^{-3}$ *M*; $MgCl_2$, $1.13 \cdot 10^{-2}$ *M*; amorphous insulin, 49 μg/ml; crystalline hexokinase, 19.4 μg/ml; tris-(hydroxymethyl)-aminomethane, $5.3 \cdot 10^{-2}$ *M*. The p_H of the solution was adjusted to 8.10 with 0.1 *N* NaOH and 0.1 *N* HCl before the addition of the enzyme. The final volume of the solution was 25.4 ml. Thus, 162 μ moles of ATP and 475 μ moles of D-glucosamine were present initially in the solution. The expected yield of phosphorylated amino sugar was 162 μ moles.

The solution was incubated at 30–31° for 110 minutes; the p_H was found to be 7.8

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after this time. To the solution 0.75 ml of 25% barium acetate was added. The p_H was adjusted to 8.5 by the addition of 0.3 ml of 2 *N* NaOH. After standing in ice water for 30 minutes, the mixture was centrifuged and an additional 0.3 ml of barium acetate was added to insure complete precipitation of the water-insoluble barium salts. After 1½ hours at 0°, the mixture was centrifuged and the supernatant fluid decanted. The precipitate was washed once with 3 ml of 0.3% barium acetate (p_H 7.8), and the washings were added to the first supernatant fluid. The alcohol insoluble barium salts then were precipitated from this slightly alkaline solution by the addition of 115 ml of 95% ethanol. After standing overnight at 2° the mixture was centrifuged in the cold and the precipitate washed with two 6.5 ml portions of 95% ethanol.

The precipitate was largely dissolved by the addition of 8 ml of water and 7.0 ml of 0.2 *N* HNO_3 (final p_H 2.0, glass electrode); a small amount of insoluble material was removed by centrifugation. To the supernatant fluid 0.35 ml of 15% Hg $(NO_3)_2$ in 0.5 *N* HNO_3 was added dropwise. After standing in the cold for a few minutes, the voluminous white precipitate was removed by centrifugation. It was washed once with 2 ml of 3% $Hg(NO_3)_2$ in 0.1 *N* HNO_3, and the washings were combined with the first supernatant fluid.

The clear solution was cooled in an ice bath and H_2S was passed in to remove excess mercuric ions. The filtrate from the removal of HgS was aerated at 0° and then adjusted to p_H 8.2 with 2 *N* NaOH. A small flocculent precipitate was removed after the addition of 0.5 ml of 25% barium acetate. To this solution 80 ml of 95% ethanol were added. After standing overnight, the precipitate was removed by centrifugation and washed with absolute ethanol. Solution of the alcohol-insoluble barium salt and reprecipitation with four volumes of 95% ethanol was repeated three times (finally, all of the salt was water soluble).

An aqueous solution of the salt was subjected to various analytical procedures described below. It was found that this solution (9.0 ml) contained no inorganic phosphate, less than 0.1 μ moles/ml of "easily hydrolyzable phosphate" (10 minutes, 1 *N* H_2SO_4, 100°), and 13.7 $\pm$ 0.1 μ moles/ml of organically bound phosphate (digestion with H_2SO_4–H_2O_2). It contained 12.6 $\pm$ 0.4 μ moles/ml of reducing sugar, calculated from the reduction equivalent of D-glucosamine (reagent 60 of SHAFFER AND SOMOGYI[6]). The amount of glucosamine-phosphate recovered was 123 μ moles (76% of theory, based on ATP taken).

A second preparation of glucosamine-phosphate was done on a smaller scale in the manner indicated except that the procedure was carried through more rapidly for reasons to be discussed below. The final aqueous solution of the barium salt contained no inorganic phosphate, no 10 minute-hydrolyzable phosphate, and 8.61 μ moles/ml of organically bound phosphate. It was found to contain 8.79 μ moles/ml of reducing sugar calculated as glucosamine.

These data show that the substance isolated is the water-soluble barium salt of a glucosamine-phosphate in which the amino sugar is esterified in such a position that the phosphate group is relatively stable to acid hydrolysis. The reducing property of the ester shows that carbon atom one of the sugar moiety is not substituted.

A sample of barium salt was washed several times with absolute ethanol and absolute ether, followed by drying at room temperature *in vacuo* over P_2O_5. The salt weighed 24.25 mg. It was dried to constant weight at 78° *in vacuo* over P_2O_5. Loss in weight: Found: 9.26% H_2O. Calc. for $C_6H_{12}O_8NPBa$. 2¼ H_2O (435.0): 9.32% H_2O. The

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dry salt was subjected to microanalysis*. Calc. for $C_6H_{12}O_8NPBa$ (394.5): C, 18.26; H, 3.07; N, 3.55; P, 7.85. Found: C, 18.21; H, 3.12; N, 3.48; P, 8.02.

Attempts to prepare a crystalline hydrochloride salt of the ester were unsuccessful.

Reaction of the Ester with Sodium Periodate

The position on the carbon chain of D-glucosamine at which the phosphate group is esterified was determined by the consumption of the oxidant, sodium periodate. Since the conditions under which phosphorylated sugars and amino sugars react completely with periodate are little known[8], the oxidation by this reagent of D-glucosamine and of D-glucose-6-phosphate was investigated preliminarily. VAN SLYKE, HILLER, AND MAC FADYEN[9] have shown that the former is at least partially oxidized; the latter substance was found by VON EULER, KARRER, AND BECKER[10] to yield no formaldehyde when oxidized by periodic acid. The periodate oxidation of D-glucosamine has recently been studied by JEANLOZ AND FORCHIELLI[11] who recommended that the reaction be carried out near p_H 4.5 in a buffered medium at 5°. In the present work, the oxidation was allowed to proceed at room temperature in an unbuffered solution. The initial concentration of D-glucosamine hydrochloride was 0.881 μ mole/ml and that of the dipotassium salt of D-glucose-6-phosphate was 1.01 μ moles/ml. The initial concentration of sodium periodate was 5.77 μ moles/ml. Table I shows the rate at which each substance was oxidized and the extent of the reaction. The relatively rapid rate of oxidation of glucose-6-phosphate is similar to that observed by WOLFROM AND PLETCHER[12] for

TABLE I

RATE OF OXIDATION OF D-GLUCOSAMINE, D-GLUCOSE-6-PHOSPHATE, AND D-GLUCOSAMINE-6-PHOSPHATE BY SODIUM PERIODATE

Compound	Time	Percent of Theoretical Amount of $NaIO_4$ Consumed
D-glucosamine hydrochloride (a)	1 hour 20 minutes	61.5
	2 hours 40 minutes	68.8
	11 hours	96.7
	22 hours 20 minutes	104.9
	48 hours 45 minutes	114.5
D-glucose-6-phosphate (b)	4 hours 10 minutes	99.0
	11 hours 40 minutes	101.1
	13 hours	101.1
	20 hours	101.1
	25 hours	105.2
	40 hours 15 minutes	106.5
D-glucosamine-6-phosphate (c)	14 hours 30 minutes	95.7
	20 hours	98.6

(a) Theory: consumption of 5 moles of oxidant
(b) Theory: consumption of 4 moles of oxidant
(c) Theory: consumption of 4 moles of oxidant

* Analyses for C, H, N, performed by Dr ADELBERT ELEK, 4763 West Adams, Los Angeles California.

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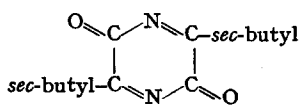
glucose-1-phosphate. With these data available for two related model compounds, the oxidation of the glucosamine-phosphate obtained above was investigated using solutions of the ester from which barium ion had been removed by sulfuric acid, and whose p_H was adjusted subsequently to 4.5. The initial concentration of the ester was 0.685 μ moles/ml and of sodium periodate was 5.77 μ moles/ml. The data in Table I show that the consumption of oxidant was four moles per mole of ester.

These data, together with the correspondence of reducing power and total organically bound phosphate, show that the enzymatically synthesized ester has the phosphate group in position six of the amino sugar. Esterification at position 3, 4 or 5 would give an ester which would consume only three moles of periodate per mole of amino sugar.

Stability of D-*glucosamine-6-phosphate*

When aqueous solutions of the barium salt of this ester were allowed to stand at p_H 8 at or below room temperature, they were found to show gradually increasing absorption at 273 mμ. The absorption spectrum of these solutions had a minimum at 241 mμ and an ill-defined shoulder with small absorption near 300 mμ. No other maxima or minima became evident on long standing. In particular, no absorption maximum occurred near 260 mμ, thus indicating that the ester preparation was substantially free of any nucleotides related to ATP. The solutions gained specific absorption at 273 mμ and lost reducing power concomitantly. These changes took place more rapidly at a p_H of 8 than at a p_H of 3.5, but, once they occurred, they could not be reversed by acidification. The reaction occurring in the ester solutions can be shown to take place also in solutions of D-glucosamine adjusted to p_H 8.0. In such a solution, with an initial sugar concentration of $3.32 \cdot 10^{-2}$ M, absorption at 275 mμ appears at the rate of about 0.003 density units (log I_o/I) per minute (1 cm cell); the reaction has an approximately constant rate for at least 40 hours.

It is believed that these changes in absorption spectrum and reducing sugar values are explicable in terms of a reaction between carbon atoms one and two of two glucosamine molecules, followed by air oxidation of the dihydropyrazine initially formed to give, finally, substituted pyrazines. This is in accord with the original observations of DE BRUYN[13] and STOLTE[14]. It was shown by DE BRUYN that D-glucosamine is slowly transformed in aqueous solution to 2,5-bis-(1,2,3,4-tetrahydroxy-*n*-butyl-)-pyrazine, and that alkali and elevated temperature accelerate the reaction. In the present work, an analogous compound in which the terminal hydroxyl group of each of the side chains is phosphorylated may be one of the substances present in solution. The absorption spectra of 2-hydroxy-pyrazine and several 2-hydroxy-3,6-disubstituted pyrazines have been reported by DUTCHER AND WINTERSTEINER[15], DUTCHER[16,17], and NEWBOLD AND SPRING[18]. These substances are of interest because of their relationship to aspergillic acid which has been shown by DUTCHER to be a pyrazone derivative. The colorless, quinone-like compound (I) obtained by DUTCHER[17]



(I)

by bromine oxidation of desoxyaspergillic acid has a broad absorption maximum at

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285 mμ with a molecular extinction coefficient of over 22,000. The less oxidized, substituted 2-hydroxy-pyrazines have absorption maxima at considerably longer wave lengths than the substance(s) present in old glucosamine and glucosamine-6-phosphate solutions. Therefore, it is possible that the substituted pyrazine demonstrated by DE BRUYN is not the only substance present in solutions of D-glucosamine. Further oxidation may occur to yield products similar to (I) above.

DISCUSSION

Crystalline yeast hexokinase is known to catalyze the phosphorylation by ATP of D-glucose, D-fructose, and D-mannose[2,3]. D-glucosamine has now been shown to be an additional substrate for hexokinase. The phosphorylation of all of these sugars occurs at carbon atom six. That the enzymatic reaction is not wholly unaffected, however, by the nature of the substituent on carbon atom two is shown by the inability of the brain acetone powders of HARPUR AND QUASTEL[1] to phosphorylate N-acetyl-D-glucosamine and by the present finding that this substance appears to be inactive in the yeast hexokinase system also.

The initial velocity of the phosphorylation of D-glucosamine (Fig. 1) shows that the turnover number for this substrate is approximately 12,000 moles/10^5 grams hexokinase/minute at p_H 7.8 and 30°. This is 75% of the turnover number which has recently been obtained for glucose using crystalline hexokinase at a p_H of 8.1 and 30°. Although the effect of insulin in the glucosamine phosphorylating system has not been specifically investigated, it is believed that it serves only to protect the hexokinase against inactivation by dilution and does not enhance the activity of the enzyme toward this substrate. This view is in accord with the findings of BERGER, SLEIN, COLOWICK, AND CORI[2] with respect to glucose phosphorylation.

The phosphorylation of D-glucosamine by hexokinase may be important in the series of anabolic steps whereby naturally occurring polysaccharides containing this sugar are produced.

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SUMMARY

D-glucosamine has been shown to be converted to a phosphorylated amino sugar by ATP in the presence of crystalline yeast hexokinase and magnesium ions. The rate of the reaction is approximately that observed for D-glucose. The turnover number of the enzyme for glucosamine is 12,000 moles/10^5 grams hexokinase/minute at p_H 7.8 and 30°.

The enzymatically synthesized ester of glucosamine was isolated as the barium salt and shown to have full reducing power and to have an acid-stable phosphate group. The ester consumes four moles of sodium periodate per mole of compound. These facts, together with its elemental composition, show that it is D-glucosamine-6-phosphate. Aqueous solutions of the ester are not stable; with time, one or more substances are formed which have characteristic absorption spectra; simultaneously, the reducing power disappears.

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RÉSUMÉ

Nous avons montré que la D-glucosamine est transformée en un sucre aminé phosphorylé par l'ATP en présence d'hexokinase de levure cristalline et d'ions magnésium. La vitesse de la réaction est approximativement celle observée pour le D-glucose. Dans le cas de la glucosamine l'enzyme transforme 12,000 mols/10^5 grammes d'hexokinase/minute à p_H 7.8 et 30°.

Nous avons isolé comme sel de barium l'ester de la glucosamine synthétisé par voie enzymatique; le pouvoir réducteur de cet ester était complet et il contenait un groupe phosphate stable en milieu acide. Une mole d'ester consomme quatre moles de periodate de sodium. Ces faits, ainsi que la composition élémentaire, montrent qu'il s'agit de D-glucosamine-6-phosphate. Les solutions aqueuses de l'ester ne sont pas stables; avec le temps une ou plusieurs substances à spectre d'absorption caractéristique prennent naissance; en même temps le pouvoir réducteur disparaît.

ZUSAMMENFASSUNG

Es wurde gezeigt, dass D-Glucosamin durch ATP in Gegenwart von kristallisierter Hefehexokinase und Magnesiumionen in einen phosphorylierten Aminozucker verwandelt wird. Die Reaktionsgeschwindigkeit ist ungefähr die gleiche wie für D-Glucose. Die Umsatzzahl des Enzyms für Glucosamin ist 12,000 Mol/10^5 Gramm Hexokinase/Minute bei p_H 7.8 und 30°.

Der enzymatisch synthetisierte Glucosaminester wurde als Bariumsalz isoliert und es wurde gezeigt, dass er vollständiges Reduktionsvermögen und eine säurefeste Phosphatgruppe hat. Der Ester verbraucht vier Mol Natriumperjodat pro Mol Verbindung. Diese Tatsachen zeigen, zusammen mit der Elementaranalyse, dass es sich um D-Glucosamin-6-phosphat handelt. Wässrige Lösungen des Esters sind nicht beständig; mit der Zeit bilden sich eine oder mehrere Substanzen mit charakteristischen Absorptionsspektren; gleichzeitig verschwindet das Reduktionsvermögen.

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