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THE BIOSYNTHESIS OF GLUCOSAMINE *

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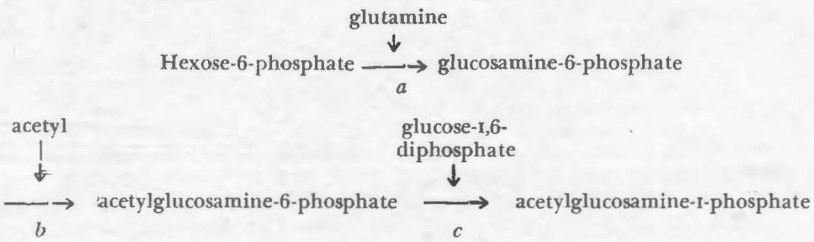
After the isolation of uridine-diphosphate-glucose (UDPG) [1], a very similar compound (UDPG) containing acetylglucosamine instead of glucose was found in yeast [2]. Considering the structural similarity of the two compounds and the coenzymic function of UDPG in the transformation of galactose-1-phosphate into glucose-1-phosphate, it has been considered that UDPG might be involved in the metabolism of hexosamine phosphates. Part of the plan of investigation consisted in a search for enzymes in some organism with a high hexosamine metabolism. Since molds should synthesize large amounts of glucosamine in order to build their cell walls, which contain chitin, experiments have been carried out with *Neurospora crassa*. While no information on a coenzymic function of UDPG has been obtained several enzymes have been found. Besides a chitinase, the *Neurospora* extracts were found to contain the enzymes required for the following sequence of reactions:



Reaction *a* and some preliminary studies on reaction *b* will be dealt with in this paper. As to reaction *c*, it has been detected by using synthetic acetylglucosamine-1-phosphate. *Neurospora* extracts were found to catalyze the transformation of this substance into acetylglucosamine-6-phosphate, but no such activity could be detected in rabbit muscle extracts. The interconversion of the acetylglucosamine phosphates is acelerated by glucose-1,6-diphosphate, a fact which bears a resemblance to its action on the mannose [3] and on the ribose-phosphates [4]. Further studies designed to decide whether the phosphoglucomutase and phosphoacetylglucosaminemutase actions are due to one or two enzymes, and to clarify the mechanism of the stimulation by glucosediphosphate are being carried out.

The synthesis of glucosamine-6-phosphate can also be brought about by a mechanism different from reaction *a*. Thus Harpur and Quastel [5] discovered that glucosamine is phosphorylated by ATP in the presence of brain extracts, and from further studies by Brown [6] and Grant and Long [7], it has been concluded that the phosphorylation is catalysed by hexokinase and that the reaction product is glucosamine-6-phosphate. It is difficult to decide whether this synthesis is a physiological process or simply an unspecific effect.

Similar events have been found to occur with galactosamine. Liver and yeast extracts containing galactokinase were found to phosphorylate galactosamine to a product which appears to be galactosamine-1-phospate [8]. In

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this case, as with glucosamine, the corresponding hexose inhibits the phosphorylation of the hexosamine.

The formation of glucosamine by a process such as reaction *a* would explain the results of TOPPER AND LIPTON [9], who found that in *Streptococcus* the glucosamine formed from glucose-1-^{14}C contained nearly all the label in the 1-position.

METHODS

Analytical. The following methods were used: BLIX [10] for glucosamine. KUNITZ AND MCDONALD [11] for protein. Glutamate was estimated with ninhydrin after paper chromatography with phenol [12]. Amide nitrogen by estimation of the ammonia liberated after heating eleven minutes at 100° in 1 *N* acid [13]. Ammonia by distillation in Conway units [14] and nesslerization.

For the estimation of acetylglucosamine the method of MORGAN AND ELSON [15] was slightly modified in order to make it less sensitive to buffers and to reduce the time needed for colour development. The *p*-dimethylamino benzaldehyde (DAB) reagent was prepared by adding 0.5 g of DAB to 10 ml of concentrated HCl and completing to 100 ml with glacial acetic acid. The analytical procedure was as follows: the neutralyzed unknowns and standards containing 0.1-0.5 μmoles of acetyl glucosamine were taken to 0.5 ml with water. After adding 0.1 ml of 1 *M* sodium carbonate the tubes were heated 5 minutes in a boiling water bath. After cooling 2.5 ml of the DAB reagent were added and mixed immediately with a suitably glass rod. The optical density at 544 mμ was measured after 3 to 5 minutes with a Beckman spectrophotometer. The colour increases during 2 minutes and begins to decrease slowly after 3 minutes. If the time elapsing between the addition of the DAB reagent and the colorimetric reading is equal in all the samples a good proportionality between concentration of acetylglucosamine and optical density is obtained.

Preparation of the enzyme. A wild type *Neurospora crassa* E-5297a was grown for three days on "minimal medium" [16] at 30° under forced aeration. The mycelium was separated by filtration, washed with water, lyophylized and stored over calcium chloride in an evacuated dessiccator at 5°. Extraction of the dried mycelium was effected by homogenizing 0.8 g in 16 ml of water at 0°, followed by centrifugation. The supernatant containing about 40 mg of protein per ml is referred to as crude extract.

Partial purification was carried out as follows: 6.5 ml of acetone were added to 13 ml of the crude extract at 0°. The inactive precipitate was centrifuged off at 0°. To the supernatant 3.9 ml of acetone were added. The precipitate was separated by centrifugation, washed three times with acetone and dried in an evacuated desiccator. The yield was about 60 mg of a white powder.

The formation of "glucosamine" was found to be greater in the presence of 8-hydroxyquinoline, and this fact was attributed to protection of the enzyme from metal inactivation. Therefore, 8-hydroxyquinoline was added to the acetone used in the purification (about 10 mg %) and the buffer (pH 6.5) used for dissolving the enzyme was saturated with 8-hydroxyquinoline.

The enzyme in solution was found to lose activity in a few hours at 5° and in a few days at −10°.

The ratio: μmoles of glucosamine formed/mg protein per hour, was about 0.04 for the crude enzyme and usually about 0.3 for the acetone fractionated enzyme.

Acetylation experiments. The enzyme preparation used was a crude extract which had been dialyzed about two hours against running water. The enzyme system was similar to that used by KAPLAN AND LIPMANN [17]. The CoA solution was an aqueous extract of rat liver. In every case controls in which the reaction was stopped at time = 0 were run simultaneously. The reaction was stopped by immersing the tubes in boiling water followed by centrifugation.

Acetylglucosamine was estimated in the supernatant as described above. In some cases the phosphoric esters were precipitated by adding 0.3 ml of 5 % zinc sulphate and 0.3 *N* barium hydroxide until the suspension gave a rose colour with phenolphthalein.

The formation of "glucosamine". Incubation of hexose-6-phosphate with glutamine and the enzyme gave rise to an increase in the glutamine content. As shown in Fig. 1, hardly any increase took place when glutamine or hexose-6-phosphate were omitted.

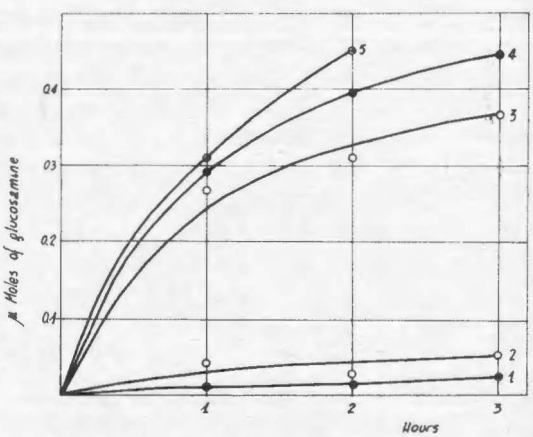

FIG. 1. — The formation of glucosamine. Incubation at 30° of 1 mg of purified enzyme and 0.05 ml of trishydroxymethylaminoethane acetate buffer of pH 6.4 with substrates. Total volume, 0.2 ml.

Curve 1, 0 μmoles of hexose-6-phosphate + 2 μmoles of glutamine
Curve 2, 2 μmoles of hexose-6-phosphate + 0 μmoles of glutamine
Curve 3, 1 μmoles of hexose-6-phosphate + 5 μmoles of glutamine
Curve 4, 4 μmoles of hexose-6-phosphate + 2 μmoles of glutamine
Curve 5, 4 μmoles of hexose-6-phosphate + 5 μmoles of glutamine

The results of an analysis of the chemical changes occurring during the reaction appear in Table 1. The increase in "glucosamine" was approximately equal to the decrease in amide nitrogen of glutamine and to the increase in glutamate. There occurred also a small increase in ammonia in the complete system as well as in the controls without glutamine.

An experiment carried out at different temperatures appears in Table II. At 37° glucosamine formation was faster than at 30° in the beginning, but slower afterwards.

In many experiments it was observed that the enzyme solutions became rapidly inactivated at room temperature. On the other hand, in the experiments of Fig. I the enzyme in the presence of the substrates was still active after 3 hours at 30°. An experiment was therefore carried out in order to ascertain which of the substrates exerted a stabilizing action. Samples of the enzyme were preincubated 30 minutes at 30° with or without substrate, and then the enzyme system was completed.

The glucosamine formed in one hour was as follows (the amount formed during preincubation was subtracted):

Preincubated without substrate	0.50
Preincubated with glutamine	0.76
Preincubated with hexose-6-phosphate	0.70
No preincubation	0.80

Thus both substrates, and specially glutamine, exerted a considerable stabilizing action.

Specificity. Glucose-6-phosphate could be replaced by fructose-6-phosphate, but not by any of the following substances: maltose, glucose, mannose, fructose, fructose-1,6-diphosphate, glucose-1,6-diphosphate, α-galactose-1-phosphate, fructose-1-phosphate, glucose-2-phosphate, xylose-5-phosphate, dihydroxyacetone or glyceraldehyde.

The enzyme preparation was found to contain considerable amounts of the enzyme which catalyzes the interconversion of fructose-6-phosphate into glucose-6-phosphate. The activity of this isomerase was estimated by measuring the disappearance of fructose phosphate with ROE'S [18] method. It was found that under the conditions used for measuring glucosamine formation the equilibrium values for the glucose-fructose esters was attained in about 5 minutes. Therefore, it has not been possible to decide whether the reactant

TABLE I

Balance experiment

The aliquots of the complete system taken for analysis contained: 1,3 μmoles of glutamine, 1.3 μmoles of glucose-6-phosphate and 0.6 mg of purified enzyme and citrate buffer. pH 6.4. Total volume, 0.1 ml. Incubated 3 hours at 30°.

	Δ *"glucosamine"*	Δ *amide*	Δ NH_3	Δ *glutamate*
Complete system	0.42	—0.39	0.17	0.36
No glutamine	0.03	—0.07	0.14	0.08
No hexose-6-phosphate	0.03	—0.04	0 22	0.10

TABLE II

Formation of "glucosamine" at different temperatures
Complete system as in Table I. Results in μmoles.

	Time of incubation (minutes)			
Temperature	30	60	120	180
24°	0.08	0.20	—	0.46
30°	0.13	0.24	0.40	0.52
37°	0.16	0.20	0.34	0.40

is glucose-6-phosphate or fructose-6-phosphate.

"Glucosamine" was formed when glucose-1-phosphate was used instead of hexose-6-phosphate with the crude enzyme, but not with the purified preparations. Under the conditions of the test and with the purified enzyme the phosphoglucomutase activity was very weak.

The substances which were tested with negative results as possible substitutes for glutamine were the following: asparagine, glutamic and aspartic acids, arginine, putrescine, urea, ammonium acetate, alanine, glycine, butyramide, serine, cysteine, lysine, ornithine, valine, leucine and citrulline. Pairs such as ammonium salts with ATP, asparagine and glutamate, etc., also gave negative results.

pH optimum. As shown in Fig. 2, the reaction has a sharp pH optimum at pH 6.4-6.8.

Study of the "glucosamine" ester. 100 μmoles each of glutamine and hexosemonophosphate plus 50 mg of enzyme in 10 ml of 0.025 *M* tris-acetate buffler (pH 6.4) were incubated 3 hours at 30°. The proteins were removed by heat coagulation. Barium acetate was added to the clear liquid and the pH was adjusted to 8. The mixture was centrifuged and the precipitate was washed twice with 1 ml of water. Three volumes of ethanol were added to the pooled supernatants. The precipitate was redissolved in 10 ml of water, a small precipitate centrifuged off and three volumes of ethanol were again added. The precipitate was then dried with ethanol and ether. Yield, 20 mg. These were dissolved in 2 ml of water. The solution contained 41 μmoles of total phosphate, 36 μmoles of reducing substance calculated as glucose, and 5.5 μmoles of "glucosamine". Direct paper chromatography of this ester mixture in different solvents gave irregular results, so that it was decided to remove the phosphate group.

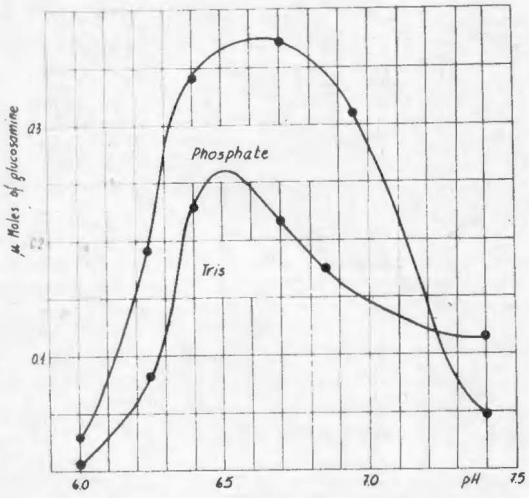

FIG. 2. — pH optimum curve. System composed of 2 μmoles each of hexose-6-phosphate plus 1 mg of enzyme and 0.1 ml of 0.1 *M* phosphate or trishydroxymethylaminoethane acetate buffers. Incubated 2 hours at 30°. The pH was determined on aliquots with a glass electrode.

0.5 ml of the above solution was made 0.01 *M* in respect to Mg^{+2}, and 10 mg of a kidney phosphatase preparation and a drop of toluene were added. After 16 hours at 37°, about 70 % of the phosphate was hydrolysed. The mixture was then deproteinized with trichloroacetic acid, washed with ether and used for paper chromatography. A sample of glucosamine-6-phosphate was run simultaneously. One of the solvents used was a mixture of ethyl acetate-pyridine-ammonia [2] with which it is possible to separate glucosamine from galactosamine. The other solvent was phenol-water [19] with ammonia. Phenol without ammonia was used with paper which had been immersed in 0.1 *M* zinc sulphate and dried in air. This procedure was based on a previous observation which disclosed that zinc ions greatly retard the migration of hexosamines but only have a small influence on others sugars. It was also observed that with an alkaline solvent there was no retardation by zinc ions.

The results of the chromatography are shown in Table III. The ex-ester sugar mixture gave spots which migrated like glucose and glucosamine. The position of the substances was revealed with the aniline phthalate reagent [20], and that of hexosamines was checked with the modified ELSON AND MORGAN reagent [19]. Besides glucose and glucosamine, the ex-ester mixture contained small amounts of another hexose which migrated like mannose. In some cases a very faint spot with the *R-glucose* value of fructose was observed. The presence of these sugars is not surprising since the sample of hexose-6-phosphate used was obtained by the action of yeast enzymes.

TABLE III

Paper chromatography of the "glucosamine" ester after treatment with phosphatase

	Ethyl acetate-pyridine-NH_3	*Rglucose Phenol-NH_3*	*Phenol-SO_4Z treated paper*
Ex-"glucosamine" ester	0.99, 0.62, 1.39	1.0, 1.73, 1.54	1.01, 0.13, 1.19
Ex-glucosamine-6-phosphate *	0.61	1.69	0.14
Glucosamine	0.61	1.69	0.14
Galactosamine	0.36	1.87	0.15
Fructose	1.42	1.47	1.45
Mannose	1.35	1.47	1.15

* Prepared from glucosamine with ATP and hexokinase 6.

Acetylation. As shown in Table IV, *Neurospora* extracts, when suitably supplemented are able to bring about the acetylation of glucosamine. These extracts are also able to catalyze the phosphorylation of glucosamine (Table V) and contain phosphatase. The result of the action of this set of enzymes is that starting with free glucosamine or with glucosamine-6-phosphate, the reaction products are similar. Most of the acetylglucosamine appears free and a part precipitates with zinc suplphate-barium hydroxide as would acetylglucosamine phosphate (Table VI). If acetylglucosamine phosphate was formed, it could not have arisen by phosphorylation of acetylglucosamine, since this process is not catalyzed by the extracts (Table V). Therefore, it seems logical to conclude that gluco-

TABLE IV

The acetylation of glucosamine

Complete system: 2 μmoles of glucosamine, 4 μmoles of ATP, 0.065 ml of 1 *M* sodium acetate, 0.05 ml of 0.1 *M* cysteine, 0.05 ml of 0.2 *M* sodium citrate of pH 7, 0.1 ml of CoA solution, 0.05 ml of 0.1 *M* magnesium chloride and 0.2 ml of crude dialyzed enzyme. Final volume, 0.7 ml. Incubation 2 hours at 37°.

	μ moles of acetylglucosamine formed
Complete system	0.38
No glucosamine	0.025
No CoA	0.075
No ATP	0.025
No Mg++	0.070

TABLE V

The phosphorylation of glucosamine

Complete system as in Table III, but without CoA. The difference in glucosamine or acetylglucosamine content between samples incubated with and without ATP was considered to be due to phosphorylation. The estimations were carried out after precipitation of proteins and phosphoric esters with zinc sulphate and barium hydroxide.

Substrate	*μ moles of substrate phosphorylated*	
	No Mg ++	*With Mg ++*
Glucosamine	0.8	1.80
Acetylglucosamine	0	0

TABLE VI

The acetylation of glucosamine phosphate

Complete system as in Table III. The acetylglucosamine content of the supernatants after zinc sulphate-barium hydroxide precipitation was considered as free acetylglucosamine.

Substrate	*Time of incubation (minutes)*	*μmoles of acetylglucosamine formed*	
		Total	*Free*
Glusosamine	30	0.26	0.18
	60	0.40	0.28
	120	0.38	0.25
Glucosamine-6-phosphate	120	0.60	0.49

TABLE VII

The acetylation of the "glucosamine" ester

The "glucosamine" ester was obtained by incubation during 3 hours at 30° of 2 μmoles each of glutamine and hexose-6-phosphate with the purified enzyme. The controls contained the same substances plus glucosamine (0.5 μmoles) or glucosamine-6-phosphate (1 μmol), and the reaction was stopped at $t = 0$. The acetylating system described in Table III was then added.

	μ moles of acetylglucosamine formed
"Glucosamine" ester	0.21
Control with glucosamine	0.20
Control with glucosamine-6-phosphate	0.31

samine-6-phosphate can be acetylated directly to acetylglucosamine phosphate.

Table VII shows the results of the action of the acetylating system on the "glucosamine" ester formed from hexose phosphate and glutamine. This substance gave rise to acetylglucosamine, as did glucosamine or glucosamine-6-phosphate.

Discussion

The substance formed from hexose-6-phosphate and glutamine gives the Elson and Morgan and Dische[21] reactions for hexosamines. It can be prepared as the barium salt admixed with hexosemonophosphates. It behaves like glucosamine-6-phosphate when incubated with the acetylating system of *Neurospora*, and after dephosphorylation with phosphatase glucosamine can be identified by paper chromatography with selected solvents.

All this is considered as evidence proving that the product is glucosamine phosphate. The ester gives positive reactions for hexosamines, so that a 1-ester can be excluded, and since a migration of the phosphate during the reaction appears unlikely, the product should be glucosamine-6-phosphate.

No evidence for the necessity of a colactor for the formation of glucosamine phosphate was obtained. Thus, no stimulation was obtained by the addition of different ions, ATP, pyridoxal phosphate, UDPAG, etc. The mechanism of the reaction cannot be discussed until it is decided whether the reactant is fructose-6- or glucose-6-phosphate, In order to settle this point it will be necessary to obtain enzyme preparations free from isomerase.

Further investigation will also be necessary in order to decide whether the acetylation step takes place on free glucosamine, on glucosamine-6-phosphate or on both. In connection with this point, it may be mentioned that Chou and Soodak[22] extrated an enzyme from pigeon liver which catalyzed the acetylation of free glucosamine and galactosamine, but that the corresponding phosphates were not tested.

SUMMARY

A partially purified enzyme has been prepared from *Neurospora crassa* which catalyzes the formation of glucosamine phosphate from hexose-6-phosphate and glutamine. The glucosamine phosphate was identified by colour reactions, by dephosphorylation and paper chromatography and by its behaviour towards an acetylating system.

Quantitative analysis of amide nitrogen, glutamate, and hexosamine agreed with the following equation:

Hexose-6-phosphate + glutamine → glucosamine-6-phosphate + glutamate.

Crude *Neurospora* extracts were found to phosphorylate glucosamine in the presence of ATP and, when suitably supplemented, to acetylate glucosamine or glucosamine phosphate.

RÉSUMÉ

Les auteurs out préparé et partiellement purifié à partir de *Neurospora crassa*, un enzyme qui catalyse la formation de glucosamine phosphate à partir d'hexose-6-phosphate et de glutamine. Le glucosaminephosphate a été identifié par ses réaction colorées, par la déphosphorylation, par la chromatographie sur papier et par son comportement en présence d'un système acétylant.

L'analyse quantitative de l'azote amidé, du glutamate et de l'hexosamine est en accord avec l'équation suivante:

Hexose-6-phosphate + glutamate → Glucosamine-6-phosphate + glutamate.

Les extraits bruts de *Neurospora* phosphorylent la glucosamine en présence d'ATP et écatylent la glucosamine et le glucosaminephosphate, quand on les supplémente convenablement.

ZUSAMMENFASSUNG

Ein teilweise gereinigtes Enzym, das die Bildung von Glucosaminphosphat aus Hexose-6-phosphat und Glutamin katalysiert, wurde aus *Neurospora crassa* dargestellt. Das Glucosaminphosphat wurde durch Farbreaktionen, Desphosphorylierung und Papierchromatographie und durch sein Verhalten gegenüber einem acetylierten System identifiziert.

Die quantitative Analyse von Amidstickstoff, glutaminsaurem Salz und Hexosamin stimmte mit der folgenden Gleichung überein:

Hexose-6-phosphat + Glutamin → Glucosamin-6-phosphat + Glutaminsaures Salz.

Es wurde gefunden, dass rohe *Neurospora*-extrakte Glucosamin in Gegenwart von ATP phosphorylieren und, wenn sie geeignet ergänzt werden, Glucosamin oder Glucosaminphosphat acetylieren.

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