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Pathway of Uridine Diphosphate N-Acetyl-D-Glucosamine Biosynthesis in Phaseolus aureus[1]

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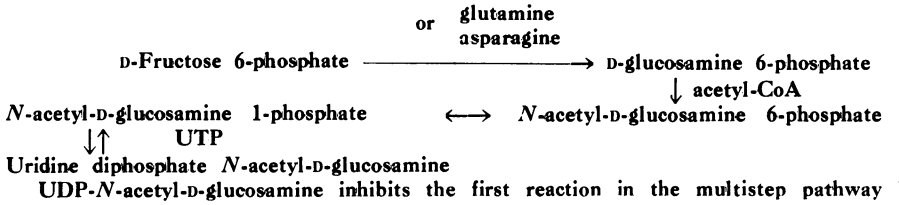
Abstract. Studies with extracts obtained from mung beans (*Phaseolus aureus*) showed that UDP-*N*-acetyl D-glucosamine is formed from D-fructose 6-phosphate by a series of the following enzymic reactions:



UDP-*N*-acetyl-D-glucosamine inhibits the first reaction in the multistep pathway leading to its biosynthesis.

N-Acetyl D-glucosamine is known to exist in polymeric form in chitin, a compound that constitutes the exoskeletons of crustaceans and the cell walls of fungi, in heteropolysaccharides such as hyaluronic acid, and in the peptidoglycan of bacterial cell walls. In the past, higher plants were considered to be devoid of D-glucosamine or *N*-acetyl D-glucosamine either in free or combined form. However, it has been reported within the last decade that certain plants and especially pollen grains contain a substance which on hydrolysis produces D-glucosamine (19, 26). This compound was also shown to be present in hydrolyzates of soybean glycoprotein (15, 23, 48) and in glycoproteins of a number of other plants (35). It was isolated in crystalline form from the hydrolysis products of glycolipids of higher plants (8). Extracts of pineapple plant tissue have been shown to give an Elson-Morgan test, indicating the presence of free amino sugar (41).

Nucleotide-bound sugars have been shown to occur in higher plants. Thus, UDP-*N*-acetyl-D-glucosamine was isolated from mung-bean seedlings (*Phaseolus aureus*) (43) and the same sugar nucleotide was identified in extracts of barley plants (3). UDP-*N*-Acetyl-D-galactosamine was also later isolated from Dahlia tubers (17). Furthermore, it was shown that an enzyme which catalyzes the formation of UDP-*N*-acetyl-D-glucosamine from UTP and *N*-acetyl α-D-glucosamine 1-phosphate is present in mung bean seedlings (19).

Lowther and Rogers (27) found that the nitrogen atom of D-glucosamine in streptococcal suspensions is derived from L-glutamine. Leloir and Cardini (24) demonstrated that cell-free extracts of *Neurospora crassa* catalyzed the conversion of hexose phosphate and L-glutamine to a hexosamine phosphate which was identified as D-glucosamine 6-phosphate, and that the hexosamine nitrogen is derived from L-glutamine. Alternatively, crude pig kidney preparations were shown by Leloir and Cardini (25) to contain glucosamine 6-phosphate deaminase, later purified by Comb and Roseman (10), which catalyzes the reversible conversion of D-glucosamine 6-phosphate into fructose 6-phosphate and ammonia, thus affording a pathway for the biosynthesis of glucosamine in which the amino group is derived from ammonia. Fructose 6-phosphate was shown to be the amino group acceptor in microbial and mammalian systems (14), rather than D-glucose 6-phosphate (32, 33).

It has been demonstrated that acetylation of D-glucosamine by pigeon liver extracts occurs by acetyl coenzyme A (9), and that *Neurospora crassa* extracts acetylate D-glucosamine 6-phosphate (11).

A specific phosphoacetylglucosamine mutase was found by Reissig (36) in extracts of *Neurospora crassa*, catalyzing the reversible conversion of *N*-acetyl-D-glucosamine 6-phosphate to *N*-acetyl-D-glucosamine 1-phosphate. The latter compound serves in the presence of UTP as a substrate for the pyro-

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phosphorylase reaction. UDP-*N*-acetylglucosamine pyrophosphorylase activity was detected in liver (28, 42) and in yeast (16). Subsequently, pyrophosphorylase from calf liver and *Staphylococcus aureus* was purified and studied by Strominger and Smith (46).

The present investigation is concerned with the pathway leading to the formation of UDP-*N*-acetyl D-glucosamine in mung beans (*Phaseolus aureus*).

Materials and Methods

Materials. L-Arabinose and D-glucosamine hydrochloride were purchased from the Pfanstiehl Chemical Company, inorganic pyrophosphatase from the Worthington Biochemical Corporation. Bovine serum albumin, wheat germ acid phosphatase and uridine diphosphate *N*-acetyl-D-glucosamine were purchased from the Sigma Chemical Company. Lyophilized *Crotalus adamanteus* venom, purchased from Ross Allen's Reptile Institute, was used as the source of phosphodiesterase. *N*-Acetylglucosamine 1-P was prepared from uridine diphosphate *N*-acetylglucosamine by the action of venom phosphodiesterase at pH 8.5, and was isolated by electrophoresis in Buffer (I). D-Lyxose was purchased from Eastman Organic Chemicals, acetyl-1-^{14}C coenzyme A (specific radioactivity 54.4 μc/μmole) from the New England Nuclear Corporation. The following compounds were obtained from the Nutritional Biochemical Corporation: L-glutamine and L-asparagine, the *N*-acetyl derivatives of D-glucosamine, D-galactosamine and D-mannosamine, D-fructose 6-P (barium salt) and D-glucosamine 6-P (barium salt). The barium was removed from D-fructose 6-P by Dowex 50 (H^+) and the solution adjusted to pH 6.5 with 1 N NaOH. D-Glucosamine 6-P was freed from the barium with 0.5 N H_2SO_4, and the acidic solution kept frozen (6). The following were purchased from Calbiochem: D-glucose-1,6-diP, 3-P glycerate, 3-P-glycerate kinase and glyceraldehyde-3-P-dehydrogenase. Other nucleotides were commercial preparations.

Analytical Methods. Reducing sugars were detected on paper with the aniline phosphate spray reagent (1). Amino sugars and their derivatives were made visible by a ninhydrin spray (4), or by a modified chlorination reaction (34) of Rydon and Smith (38). The *N*-acetyl-hexosamines, after chromatography on borate-treated paper (7), were detected with 0.5 N NaOH in ethanol (44), or with 0.5 NaOH in ethanol-1-propanol (6:4, v/v) (40). Sugar phosphate derivatives were located with the Bandurski and Axelrod spray reagent (2), nucleotides and derivatives with an ultraviolet lamp. Protein was determined by the biuret method of Gornall *et al.* (18), using bovine serum albumin as standard.

Electrophoresis and Chromatography. Paper electrophoresis was carried out on Schleicher and Scheull No. 589 Orange Ribbon paper at about 45 v/cm with the following buffer systems: Buffer (I) 0.2 M ammonium formate, pH 3.6; Buffer (II) 0.01 M citrate—0.02 M phosphate (pH 6.0) a saturated solution of picrate-caffeine serving as a marker.

Paper chromatography was carried out on Whatman No. 1 filter paper at room temperature with the following solvent systems: (A) *n*-butanol-pyridine-water (6:4:3, v/v); (B) ethanol-1 M ammonium acetate, pH 3.8 (7.5:3, v/v); (C) ethanol-1 M ammonium acetate, pH 7.5 (7.5:3, v/v); (D) isobutyric acid-1 M ammonium hydroxide (5:3, v/v); (E) *n*-butanol-pyridine-0.1 N HCl (5:3:2); (F) *n*-butanol-pyridine-water-acetic acid (60:40:30:3).

Preparation of Enzyme Extracts. L-Glutamine D-Fructose 6-Phosphate Amidotransferase (EC 2.6.1.16). Mung bean seeds were soaked overnight in distilled water; they were rinsed several times with distilled water, blotted with paper towels and decoated. The decoated beans, 16 g, were chilled at 5° for 2 hours, ground in a mortar with acid-washed sand in 20 ml of 0.05 M phosphate buffer made 0.002 M in EDTA, pH 6.6, and filtered through 2 layers of cheesecloth. The extract was then centrifuged at 12,000 × *g* for 12 minutes, and the supernatant solution brought to 30% saturation in ammonium sulfate made 1 mM in EDTA. After stirring for 1 hour in an ice bath before centrifuging as above, the 30% saturated ammonium sulfate precipitate was taken up in 2 ml of the grinding buffer and was immediately used as the source for amidotransferase activity.

D-Glucosamine 6-Phosphate Transacetylase (EC 2.3.1.4). Mung bean seeds were soaked overnight in distilled water, rinsed several times, decoated and treated as before. In this case, however, 0.05 M cacodylate buffer containing 2 mM EDTA, pH 7.2, was used for grinding and suspension of the 30% saturated ammonium sulfate precipitate. This fraction was used as an enzyme source in subsequent experiments.

Acetylglucosamine Phosphomutase (EC 2.7.5.2). Three-day old whole mung bean seedlings, germinated with aeration, were shaken several times in cold distilled water to free them from the seed coat remnants. All subsequent operations were performed at 0 to 5°. The method for extraction closely resembled those used in this laboratory by previous workers. Lots of 100 g seedlings were ground in a chilled mortar with 100 ml of ice-cold 0.01 M phosphate, pH 7.3-0.5 M sucrose-0.01 M mercaptoethanol. After filtration through 2 layers of cheesecloth, the homogenate was centrifuged at 30,000 × *g* for 20 minutes, the sediment discarded, and the supernatant liquid made 50% saturated by the addition of solid ammonium sulfate. The precipitate was separated by centrifugation, dissolved in 3 ml of 0.05 M tris, pH 7.3-0.05 M mercaptoethanol buffer, and dialyzed overnight against 1 liter of the same buffer. After a small amount of insoluble precipitate was removed by centrifugation, this fraction was used immediately as the enzyme source.

UDP-N-Acetylglucosamine Pyrophosphorylase

(*EC 2.7.7.23*). The extract containing mutase activity (see above) was further fractionated with $(NH_4)_2SO_4$, and the fraction precipitating between 40 and 50 % saturation was dissolved in a minimum volume of 0.05 M tris, pH 7.3-0.05 M mercaptoethanol buffer, dialyzed overnight in the same buffer, and used as enzyme source.

Enzyme Assays. Enzymic formation of hexosamine-6-P and of *N*-acetyl-D-glucosamine-6-P was followed using a modified Elson-Morgan test (14, 37) with D-glucosamine and *N*-acetyl-D-glucosamine as standards, respectively. For hexosamine-6-P determination, the indole-hydrochloric acid reaction method (13) was also used.

L-Glutamine D-Fructose 6-Phosphate Amidotransferase. The standard reaction mixture contained 0.1 ml of 0.15 M L-glutamine, 0.1 ml of 0.1 M D-fructose-6-P, 0.1 ml of 0.3 M phosphate buffer made 0.01 M in EDTA, pH 6.6, and 0.2 ml of enzyme extract in a total volume of 0.5 ml. The mixture was incubated at 30° for 3 hours, then placed in boiling water for 2 minutes, cooled and frozen. After thawing, the mixture was centrifuged at 10,000 × *g* for 10 minutes. Aliquots of the clear supernatant solution were analyzed for hexosamine phosphate (14). A control which was run simultaneously with a mixture of the same composition, but which was kept in boiling water for 2 minutes prior to incubation under the same conditions was subtracted. With crude extracts the incubation mixtures were sometimes doubled proportionately in order to have larger aliquots for hexosamine phosphate determinations. One unit of enzyme activity is defined as that quantity catalyzing the formation of 1 μmole of hexosamine phosphate per hour under the conditions of assay. Specific activity is expressed as activity per mg of protein.

D-Glucosamine 6-Phosphate Transacetylase. The standard reaction mixture contained 25 μl (1.6 μmoles) of D-glucosamine-6-P, 25 μl of 0.3 M cacodylate buffer made 0.01 M in EDTA, pH 7.2, 100 μl of enzyme extract, and 5 μl of acetyl-1-^{14}C coenzyme A, in a total volume of 155 μl. Two controls were simultaneously run: one lacking the glucosamine-6-P substrate, and another containing the complete reaction mixture, but which was placed in boiling water for 2 minutes prior to incubation (the mixture was heated, cooled, and acetyl-1-^{14}C coenzyme A was added). The mixtures were incubated for 3 hours at 28 to 30°. They were then frozen and, after thawing, subjected to electrophoresis in Buffer (I). The procedure used to measure the extent of acetyl-1-^{14}C incorporation into D-glucosamine-6-P and for characterization of the labeled product was as follows: the papers were scanned after electrophoresis with a monitor and the radioactivity located near the picrate spot indicators was marked. Horizontal strips, 0.5 cm wide, with a mean $R_{picrate}$ of 0.70 were cut out and dried *in vacuo* over NaOH-H_2SO_4 for 12 to 16 hours. The strips were then eluted with water and the eluates evaporated *in vacuo* over NaOH-H_2SO_4. The residues were dissolved in 0.1 ml of 0.05 M citrate buffer, pH 5.5, small amounts of wheat germ acid phosphatase were added, and the mixtures were incubated at 30° for 6 hours, then evaporated to dryness *in vacuo*. After suspending the residues in 1 ml of water, 0.5 to 1.0 ml of mixed-bed resin, 20 to 50 mesh Bio-Rad AG 501-X8 (D) was added. The mixtures were filtered through paper with vacuum after stirring intermittently for 30 minutes and the resin washed several times with water. The filtrates and washings were combined and evaporated *in vacuo* over P_2O_5 to dryness. The residues were dissolved in 15 to 40 ml of water and aliquots of these solutions were spotted on paper together with unlabeled *N*-acetyl-D-glucosamine serving as an internal marker. The borate-treated papers were subjected to chromatography in Solvent (A). Horizontal strips, 0.5 cm wide, corresponding to the standard *N*-acetylglucosamine were cut from the paper, placed in vials with 10 ml of liquifluor solution prepared by adding 145 ml of liquifluor (a mixture of 50 g POP and 0.625 g POPOP, New England Nuclear Corporation) to 3 kg toluene, and counted in a Packard Tri-Carb liquid scintillation counter Model 3003 (efficiency about 50 %).

Acetylglucosamine Phosphomutase. The standard reaction mixture for following mutase activity in the direction of *N*-acetylglucosamine-6-P formation was similar to that used for measuring L-glutamine D-fructose 6-phosphate amidotransferase activity. The modification of Reissig *et al.* (37) of the Elson-Morgan test was used. A control containing the complete reaction mixture but lacking the enzyme extract was subtracted.

UDPAG[2] *Pyrophosphorylase.* Pyrophosphorylase activity was followed in the direction of pyrophosphorolysis by using the PP_i-dependent formation of UTP according to the method described by Verachtert *et al.* (47). The standard assay mixture, in a cuvette with a 1 cm light path, contained 1.0 μmole of Mg^{2+}, 1.0 μmole of PP_i, 0.5 μmole of hydrazine sulfate, 0.6 μmole of 3-P-glycerate, 0.12 μmole of NADH, 0.2 μmole of UDPAG, excess 3-P-glycerate kinase and glyceraldehyde-3-P dehydrogenase, and enzyme extract in a total volume of 0.5 ml with 0.05 M triethanolamine buffer (pH 7.8) at 25°. Oxidation of NADH was followed at 340 mμ using a Zeiss model PMQ II spectrophotometer. A control containing the complete reaction mixture but with a heat-denatured enzyme was subtracted. Verachtert *et al.* (47) consider the possibility that phosphatases present in crude extracts may compete with 3-P-glycerate kinase for the nucleoside triphosphates formed and consequently would result in a low estimate of the sugar 1-phosphate nucleotidyltransferase level. In our experiments, electrophoresis

[2] The abbreviations used are: UDPAG, uridine diphosphate N-acetyl-D-glucosamine; UDPG, uridine diphosphate D-glucose.

in Buffer (I) and chromatography in several solvent systems of an incubated reaction mixture containing 0.5 μmole of UDPAG, 1.0 μmole of PP_i, 0.5 μmole of Mg^{2+}, 10 μmoles of NaF and 20 μmoles of enzyme extract in 0.1 M tris buffer, pH 7.8, showed a U.V.-absorbing compound that cochromatographed with authentic UTP, and a barely detectable U.V.-absorbing compound which cochromatographed with authentic UDP. Evidently NaF does not completely inhibit phosphatase activity, but no attempt was made to estimate the extent of this activity.

In the direction of UDPAG biosynthesis, the assay was not made quantitative, although excellent recoveries of the amino sugar nucleotide were obtained. The standard reaction mixture contained 2 μmoles of *N*-acetylglucosamine-1-P, 2 μmoles of UTP, 1.25 μmoles of $MgCl_2$, excess inorganic pyrophosphatase and 100 μl of enzyme extract in a total volume of 0.2 ml with 0.1 M tris buffer, pH 7.8. After incubation for 2 hours at 30° the reaction was stopped by addition of 3 volumes of warm ethanol (30). The resultant precipitate was removed by centrifugation, and the supernatant solution concentrated *in vacuo* over P_2O_5. The residue was then taken up in water and subjected to electrophoresis in Buffer (I). A compound with electrophoretic mobility of authentic UDPAG was eluted from the paper, concentrated *in vacuo* over P_2O_5 and purified by paper chromatography in solvents (B) (C) and (D). Controls were run, lacking UTP or containing a heat-denatured enzyme.

Results

Determination of Hexosamine Content of Mung Beans. Before attempting to study the path of amino sugar nucleotide biosynthesis in mung beans, it was desirable to determine the amino sugar content of these beans.

Beans were germinated up to 5 days in a cabinet of 100 % humidity. The whole seedlings were collected and, after removing the seed coats, were blotted with paper towels. They were homogenized in a Waring Blendor with 10 volumes of acetone, filtered with vacuum, and the residue air-dried. The hexosamine analyses of non-germinated beans were carried out on acetone powders prepared from beans soaked overnight in distilled water. Removal of the seed coats from the soaked beans did not alter their hexosamine content.

The acetone residues were ground in a mortar, the powdered material suspended in 10 to 15 volumes of 0.5 N HCl and allowed to reflux in a boiling water bath for 10 hours. After cooling in ice water, the mixture was centrifuged at 8000 × *g* for 20 minutes and the clear supernatant solution used for analysis.

Hexosamine was isolated from 2 ml aliquots of the supernatant liquid by passing it through a 200 to 400 mesh Dowex-50 (H^+) resin as described by Boas (5). The column was washed with water to remove neutral material, and the amino sugar was eluted with 2 N HCl. The Dowex eluate was diluted to 10 ml with water, and portions of this eluate were evaporated to dryness *in vacuo* over P_2O_5-NaOH. The residue was then dissolved in water and analyzed by the modified Elson-Morgan procedure (14). The results are given in table I. Recoveries of authentic samples of D-glucosamine hydrochloride from the resin were 90 to 92 %.

To test the method for hexosamine determination, an egg white sample from a fresh egg, the composition of which is known, was hydrolyzed and analyzed by the above procedure. The yield was 3.6 μmoles of hexosamine per 1 ml of egg white hydrolysate; the calculated yield of hexosamine from the average composition of egg white (49) is 3.4 μmoles per ml of hydrolysate, the agreement being within 6 %.[3]

Properties of L-*Glutamine* D-*Fructose 6-Phosphate Amidotransferase.* The specific activity of the 30 % saturated ammonium sulfate fraction was 0.011 μmole of hexosamine phosphate per hour per mg of protein. This fraction lost activity upon freezing and thawing.

[3] The hexosamine content of mung bean seeds as recorded in table I agrees with published values for other seeds (32). An increase in hexosamine content of approximately 30 % over the dormant beans was noticed upon continued germination up to 5 days. For the 2 day germinated seedlings it may be noted that less than 3 % of the total hexosamine could have been in free form.

Table I. *Analyses for Hexosamine Content of Germinated and Non-Germinated Mung Bean Samples*

Material[1]	μmoles of hexosamine per 100 g of material	g hexosamine per 100 g of material
Ground whole beans	320	0.057
Non-germinated beans (soaked overnight in water)	290	0.052
2 Day germinated seedlings	360	0.064
2 Day germinated seedlings, control[2]	<11	<0.002
3 Day germinated seedlings	350	0.063
4 Day germinated seedlings	430	0.077
5 Day germinated seedlings	440	0.079

[1] Air-dried acetone powders.

[2] Material was suspended in 10 volumes of 0.5 N HCl and maintained at room temperature with intermittent stirring for 16 hours.

A preparation with 0.31 unit per ml gave 0.13 and 0.020 units per ml after a first and second thawing, respectively. If nucleic acids were removed from the initial extract with $MnCl_2$, no detectable amidotransferase activity remained. The activity was also destroyed by dialysis. It appears that dialysis or complexing with Mn^{2+} might have removed a cofactor required for activity; no cofactor requirements, however, could be demonstrated for activities of enzyme extracts isolated from various sources by other workers, nor could several seemingly possible cofactors such as pyridoxal phosphate exert any stimulatory effects (14, 24).

Using a freshly prepared 30 % saturated ammonium sulfate fraction as enzyme source, the formation of hexosamine phosphate was linear at least up to 5 hours, whereas with a preparation which had been stored frozen overnight the amide transfer reaction was linear up to 3 hours, and then the rate decreased. From these experiments it may be inferred that the enzyme of the fresh preparation was not only more active but also more stable to denaturation upon prolonged incubation than one which had been frozen and thawed. The results of these experiments are shown in figure 1.

Since the rate of hexosamine phosphate formation was linear, substrate was not limiting. Previous results of incubation experiments had shown that maximum transamination was achieved with 0.02 M D-fructose-6-P and 0.015 M L-glutamine (final conc). Increasing the L-glutamine concentration to 0.03 M had little effect on the extent of the reaction. Maintaining the L-glutamine concentration at 0.03 M, the D-fructose-6-P concentration was varied from 0.02 to 0.001 M in order to observe its effects on product formation. The results are given in figure 2. From a double reciprocal plot the *Km* for D-fructose-6-P was calculated to be 9×10^{-2} M.

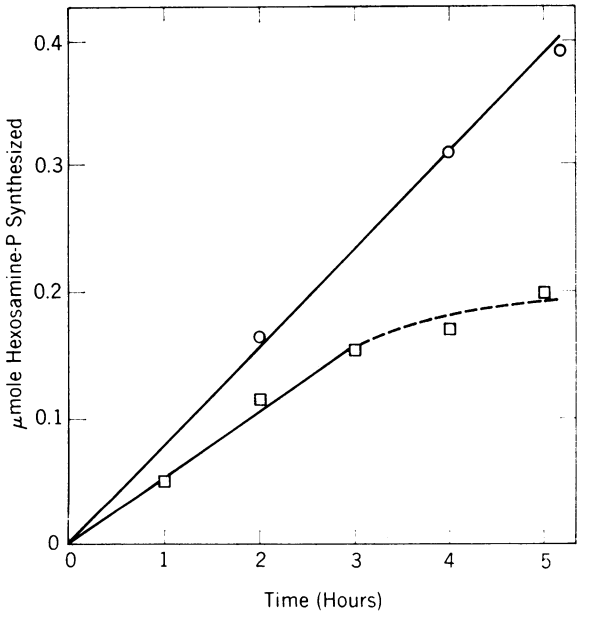

FIG. 1. Time course of hexosamine phosphate synthesis. - ○ - ○ -, enzyme source is fresh 30 % saturated ammonium sulfate precipitate; - □ - □ -, enzyme source is frozen and thawed 30 % saturated ammonium sulfate precipitate.

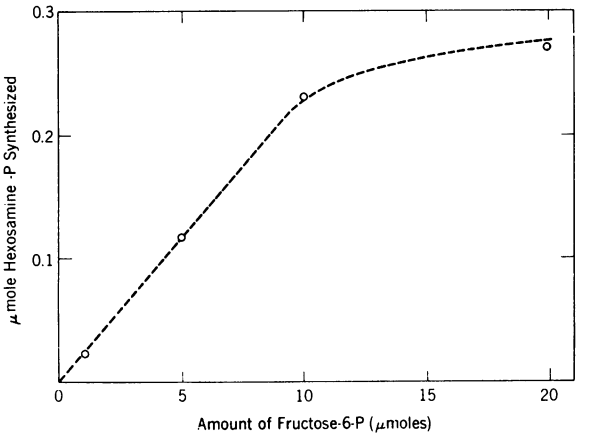

FIG. 2. Effect of D-fructose-6-P concentration on hexosamine phosphate formation. Enzyme source: 30 % saturated ammonium sulfate precipitate.

Crude mung bean extracts showed amidotransferase activity with both L-glutamine and, as far as is known for the first time, with L-asparagine. With L-glutamine as an amino group donor 2 separate preparations gave 0.044 and 0.042 units per ml of extract, respectively, and with L-asparagine 0.033 and 0.030 units per ml of extract, respectively. With either fresh or frozen samples of the 30 % saturated ammonium sulfate fraction no amidotransferase activity could be observed using L-asparagine as an amide donor. Whether there are 2 aminating systems in mung beans, one using L-glutamine and another L-asparagine, remains to be determined.

Characterization of Product from D-Fructose 6-Phosphate and L-Glutamine Incubation. In order to isolate sufficient material for characterization a large scale reaction mixture was prepared, consisting of 0.8 ml of 0.1 M D-fructose-6-P, 0.8 ml of 0.15 M L-glutamine, 0.8 ml of 0.3 M phosphate made 0.01 M in EDTA, pH 6.6, and 1.6 ml of the enzyme extract in a total volume of 4 ml. The mixture was incubated for 3 to 3 and one-half hours at 30° and, after heating in boiling water for 2 minutes, was frozen. It was then allowed to melt and centrifuged at 10,000 × *g* for 10 minutes. The pH of the clear supernatant solution which was initially 6.6 was lowered to 1.8 with 1 N HCl. A silky white precipitate formed as the pH was lowered. This mixture was frozen and then centrifuged as before. A clear supernatant solution was obtained with a pH 1.9.

Analysis of an aliquot of the incubation mixture by a modified Elson-Morgan reaction (14) showed that 1.5 to 2.0 % of the D-fructose 6-phosphate had

been transformed into hexosamine phosphate. Quantitative recovery of the hexosamine phosphate in the pH 6.6 supernatant solution was obtained in the pH 2 supernatant solution. This supernatant solution was passed through a 200 to 400 mesh Dowex-50 (H^+) column, 1.4 × 12 cm. The resin was washed with water until the total volume of liquid reached 15 ml. The hexosamine phosphate was eluted from the resin with 20 to 30 ml of water (33), the yield being 15 to 30 % as calculated from the hexosamine phosphate content of the pH 2 supernatant solution. Aliquots from 2 separate Dowex eluates were assays by the modified Elson-Morgan reaction and by the indole-hydrochloric acid method. The amounts of hexosamine phosphate per aliquot determined by the 2 methods were, in μmoles, 0.10 and 0.13, 0.066 and 0.064, respectively.

In order to ascertain the purity of the Dowex eluate, it was subjected to electrophoresis in the 2 systems, Buffer (I) and Buffer (II). In both cases approximately 0.03 μmole of hexosamine phosphate from a Dowex eluate and 0.03 μmole of standard D-glucosamine-6-P were used. The results are summarized in table II. Although the presence of neutral sugar was not excluded by these tests, contamination of the Dowex eluate does not seem likely since it would not have been retained by the resin. The 2 systems do separate the other possible contaminants, *e.g.* L-glutamine, L-glutamate, D-fructose-6-P from hexosamine phosphate. It can therefore be concluded that the product of the Dowex eluate was free from impurities.

Ninhydrin Oxidation. To check the migration of the pentose phosphate produced by oxidative deamination of hexosamine phosphate the following experiment was performed: To 20 μl of standard D-glucosamine 6-P (approx 0.05 μmole) and 20 μl of a Dowex eluate (0.05 μmole of hexosamine phosphate) in separate tubes 25 μl of ninhydrin reagent were added (45). The tubes were fitted with air condensers and placed in boiling water for 30 minutes. After cooling, the entire contents were spotted on paper and subjected to electrophoresis in Buffer (I). The results are shown in table III.

It appears that a pentose phosphate was produced by ninhydrin oxidation of the Dowex eluate, as indicated by the characteristic reddish color. To verify this observation the following experiment was performed: To 0.11 ml of Dowex eluate containing 0.3 μmole of hexosamine phosphate, 0.15 ml of ninhydrin reagent was added and the oxidation carried out as previously described. The entire mixture was stripped on paper and treated as before. A 5 cm wide strip with a mean $R_{picrate}$ = 0.94 was cut from the paper, dried overnight *in vacuo* over NaOH-H_2SO_4, then eluted with water. The eluate was evaporated to dryness *in vacuo* over NaOH-H_2SO_4, and the residue dissolved in 0.1 ml of 0.05 M citrate buffer, pH 5.5. A small amount (<0.5 mg) of wheat germ acid phosphatase was added, the mixture incubated at 30° overnight and evaporated to dryness *in vacuo*. The residue was taken up in 1 to 1.5 ml of water and mixed-bed resin, 0.5 to 1.0 ml of 20 to 50 mesh Bio-Rad AG 501-x8 (D),

Table II. *Electrophoresis of the Product Contained in the Dowex Eluate*

Electrophoresis in Buffer (II). Sprayed with ninhydrin.

Material	Migration in cm	$R_{picrate}$	Ninhydrin coloration
Picrate	14.0	1.0	...
D-Glucosamine 6-phosphate	4.8	0.34	Purple
Dowex eluate	4.9	0.35	Purple (only visible spot)

Electrophoresis in Buffer (I). Sprayed with aniline phosphate.

Material	Migration in cm	$R_{picrate}$	Aniline phosphate color
Picrate	13.2	1.0	...
D-Glucosamine 6-phosphate	0	0	Brownish
Dowex eluate	0	0	Brownish (only visible spot)

Table III. *Electrophoresis of Standard* D-*Glucosamine 6-Phosphate and the Dowex Product After Ninhydrin Oxidation*

Material	Migration in cm	$R_{picrate}$	Aniline phosphate color
Picrate	7.1		...
D-Glucosamine 6-phosphate	6.5	0.92	Reddish
	0	0	Purple before spraying
Dowex eluate	6.6	0.93	Reddish
	0	0	Purple before spraying

was added. After stirring intermittently for 30 minutes, the mixture was filtered through paper with vacuum, the resin washed several times with water and the filtrate and washings combined and evaporated *in vacuo* over P_2O_5. A control consisting of buffer and wheat germ acid phosphatase was treated in the same manner as the ninhydrin oxidation product. The residues were taken up in 40 μl of water and aliquots containing approximately 0.05 μmole of the product were chromatographed in the systems described in table IV.

The Dowex eluate was reducing as indicated by the aniline phosphate color test; it possessed an α-amino group as shown by the ninhydrin-positive reaction. Upon ninhydrin oxidation a compound which was reducing and which moved toward the anode was produced. After dephosphorylation with acid phosphatase, the compound yielded a substance which had a chromatographic mobility as that of authentic L-arabinose. Since galactosamine-6-P would produce lyxose after ninhydrin oxidation and dephosphorylation, it can be concluded that the Dowex eluate contained either glucosamine phosphate or mannosamine phosphate.

Acetylation. The results of the oxidative deamination indicated that the hexosamine phosphate was either glucosamine phosphate or mannosamine phosphate but excluded galactosamine phosphate. In order to ascertain the identity of this phosphorylated amino sugar in the Dowex eluate, the product was acetylated in a reaction mixture containing 0.05 ml of Dowex eluate (approx 0.5 μmole of hexosamine phosphate), 0.50 ml of water, 0.10 ml of saturated $NaHCO_3$, and 0.10 ml of 5 % aqueous acetic anhydride in a total volume of 0.75 ml. The mixture was incubated for 3 minutes at room temperature, then placed in boiling water for 3 minutes (14). After cooling, Dowex-50 (H^+) was added to cessation of bubbling, the mixture filtered through paper and the filtrate evaporated *in vacuo*. To the residue dissolved in 30 μl of water 0.2 ml of citrate buffer, pH 5.5, and approximately 1 mg of wheat germ acid phosphatase were added. The mixture was incubated overnight at 30° and evaporated *in vacuo*, the residue suspended in 1 ml of water and 1.5 to 2.0 ml of mixed bed resin was added. After stirring intermittently for 30 minutes, the mixture was filtered with vacuum and the resin washed several times with small amounts of water. The combined filtrate and washings were evaporated *in vacuo* over P_2O_5, the residue taken up in a small amount of water and an aliquot containing 0.05 to 0.10 μmole of the product was spotted on borate-treated paper and chromatographed. The results are presented in table V.

Table V. *Results of Chromatography of the Dowex Eluate Product After Acetylation and Phosphatase Treatment*

Developed in Solvent (A). The paper was sprayed with sodium hydroxide.

Material	Migration in cm	R_G
N-acetyl-D-mannosamine	6.0	0.19
Dowex eluate	9.3	0.30
N-acetyl-D-glucosamine	9.4	0.30
N-acetyl-D-galactosamine	6.4	0.21

The fluorescent material from the acid phosphatase reaction migrated, after sodium hydroxide treatment, with the same mobility as authentic *N*-acetyl-D-glucosamine. Standard glucosamine 6-P, when subjected to the same series of reactions, produced similar results. It may be concluded that the Dowex eluate yielded a compound which behaved as standard *N*-acetyl-D-glucosamine.

The data obtained from oxidative deamination and acetylation of the hexosamine phosphate support the conclusion that the compound produced upon incubation of D-fructose 6-P and L-glutamine with the mung bean fraction is D-glucosamine-6-P.

Properties of D-Glucosamine 6-Phosphate Transacetylase. A plot of cpm *versus* strip number for a reaction mixture and a control with heat-denatured enzyme is shown in figure 3. As can be seen, negligible counts were detected in the area corresponding to the standard *N*-acetyl-D-glucosamine in the control. An experiment was carried out in which the D-glucosamine-6-P substrate was omitted but active enzyme was used. In the area corresponding to the standard *N*-acetyl-D-glucosamine, 8000 cpm were detected for the reaction and 673 cpm for the control. Incorporation of radioactivity in this control was small in comparison to that of the complete reaction mixture.

Table IV. *Chromatography of the Ninhydrin Oxidation Product After Phosphatase Treatment*

Material	Migration in cm	R_F	Aniline phosphate color
Developed in Solvent (E)			
Lyxose	13.9	0.45	Reddish
Dowex eluate	10.4	0.34	Reddish
Arabinose	11.0	0.36	Reddish
Phosphatase control	...	...	No coloration
Developed in Solvent (F)			
Lyxose	14.3	0.45	Reddish
Dowex eluate	11.0	0.35	Reddish
Arabinose	11.9	0.38	Reddish

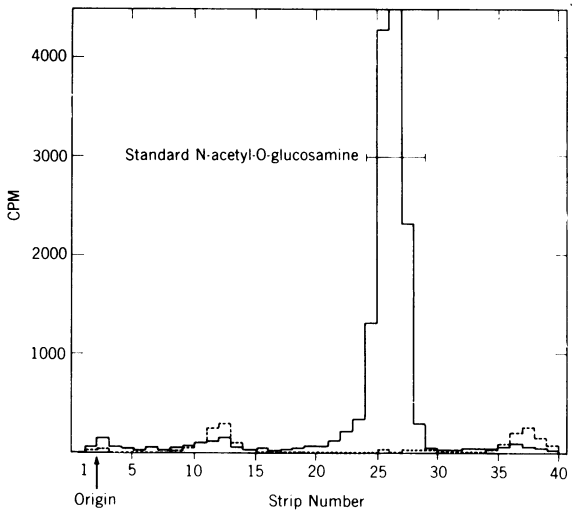

FIG. 3. Chromatography of biosynthetic *N*-acetylhexosamine phosphate after phosphatase treatment. ————, reaction; ------, control. See text for details.

It can be calculated from figure 3 that the extent of incorporation of acetyl group from acetyl CoA was approximately 4 % (348,000 cpm of acetyl CoA added to the incubation mixture and 14,000 cpm recovered under the *N*-acetyl-D-glucosamine peak.) This figure should be considered as a lower limit of transfer, because of some loss of radioactive material during the transfer of the incubation mixture to the paper for electrophoresis, loss of radioactivity from the resin treatment, and subsequent filtration. Undoubtedly, the amount of acetyl transfer was greater than the calculated 4 %. Experimental data showed that acetyl group was also transferred from acetyl coenzyme A to D-glucosamine 6-P at pH 6.6. At the lower pH, however, the amount of activity detected under the standard *N*-acetyl-D-glucosamine peak was about one-half that at the higher pH.

Properties of Acetylglucosamine Phosphomutase. The substance formed upon incubation, at pH 8, of *N*-acetyl-D-glucosamine-1-P with the enzyme extract gave the modified Elson-Morgan test in the presence of Mg^{2+} and of catalytic amounts of glucose 1,6-diphosphate. In order to demonstrate the reaction in the reverse direction, advantage was taken of the pyrophosphorylase reaction which proved to be responsible for the formation of UDP-*N*-acetyl-D-glucosamine in the mung bean seedlings. *N*-Acetyl-D-glucosamine 6-P, prepared from D-glucosamine 6-P and acetyl-1-^{14}C coenzyme A with the mung bean extract as previously described, was reacted at pH 7.8 with UTP in the presence of Mg^{2+}, catalytic amounts of D-glucose 1,6-diphosphate, excess inorganic pyrophosphatase and the enzyme extract. The reaction was stopped by the addition of 3 volumes of warm ethanol, and the precipitated protein removed by centrifugation. The supernatant solution was then concentrated *in vacuo* over P_2O_5, the residue taken up in water and chromatographed in solvent (D) together with an unlabeled UDPAG serving as an internal marker. This procedure gives a good separation of the unreacted acetyl coenzyme A (R_F 0.63) from UDPAG (R_F 0.15). Horizontal strips, 1 cm wide, were cut from the paper, placed in vials with 10 ml of liquifluor solution, and counted. Incorporation of radioactivity was observed in an area of the paper with an R_F identical with that of the standard UDPAG. The strips corresponding to the UDPAG spot were removed from the vials, washed with toluene, air-dried and eluted with water. The eluate was then lyophilized, the residue taken up in water and chromatographed in solvent (B). The spot corresponding to standard UDPAG was eluted again, subjected to electrophoresis in Buffer (I) and chromatography in solvent (C). In each case, unlabeled UDPAG was used as a standard reference, and in both chromatography and electrophoresis incorporation of radioactivity into a spot associated

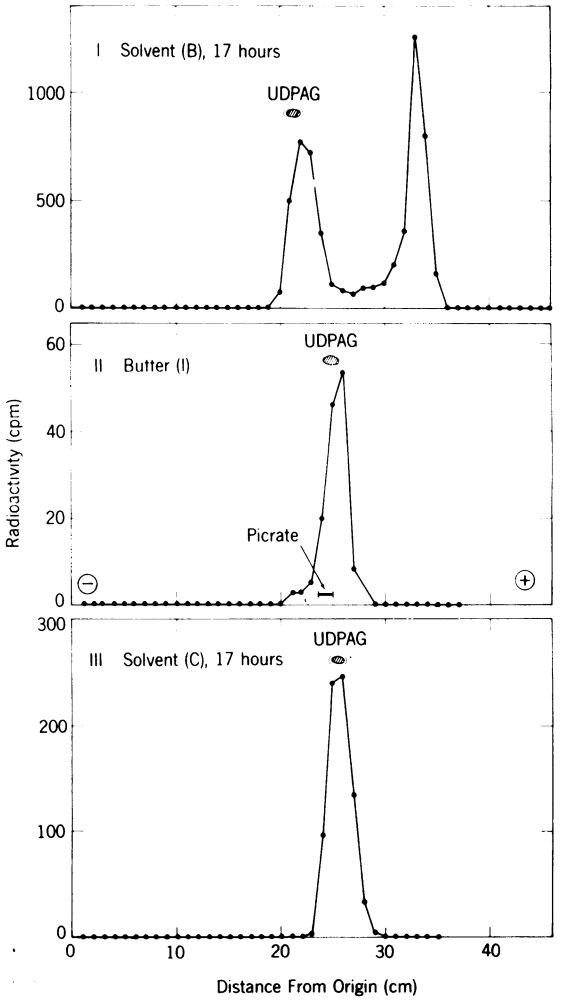

FIG. 4. Identification of the enzymatically synthesized radioactive UDPAG by paper electrophoresis and paper chromatography. Unlabelled standard UDPAG was detected by U.V.

with that of the reference UDPAG compound was observed (fig 4). No incorporation of radioactivity was observed at the UDPAG reference area when a previously-boiled enzyme extract was used, nor when UDP was substituted for UTP. By analogy with other mutase and pyrophosphorylase reactions (see also next section), these results show that the mung bean extract contains an active acetylglucosamine phosphomutase catalyzing the interconversion of *N*-acetyl-D-glucosamine-1-P and *N*-acetyl-D-glucosamine-6-P. Thus the path *N*-acetyl-D-glucosamine 6-P → *N*-acetyl-D-glucosamine-1-P → UDP-*N*-acetyl-D-glucosamine was made irreversible by hydrolyzing the pyrophosphate liberated with inorganic pyrophosphatase.

Properties of UDPAG Pyrophosphorylase. A U.V.-absorbing spot was detected in an area of the paper corresponding to authentic UDPAG ($R_{picrate}$ = 1.0) after electrophoresis in Buffer (I) of a reaction mixture incubated at 30° for 2 hours and containing 2 μmoles of *N*-acetyl-D-glucosamine-1-P, 2 μmoles of UTP, 1.25 μmoles of $MgCl_2$, 100 μl of the enzyme extract and excess inorganic pyrophosphatase in a total volume of 0.2 ml with 0.1 M tris buffer, pH 7.8. In either the absence of UTP or in the presence of a heat-denatured enzyme, no U.V.-absorbing spot with ionophoretic mobility of UDPAG was detected. The area corresponding to UDPAG on the paper was then cut out, eluted with water, the eluate concentrated *in vacuo* over P_2O_5 or lyophilized, and the residue taken up in water and chromatographed in solvents (B), (C), and (D). In all 3 cases, only 1 U.V.-absorbing spot was detected, which cochromatographed with authentic UDPAG and therefore substantiated the electrophoretic identification of this compound. The electrophoretogram from Buffer (I) contained, besides the U.V.-absorbing spot corresponding to UDPAG ($R_{picrate}$ 1.0) and UTP (unreacted excess; $R_{picrate}$ 1.53), also small U.V.-absorbing spots that migrated with authentic UDP, UMP, and uridine. The formation of all of these compounds is indicative of the presence of active phosphatase(s) in the extract.

Pyrophosphate is the second product of the reaction catalyzed by pyrophosphorylase in the direction of UDPAG synthesis. It was hydrolyzed by inorganic pyrophosphatase to orthophosphate, thus ensuring irreversibility of the reaction.

The products of the reaction catalyzed by pyrophosphorylase in the direction of pyrophosphorolysis were also identified. Thus, a reaction mixture containing 0.5 μmole of UDPAG, 1.0 μmole of PP_i, 0.5 μmole $MgCl_2$, 10 μmoles of NaF and 20 μl of enzyme extract in a total volume of 55 μl with 0.1 M tris buffer (pH 7.8) was incubated for 2 hours at 30°, then subjected to electrophoresis in Buffer (I) after protein removal as described above. A U.V.-absorbing spot with mobility exactly as that of authentic UTP ($R_{picrate}$ 1.53) appeared. A second compound, which did not absorb U.V. light, gave a positive response to both the phosphate spray and chlorinating reagent. It had the same mobility upon co-electrophoresis with an isolated *N*-acetylglucosamine-1-P ($R_{picrate}$ 0.8), which, in turn, was prepared from UDPAG by hydrolysis with *Crotalus admanteus* venom phosphodiesterase, and which was used in the presence of UTP as a substrate in the reverse reaction, namely the biosynthesis of UDPAG.

The areas of electrophoretogram corresponding to UTP and *N*-acetylglucosamine-1-P were separately cut out, eluted with water and subjected to chromatography in solvents (B) and (C) for further identification. In both solvents only 1 spot corresponding to UTP and 1 corresponding to *N*-acetylglucosamine-1-P were detected. A control containing the complete reaction mixture but with no enzyme was treated as above. In both electrophoresis and chromatography the UDPAG spot remained intact. The electrophoretogram of the complete reaction mixture also contained spots of free *N*-acetylglucosamine and of inorganic phosphate, indicating the presence of a phosphomonoesterase in the extract.

Inhibition of L-*Glutamine and* D-*Fructose 6-Phosphate Amidotransferase.* Since amidotransferase from mammalian tissue is inhibited by UDPAG and this inhibition is absent in bacteria (20), it was of interest to learn whether a mechanism of regulation of the pathway leading to UDPAG biosynthesis is operative in a higher plant. Figure 5 shows that amidotransferase of *P. aureus* is inhibited by UDPAG, although to a lesser extent than the enzyme isolated from mammalian tissues. Specifically, the amidotransferase of *P. aureus* is inhibited by UDPAG to an extent of about 60 %, independent of further increment in UDPAG concentration, whereas the enzyme from mammalian sources is inhibited to about 80 % (20, 22). UDPG does not appreciably inhibit the enzyme. Under the conditions of assay, the partially purified mung bean

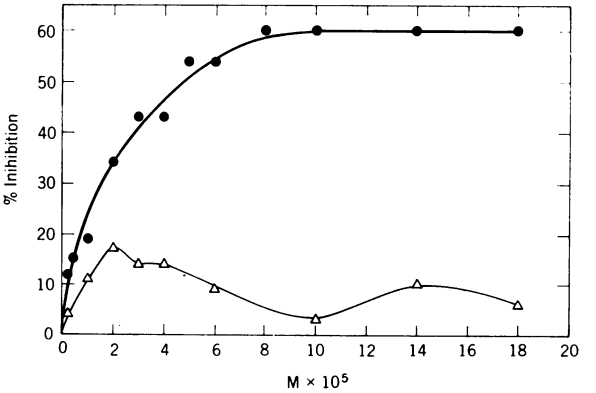

FIG. 5. Inhibition of L-glutamine D-fructose 6-phosphate amidotransferase by UDPAG. Enzyme source is fresh 30 % saturated ammonium sulfate precipitate, assayed under the standard conditions, but with UDPAG (- ● - ● -) or UDPG (-△-△-) added to the concentrations indicated.

L-glutamine D-fructose 6-phosphate amidotransferase did not catalyze the breakdown of UDPAG, even when the mixture was incubated at 30° up to 5 hours, as determined by chromatography and electrophoresis of the incubation mixture against authentic UDPAG. The kinetics of this inhibition remain to be studied.

Discussion

The results of the present investigation show that the biosynthesis in *P. aureus* of UDP-*N*-acetyl-D-glucosamine from D-fructose 6-phosphate occurs by a similar pathway to that deduced from various reactions with enzymic preparations of *Neurospora crassa* (11, 24, 36), mammalian tissues and microorganisms (14, 46); the only major difference is that with mung bean extracts, in addition to glutamine, asparagine can serve as an amino group donor for the formation of D-glucosamine 6-phosphate.

UDP-*N*-Acetyl-D-glucosamine was shown to inhibit rat liver L-glutamine D-fructose 6-phosphate amidotransferase, which catalyzes the first reaction leading to the biosynthesis of this amino sugar nucleotide (22). However, it was reported in further studies of this feedback inhibition (20) that in contrast to the enzyme from mammalian tissues, amidotransferase from 3 species of bacteria is insensitive even to high UDP-*N*-acetyl-D-glucosamine concentrations.

The present investigation demonstrates that UDP-*N*-acetyl-D-glucosamine inhibits *P. aureus* amidotransferase, thus establishing the presence of such inhibition in a higher plant.

Since a hexokinase is known to occur in mung bean mitochondria (29) and is also distributed in several other higher plants (39), it can be assumed that this enzyme is responsible for the formation from D-fructose of D-fructose-6-phosphate which is required for the subsequent reactions.

Inasmuch as UDP-*N*-acetyl-D-glucosamine is present in mung beans and D-glucosamine was found to be a constituent of glycolipids and glycoproteins in certain plants, it is most likely that the amino sugar moiety of these compounds is donated by this amino sugar nucleotide. However, since ADP-*N*-acetyl-D-glucosamine was isolated from corn grain (12), and ADP-*N*-acetylglucosamine pyrophosphorylase activity was shown to be present in this grain (31), there is a possibility that a nucleoside diphosphate D-glucosamine containing a base other than uracil is a potential *N*-acetylglucosamine donor to polymers containing this amino sugar.

Other pathways for the biosynthesis of UDP-*N*-acetyl-D-glucosamine are also possible. For example, Kornfeld and Glaser (21) showed that extracts of *Pseudomonas aeruginosa* catalyze acetylation on the amino sugar nucleotide level, not on the amino sugar level, by the following reaction: TDP-D-glucosamine + acetyl-CoA→TDP-*N*-acetylglucosamine + CoA.

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