Biochem. J. (1965) **96**, 147

Further Studies on the Regulation of Amino Sugar Metabolism in *Bacillus subtilis*

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(*Received* 16 *December* 1964)

1. Glucosamine 6-phosphate deaminase [2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating), EC 5.3.1.10] of *Bacillus subtilis* has been partially purified. Its K_m is 3·0mM. 2. Extracts of *B. subtilis* contain *N*-acetylglucosamine 6-phosphate deacetylase (K_m 1·4mM), glucosamine 1-phosphate acetylase and amino sugar kinases (EC 2.7.1.8 and 2.7.1.9). 3. Glucosamine 6-phosphate synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) is repressed by growth of *B. subtilis* in the presence of glucosamine, *N*-acetylglucosamine, *N*-propionylglucosamine or *N*-formylglucosamine. Glucosamine 6-phosphate deaminase and *N*-acetylglucosamine 6-phosphate deacetylase are induced by *N*-acetylglucosamine. Amino sugar kinases are induced by glucose, glucosamine and *N*-acetylglucosamine. The synthesis of glucosamine 1-phosphate acetylase is unaffected by amino sugars. 4. Glucose in the growth medium prevents the induction of glucosamine 6-phosphate deaminase and of *N*-acetylglucosamine 6-phosphate deacetylase caused by *N*-acetylglucosamine; glucose also alleviates the repression of glucosamine 6-phosphate synthetase caused by amino sugars. 5. Glucosamine 6-phosphate deaminase increases in bacteria incubated beyond the exponential phase of growth. This increase is prevented by glucose.

D-Glucosamine 6-phosphate is a key intermediate in the biosynthesis and degradation of amino sugars (Roseman, 1959). It is synthesized by the enzyme glucosamine 6-phosphate synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) and is degraded by glucosamine 6-phosphate deaminase (2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.10). The addition of *N*-acetylglucosamine to growing cultures of *Bacillus subtilis* causes repression of the first enzyme and induction of the second (Clarke & Pasternak, 1962). By examining various analogues of *N*-acetylglucosamine it has been shown that repression and induction are under separate metabolic control. The effect of glucose on these enzymic changes, which was briefly referred to by Clarke & Pasternak (1962), has been further studied. It is shown elsewhere (Bates & Pasternak, 1965) that glucose also affects the uptake of amino sugars.

The formation of glucosamine 6-phosphate from *N*-acetylglucosamine is believed to occur by way of *N*-acetylglucosamine kinase (EC 2.7.1.9) followed by *N*-acetylglucosamine 6-phosphate deacetylase (Roseman, 1959). We have found these enzymes and glucosamine kinase (EC 2.7.1.8) in extracts of *B. subtilis* and have measured their concentrations under various conditions of growth. Although the amino sugars in cell walls are mostly acetylated (Salton, 1964), Clarke & Pasternak (1962) were unable to detect glucosamine 6-phosphate acetylase (EC 2.3.1.4) in *B. subtilis*. The discovery of a glucosamine 1-phosphate acetylase in *Pseudomonas aeruginosa* (Kornfeld & Glaser, 1962) prompted us to examine extracts of *B. subtilis*, and we now report the occurrence of the enzyme and its metabolic control in that organism. Some of these results have been published in brief (Bates & Pasternak, 1962).

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MATERIALS AND METHODS

Chemicals. *N*-Acyl-amino sugars were prepared from D-glucosamine hydrochloride and the appropriate acid anhydride by the method of Kuhn & Bister (1958). They were purified by the addition of 10vol. of acetone to the reaction mixture, recrystallizing the precipitated material several times from acetone–water or methanol–water and passing it through a column of Amberlite IR-120 resin (H^+ form) to remove traces of glucosamine where necessary. *N*-Propionylglucosamine had m.p. 192°; Kuhn & Haber (1953) quote 182–183° (Found: C, 45·9; H, 7·2; N, 5·9. Calc. for $C_9H_{17}NO_6$: C, 45·9; H, 7·2, N, 6·0%). *N*-*n*-Butyrylglucosamine had m.p. 206°; Yoshiyuki (1958) quotes 209°

(Found: C, 48·2; H, 7·7; N, 5·7. Calc. for $C_{10}H_{19}NO_6$: C, 48·2; H, 7·6; N, 5·6%). *N*-Isobutyrylglucosamine had m.p. 228°. *N*-Succinylglucosamine was difficult to crystallize. *N*-Formylglucosamine was prepared from D-glucosamine hydrochloride and ethyl formate by the method of Meyer zu Reckendorf & Bonner (1961), purified by passage through Amberlite IR-120 resin and recrystallized from methanol. It had m.p. 154–158°. All compounds except *N*-formylglucosamine gave the same colour yield as *N*-acetylglucosamine in the Levvy & McAllan (1959) assay method for acetylated amino sugar. *N*-Acetyl-6-deoxy-D-glucosamine (*N*-acetylchinovosamine) was prepared by the method of Morel (1958). The m.p. was 202–203° (decomp.); that quoted by Kuhn & Haber (1953) is 210–211° (decomp.) (Found: C, 46·7; H, 7·3; N, 7·0. Calc. for $C_8H_{15}NO_5$: C, 46·8; H, 7·3; N, 6·8%). *N*-Acetyl-6-deoxy-6-iodoglucosamine was prepared by treating 1,3,4-tri-*O*-acetyl-*N*-acetyl-6-deoxy-6-iodoglucosamine with ammonia (Morel, 1958). It melted at 169–171° (Found: C, 28·9; H, 4·5; I, 38·2; N, 4·2. $C_8H_{14}INO_5$ requires: C, 29·0; H, 4·2; I, 38·4; N, 4·2%). Both compounds behaved like *N*-acetylglucosamine in the assay for acetylated amino sugar, though the colour developed at different rates. D-Glucosamine 6-phosphate (barium salt; obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.), *N*-acetylglucosamine 6-phosphate and acetyl-CoA were prepared and purified as described by Clarke & Pasternak (1962). *N*-Propionylglucosamine 6-phosphate was prepared from D-glucosamine 6-phosphate and propionic anhydride (Kuhn & Bister, 1958). It behaved like *N*-acetylglucosamine in the acetylated amino sugar assay. α-D-Glucosamine 1-phosphate was prepared by the method of Maley, Maley & Lardy (1956). It had $[\alpha]_D^{20}+90°$ (*c* 1·98 in water); Maley *et al.* (1956) quote $[\alpha]_D+100°$ (*c* 1·98 in water).

Preparation of enzyme extracts. B. subtilis N.C.T.C. 1379 was grown and extracted as described by Clarke & Pasternak (1962), except that glucose was omitted from the standard broth plus glutamate growth medium. Glucose, and other compounds, were added separately as indicated. In some experiments cells were suspended in 0·01 M-tris–0·02 M-β-mercaptoethanol, pH 7·5, and disrupted by passage through a French pressure cell (American Instrument Co. Inc., Silver Spring, Md., U.S.A.). The high-speed supernatant was fractionated to separate glucosamine 6-phosphate synthetase from glucosamine 6-phosphate deaminase, as described by Clarke & Pasternak (1962).

Assay of enzymes. Glucosamine 6-phosphate synthetase and glucosamine 6-phosphate deaminase were assayed as described by Clarke & Pasternak (1962). The incubation mixture for the spectrophotometric assay of the deaminase (Clarke & Pasternak, 1962) contained 0·5 μmole of glucosamine 6-phosphate, 0·12 μmole of NADP, 2 μmoles of $MgCl_2$, 40 μmoles of tris–HCl buffer, pH 8, 0·56 unit of glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) and extract (approx. 0·6 mg. of protein) in a final volume of 0·5 ml. The extinction at 340 mμ was measured in a model DB spectrophotometer (Beckman Instruments, Queensway, Glenrothes, Fife) coupled to a model SR recorder (E. H. Sargent and Co., Chicago 30, Ill., U.S.A.).

N-Acetylglucosamine 6-phosphate deacetylase was assayed by measuring the disappearance of acetylated amino sugar from an incubation mixture containing 0·25 μmole of *N*-acetylglucosamine 6-phosphate, 50 μmoles of tris–HCl buffer, pH 8·5, and extract (1–2 mg. of protein) in a final volume of 0·5 ml. The reaction was stopped by the addition of 0·1 ml. of 20% (w/v) trichloroacetic acid. Alternatively, the enzyme extract (which contains glucosamine 6-phosphate deaminase and glucose phosphate isomerase) was assayed spectrophotometrically as described above, with 0·5 μmole of *N*-acetylglucosamine 6-phosphate in place of glucosamine 6-phosphate.

Amino sugar kinases were assayed by measuring the appearance of ^{14}C-labelled amino sugar phosphates from ^{14}C-labelled amino sugars (cf. Buttin, Jacob & Monod, 1960). The incubation mixture contained 175 mμmoles (0·25 μc) of [1-^{14}C]glucosamine or 50 mμmoles (0·25 μc) of *N*-acetyl-[1-^{14}C]glucosamine (Bates & Pasternak, 1965), 5 μmoles of ATP, 5 μmoles of $MgCl_2$, 50 μmoles of tris–HCl buffer, pH 7·9, 2 mg. of serum albumin and extract prepared with the French pressure cell (2–4 mg. of protein) in a final volume of 0·5 ml. The reaction was stopped by the addition of 0·5 ml. of 0·5 M-barium acetate and 4 ml. of ethanol. The precipitated phosphates (and protein) were filtered after 10 min. at 0° through an Oxoid membrane filter and washed five times with 5 ml. of ethanol–water (4:1, v/v) and once with 5 ml. of ethanol. For glucosamine kinase the ethanol–water washes contained 5 mM-glucosamine to remove adsorbed [^{14}C]glucosamine. The dried filters were pasted on to aluminium planchets and the radioactivity was measured in a type 6051 LA Lobetamat Low Background Sample Changing Assembly (Isotope Developments Ltd., Beenham, Berks.). Self-absorption was negligible. The formation of 0·1 mμmole could be detected by this method.

Glucosamine 1-phosphate acetylase was assayed (Kornfeld & Glaser, 1962) by incubating 0·4 μmole of glucosamine 1-phosphate, 0·5 μmole of acetyl-CoA, 1 μmole of $MgCl_2$, 2 μmoles of β-mercaptoethanol, 50 μmoles of tris–HCl buffer, pH 8·5, and extract (1–3 mg. of protein) in a final volume of 0·45 ml. The reaction was stopped by the addition of 0·03 ml. of N-HCl and heating at 100° for 10 min. (which removes P_i from *N*-acetylglucosamine 1-phosphate), followed by the addition of 0·02 ml. of 50% (w/v) trichloroacetic acid to remove protein. The formation of acetylated amino sugar was measured.

Other analyses. Glucose, amino sugars, acetylated amino sugars and protein were measured as described by Clarke & Pasternak (1962). Radioactivity in effluent fractions after ion-exchange chromatography was measured by drying 0·2 ml. on glass cover slips embedded in aluminium planchets and counting in a Nuclear–Chicago gas-flow automatic sample-changing counter or a type 6051 LA Lobetamat Low Background Sample Changing Assembly (Isotope Developments Ltd.).

Microanalyses were performed by Dr A. Bernhardt (Microanalytisches Laboratorium, Mülheim, Germany).

RESULTS

Glucosamine 6-phosphate deaminase. A 60-fold purification was obtained by fractionation with protamine sulphate (Clarke & Pasternak, 1962) followed by chromatography on DEAE-cellulose. The enzyme was eluted in a single peak by 0·05–0·15 M-sodium chloride in 5 mM-tris–hydrochloric acid buffer, pH 7·5. Although *N*-acetylglucosamine 6-phosphate deacetylase was eluted in the same

fractions, the ratios of the activities of the two enzymes were not constant, suggesting that a partial separation had been achieved. The purified deaminase was stimulated less than twofold by 0·2mM-*N*-acetylglucosamine 6-phosphate. The K_m in crude extracts, measured by the method of Lineweaver & Burk (1934), is 3mM at pH 8·0. High concentrations of fructose 6-phosphate and ammonia inhibit the deamination of glucosamine 6-phosphate (0·5mM) by crude extracts (17% inhibition by 5mM- and more than 98% inhibition by 50mM-fructose 6-phosphate; 86% inhibition by 100mM-ammonium sulphate), but since the reaction is reversible (Comb & Roseman, 1958) such an effect is not unexpected.

Structural requirements for repression of glucosamine 6-phosphate synthetase and induction of glucosamine 6-phosphate deaminase. Table 1 shows that glucosamine, *N*-propionylglucosamine and *N*-formylglucosamine, in addition to *N*-acetylglucosamine (Clarke & Pasternak, 1962), repress glucosamine 6-phosphate synthetase when added to the growth medium. Only *N*-acetylglucosamine induces the deaminase, suggesting that inducer and repressor are different compounds. Their structures cannot be deduced from these experiments since all the compounds listed in Table 1 are removed from the medium, and hence presumably metabolized, during growth. No gratuitous inducer or repressor was discovered; *N*-isobutyrylglucosamine, *N*-succinylglucosamine, galactosamine, *N*-acetylgalactosamine, *N*-acetylmannosamine, *N*-acetyl-6-deoxyglucosamine, *N*-acetyl-6-deoxy-6-iodoglucosamine, glucosamine 6-phosphate or *N*-acetylglucosamine 6-phosphate, which were not removed from the medium, exerted no control effects. These results may reflect the structural requirements of a permease, kinase or other enzyme, rather than that of the inducing or repressing machinery itself. The induction of deaminase is detectable at an initial concentration of 0·1mM-*N*-acetylglucosamine in the medium and is maximal at 1mM. Repression of synthetase by *N*-acetylglucosamine, glucosamine, *N*-formylglucosamine or *N*-propionylglucosamine shows a similar dependence on concentration between 0·1 and 1·0mM. If cultures are incubated for longer times amino sugar in the medium is exhausted and the enzyme concentrations approach those of control cultures. After very long periods of incubation in the absence of added amino sugar, however, the deaminase activity rises appreciably. Thus after 36hr. growth in the presence of broth and glutamate the specific activity was 11mμmoles/mg. of protein/min. compared with 3mμmoles/mg./min. at 8hr. In the presence of 100mM-glucose the activities at 8 and 36hr. were 2 and 5mμmoles/mg. of protein/min. respectively. The pH, which tended to rise in the absence of glucose and to fall in its presence, was kept constant by frequent titration. It was noted that, when glucose was present, a compound displaying the Levvy & McAllan (1959) reaction of acetylated amino sugar began to accumulate after 20hr.

Alleviation of control effects by sugars. The induction of glucosamine 6-phosphate deaminase is severely diminished by the addition of glucose to the growth medium, as noted above and by Clarke & Pasternak (1962). The effect is not confined to reversal of deaminase because repression of synthetase is released at the same time (Table 2). This may be because those sugars that exhibit an effect also prevent removal of amino sugar from the growth medium (Table 2 and Bates & Pasternak, 1965). D-Galactose, D-arabinose, D-ribose, lactose, succin-

Table 1. *Repression of glucosamine 6-phosphate synthetase and induction of glucosamine 6-phosphate deaminase by amino sugars*

Cultures of *B. subtilis* that had been grown in the presence of the compounds indicated were harvested (approx. 0·4mg. dry wt. of cells/ml.), disrupted by ultrasonic oscillation or a French pressure cell and assayed as described by Clarke & Pasternak (1962). The values quoted are means of two or more experiments.

Addition to growth medium	Amino sugar remaining at harvesting (mM)	Glucosamine 6-phosphate synthetase (arbitrary units)	Glucosamine 6-phosphate deaminase (arbitrary units)
None	—	1·00*	1·0†
Glucosamine (5mM)	3·4	0·11	2·1
N-Acetylglucosamine (5mM)	0·5	0·10	26·9
N-Propionylglucosamine (5mM)	4·9	0·23	2·0
N-Formylglucosamine (5mM)	>4·9	0·38	1·5
N-*n*-Butyrylglucosamine (5mM)	>4·9	1·01	1·8

* Specific activity 10·4mμmoles/mg. of protein/min.
† Specific activity 7·0mμmoles/mg. of protein/min.

Table 2. *Reversal of control effects by sugars*

Cultures of *B. subtilis* that had been grown in the presence of the compounds indicated were harvested (0·35–0·4mg. dry wt. of cells/ml.), disrupted by ultrasonic oscillation or a French pressure cell and assayed as described by Clarke & Pasternak (1962). The values quoted are means of two or more experiments.

Addition to growth medium	Amino sugar remaining at harvesting (mM)	Glucosamine 6-phosphate synthetase (arbitrary units)	Glucosamine 6-phosphate deaminase (arbitrary units)
None	—	1·00	1·0
Glucose (10mM)	—	1·00	0·8
N-Acetylglucosamine (5mM)	0·5	0·10	26·9
N-Acetylglucosamine (5mM)+glucose (10mM)	4·6	0·48	2·4
N-Acetylglucosamine (5mM)+fructose (10mM)	>4·9	0·55	2·1
N-Acetylglucosamine (5mM)+mannose (5mM)	3·7	0·14	15·5
N-Acetylglucosamine (5mM)+sucrose (5mM)	4·5	0·32	7·0
Glucosamine (5mM)	3·4	0·11	2·1
Glucosamine (5mM)+glucose (10mM)	4·6	1·07	0·9

Table 3. N-*Acetylglucosamine 6-phosphate deacetylase in* B. subtilis

Cultures that had been grown in the presence of the compounds indicated were harvested (approx. 0·4mg. dry wt. of cells/ml.) and disrupted in a French pressure cell, and the high-speed supernatant fractions assayed as described in the Materials and Methods section. The values quoted are means of two or more experiments.

Addition to growth medium	Enzymic activity (arbitrary units)
None	1·0*
Glucosamine (5mM)	1·3
N-Acetylglucosamine (5mM)	22·0
N-Propionylglucosamine (5mM)	1·3
Glucose (5mM)	0·9
N-Acetylglucosamine (5mM)+glucose (5mM)	1·6

* Specific activity 0·61 mμmole/mg. of protein/min.

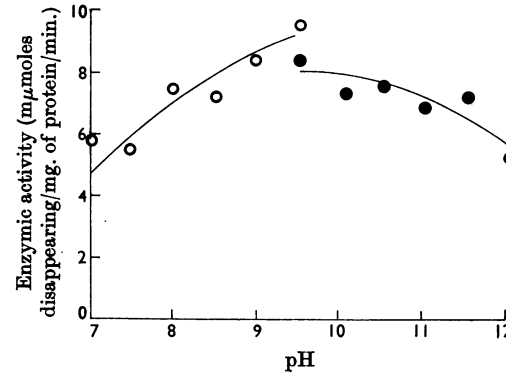


Fig. 1. Effect of pH on *N*-acetylglucosamine 6-phosphate deacetylase. An extract (1mg. of protein) of *B. subtilis* grown in the presence of 10mM-*N*-acetylglucosamine was incubated with 0·2μmole of *N*-acetylglucosamine 6-phosphate and 50μmoles of tris–HCl buffer (○) or glycine–HCl buffer (●) in a final volume of 0·45ml. for 10min. at 37°, and the disappearance of acetylated amino sugar was measured as described in the Materials and Methods section.

ate, DL-lactate, acetate and glycerol were inactive. Since the synthesis of glucosamine 6-phosphate deaminase could not be diminished without simultaneously affecting the uptake of *N*-acetylglucosamine, it is not possible to decide whether true catabolite repression (Magasanik, 1961) operates.

N-*Acetylglucosamine 6-phosphate deacetylase*. This enzyme is present in extracts of *B. subtilis*. It is induced by *N*-acetylglucosamine to about the same extent (22 times the basal concentration) as glucosamine 6-phosphate deaminase, and the induction is repressible by glucose (Table 3). No accumulation of glucosamine 6-phosphate could be detected in crude extracts, but this may be because glucosamine 6-phosphate deaminase is much more active in such extracts than the deacetylase. Attempts to separate the two enzymes were not successful (see above). It is unlikely that *N*-acetylglucosamine 6-phosphate is degraded directly to fructose 6-phosphate, ammonia and acetate by an enzyme similar to that listed in the Report of the Commission on Enzymes (EC 5.3.1.11) because *N*-acetylglucosamine, which is presumably degraded via *N*-acetylglucosamine 6-phosphate, induces both deaminase and deacetylase in *B. subtilis* and *Escherichia coli*. Moreover, in extracts of *E. coli*, in which the activity of deacetylase greatly exceeded that of deaminase, accumulation of non-acetylated amino sugar from *N*-acetylglucosamine 6-phosphate occurred. Also, mutants of *E. coli* that are unable to use *N*-acetylglucosamine as nitrogen or carbon source because they lack glucosamine 6-phosphate deaminase had normal

concentrations of *N*-acetylglucosamine 6-phosphate deacetylase (C. J. Bates, R. J. White & C. A. Pasternak, unpublished work). The pH–activity curve of the partially purified deacetylase is shown in Fig. 1. The K_m measured by the method of Lineweaver & Burk (1934) was 1·4mM in crude extracts at pH 8·0. The enzyme is not inhibited by sodium acetate, ammonium sulphate, *N*-propionylglucosamine or *N*-acetylglucosamine (each at 5mM). *N*-Propionylglucosamine 6-phosphate was deacylated at 70–75% of the rate of *N*-acetylglucosamine 6-phosphate by normal or induced enzyme preparations.

Amino sugar kinases. Extracts of *B. subtilis* grown in the absence of amino sugar exhibited very little kinase activity. The addition of amino sugar to growing cultures gave measurable, though very variable, amounts of glucosamine and acetylglucosamine kinase (Table 4), most of which was in the high-speed supernatant fraction. The rate was constant over the first hour. The omission of Mg^{2+} decreased the activity by more than 90%, but the omission of ATP did not. The activity in the absence of ATP was not decreased by dialysis but was abolished by the addition of sodium fluoride (0·01M) (which decreased the rate in the presence of ATP by 50%) or by preincubation of the extract for 60min. at 37°. The products of the reaction, which were difficult to identify because of the presence in extracts of *N*-acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase, were examined as follows. [1-^{14}C]-Glucosamine (1·8μmoles, 1μC) or *N*-acetyl[1-^{14}C]-glucosamine (0·9μmole, 2μC) was incubated with 100μmoles of ATP, 100μmoles of magnesium chloride, 500μmoles of tris–hydrochloric acid buffer, pH 7·6, 5m-moles of ammonium acetate and glucosamine-grown extract (40mg. of protein) in a final volume of 5ml. for 80min. A sample was removed for assay as described above; the yield was 20% with glucosamine and 10% with *N*-acetylglucosamine. To the remainder was added 2ml. of 50% (w/v) trichloroacetic acid, protein was removed by centrifugation and the supernatant was extracted with ether until the pH was neutral. It was then freeze-dried, extracted three times with ethanol to remove ammonium acetate and residual amino sugars, diluted to 50ml. and adsorbed on a column (40cm. × 0·8cm. diam.) of Dowex 1 (Cl^- form; 200–400 mesh) in the presence of unlabelled glucosamine 6-phosphate and *N*-acetylglucosamine 6-phosphate (each 2μmoles). Gradient elution was achieved by adding 150ml. of 0·05N-acetic acid dropwise to a reservoir containing 150ml. of water. When 75 fractions (each 2ml.) had been collected, 150ml. of 0·15N-hydrochloric acid was dripped into the reservoir. The amino sugar content and radioactivity of each sample were measured. Only one peak of radioactivity (I), which coincided with glucosamine 6-phosphate (tubes 24–30), was eluted by acetic acid. This contained 54% of the radioactivity added to the column with [^{14}C]glucosamine and 4% with *N*-acetyl[^{14}C]glucosamine. Three peaks of radioactivity (II, III and IV), the first of which (II) coincided with *N*-acetylglucosamine 6-phosphate (tubes 90–93), were eluted by hydrochloric acid. Peak II contained 18% of the radioactivity with [^{14}C]glucosamine and 53% with *N*-acetyl[^{14}C]glucosamine. The radioactive fractions corresponding to peaks I and II were each pooled and hydrolysed by Polidase (Benson, Bassham & Calvin, 1951) and by 6N-hydrochloric acid for 8hr. at 100°. Enzymic and acidic hydrolysis of peak I gave only material behaving like [^{14}C]glucosamine. Acidic hydrolysis of peak II gave more than 50% of material behaving like [^{14}C]glucosamine. Enzymic hydrolysis of peak II gave a compound with the R_F of *N*-acetylglucosamine in butan-1-ol–pyridine–water (6:4:3, by vol.), though glucose and fructose have a similar R_F. In summary, all the radioactivity

Table 4. *Amino sugar kinases in* B. subtilis

Cultures that had been grown in the presence of the compounds indicated were harvested (0·4–0·5mg. dry wt. of cells/ml.) and disrupted in a French pressure cell, and the high-speed supernatant fractions assayed as described in the Materials and Methods section. The values quoted are the means of two or more experiments.

Addition to growth medium	Phosphorylation of glucosamine (arbitrary units)	Phosphorylation of *N*-acetylglucosamine (arbitrary units)
None	1·0*	1·0*
Glucose (10mM)	9·0	5·2
N-Acetylglucosamine (10mM)	10·7	21·0
Glucosamine (10mM)	14·0	7·0
N-Acetylglucosamine (10mM)+glucose (10mM)	5·0	9·5
Glucosamine (10mM)+glucose (10mM)	13·7	1·7

* Specific activity 0·04mμmole/mg. of protein/min. in each case (mean of seven determinations ranging from 0·002 to 0·3mμmole/mg. of protein/min.).

Table 5. *Inhibition of amino sugar kinases of* B. subtilis

An extract (2·0 mg. of protein) of a culture (0·37 mg. dry wt. of cells/ml.) grown with 2·5 mM-*N*-acetylglucosamine was incubated with 50 mμmoles of *N*-acetyl[^{14}C]glucosamine or 175 mμmoles of [^{14}C]glucosamine, 5 μmoles of ATP, 5 μmoles of $MgCl_2$, 50 μmoles of tris–HCl buffer, pH 7·9, 2 mg. of serum albumin and 5 μmoles of unlabelled amino sugar or sugar as indicated in a final volume of 0·5 ml. for 20 min., and ^{14}C-labelled phosphates were assayed as described in the Materials and Methods section.

Addition to incubation mixture	Phosphorylation of glucosamine (arbitrary units)	Phosphorylation of *N*-acetylglucosamine (arbitrary units)
None	100*	100†
Glucosamine	—	34
N-Acetylglucosamine	39	—
N-Formylglucosamine	69	7
N-Propionylglucosamine	63	< 5
Glucose	< 2	28
Fructose	7	53

* Yield 3·7 mμmoles of phosphorylated products.
† Yield 2·6 mμmoles of phosphorylated products.

of peak I is present as glucosamine 6-phosphate and most of that in peak II is *N*-acetylglucosamine 6-phosphate. Peaks III and IV were not identified. Since mild hydrolysis (2N-hydrochloric acid for 4 hr. at 100°) gave no [^{14}C]glucosamine, *N*-acetyl-[1-^{14}C]glucosamine 1-phosphate is unlikely to have been present.

The selective inhibition of glucosamine phosphorylation by glucose and that of *N*-acetylglucosamine phosphorylation by *N*-propionylglucosamine and *N*-formylglucosamine, as well as the lack of competition between glucosamine and *N*-acetylglucosamine (Table 5), suggests that two separate kinases (cf. Asensio, 1960) are involved: one phosphorylates glucose and glucosamine, the other the acyl-glucosamines. Pattabiraman & Bachhawat (1961*a*) have found *N*-propionylglucosamine, but not glucosamine, to be phosphorylated by an *N*-acetylglucosamine kinase from brain. Nevertheless, the results in Table 4 show that the phosphorylation of glucosamine and *N*-acetylglucosamine is induced simultaneously, so that the situation is not clear-cut.

Glucosamine 1-phosphate acetylase. Extracts of *B. subtilis* contain low amounts of the enzyme in the supernatant fraction (Table 6). The rate was constant over 20 min. Glucosamine and glucosamine 6-phosphate are acetylated at less than 15% of the rate with glucosamine 1-phosphate. The concentration of the enzyme was not greatly altered by the addition of glucose or amino sugars to the growth medium (Table 6). The product of the reaction gave the colour test of acylated amino sugar only after hydrolysis with hydrochloric acid as described in the Materials and Methods section. It is therefore not *N*-acetylglucosamine or *N*-acetylglucosamine 6-phosphate.

Table 6. *Glucosamine 1-phosphate acetylase in* B. subtilis

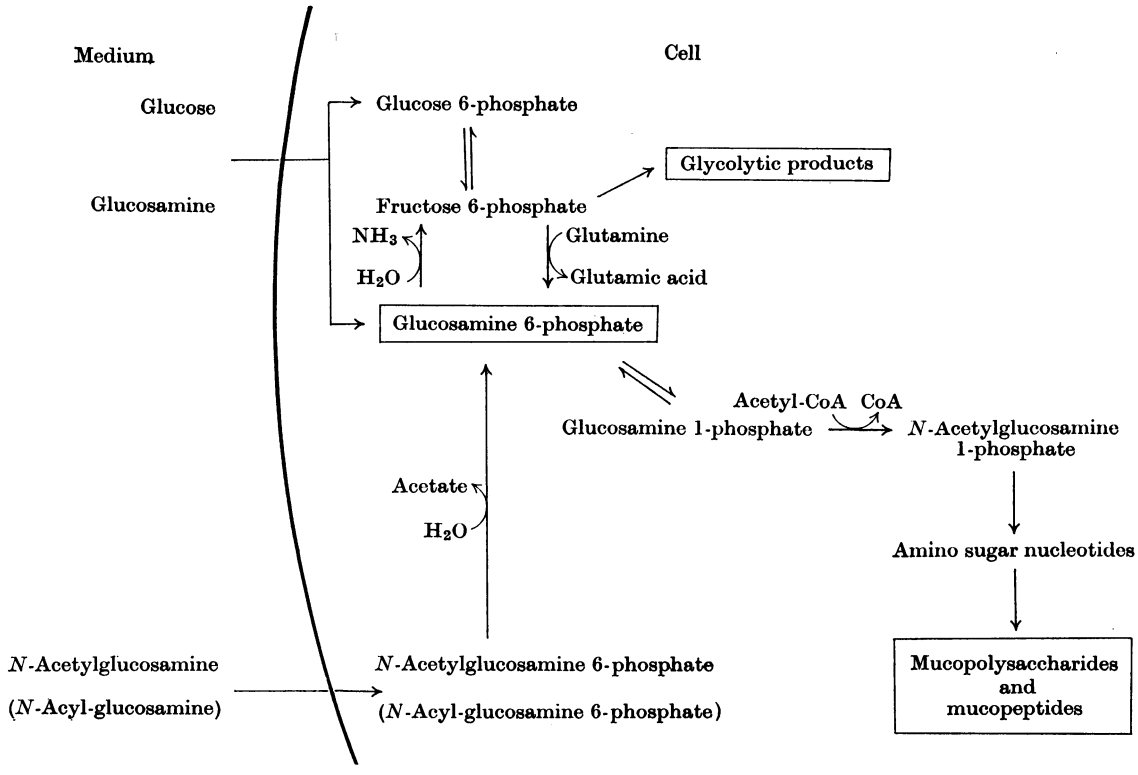
Cultures that had been grown in the presence of the compounds indicated were harvested (0·3–0·5 mg. dry wt. of cells/ml.) and disrupted in a French pressure cell, and the high-speed supernatant fractions assayed as described in the Materials and Methods section.

Addition to growth medium	Enzymic activity (arbitrary units)
None	1·00*
Glucose (10 mM)	2·54
Glucosamine (10 mM)	1·48
N-Acetylglucosamine (10 mM)	1·49
Glucosamine (10 mM) + glucose (10 mM)	1·20
N-Acetylglucosamine (10 mM) + glucose (10 mM)	2·70

* Specific activity 0·46 mμmole/mg. of protein/min.

DISCUSSION

The amino sugars contained in mucopeptide and other polymers of *B. subtilis* can be derived from glucose or amino sugars (Bates & Pasternak, 1965) present in the growth medium. Our results suggest that the metabolic pathways are as depicted in Scheme 1. *N*-Acetylglucosamine 6-phosphate does not appear to be an intermediate in the synthesis of amino sugars since it is glucosamine 1-phosphate and not glucosamine 6-phosphate that becomes acetylated *in vitro* (Table 6). The loss of the acetyl group during the incorporation of *N*-[^{14}C]acetylglucosamine (Bates & Pasternak, 1965) is in accord with this hypothesis. The branch point between synthesis and utilization of preformed amino sugar



Scheme 1. Amino sugar metabolism in *B. subtilis*.

is thus at glucosamine 6-phosphate. In the present experiments the regulation of the two pathways has been studied under conditions in which the overall metabolism and energy supply were disturbed as little as possible, by supplying cultures with L-glutamate and nutrient broth.

The synthetic route is controlled by the concentration of glucosamine 6-phosphate synthetase, which is repressed by those amino sugars capable of being metabolized by the organism (Table 1). The physiological significance of this is seen by the fact that 7 times as much [^{14}C]glucosamine and 10 times as much *N*-acetyl[^{14}C]glucosamine are incorporated by repressed (glucosamine-grown) cells as by control (glucose-grown) ones (Bates & Pasternak, 1965). It would be to the advantage of the cell to use preformed amino sugars, since energy is required for the glucosamine 6-phosphate-synthetase reaction (in that ATP is used for the resynthesis of glutamine from glutamic acid). It has been suggested (Comb & Roseman, 1958; Pattabiraman & Bachhawat, 1961*b*) that the deamination of glucosamine 6-phosphate by glucosamine 6-phosphate deaminase, which does not involve a 'high-energy' bond, could operate backwards *in vivo*. The fact that repression of glucosamine 6-phosphate synthetase is accompanied by the induction of glucosamine 6-phosphate deaminase makes this unlikely for *B. subtilis*.

The utilization of exogenous amino sugars is controlled by induction of all the enzymes leading to glucosamine 6-phosphate. Thus glucosamine induces a kinase (Table 4), and *N*-acetylglucosamine induces a kinase (Table 4) and a deacetylase (Table 3). The induction of glucosamine 6-phosphate deaminase by acetylglucosamine (Clarke & Pasternak, 1962, and Table 2) makes it possible for the cell to use exogenous amino sugars as a source of cellular material and energy in addition to incorporating it into polymers, though the extent to which such degradation occurs in the presence of glutamate and broth is doubtful. Sugars such as glucose prevent the synthesis of deacetylase and deaminase, which are induced by *N*-acetylglucosamine (Tables 2 and 3), but it is not yet clear whether this is a direct effect or a consequence of diminished uptake of *N*-acetylglucosamine (Bates & Pasternak, 1965). The anabolic route from glucosamine 6-phosphate onwards, which is common to the synthetic and 'salvage' pathways, would not be expected to be under the control of exogenous amino sugar. This

is confirmed by the finding that the synthesis of glucosamine 1-phosphate acetylase is unaffected by the addition of amino sugars to the growth medium (Table 6). Whether glucosamine 1-phosphate or a nucleotide amino sugar (cf. Kornfeld & Glaser, 1962) is the substrate for this enzyme *in vivo* remains to be determined.

It is surprising from the foregoing that glucosamine, *N*-propionylglucosamine and *N*-formylglucosamine, which repress glucosamine 6-phosphate synthetase, do not induce glucosamine 6-phosphate deaminase (Table 1). The *N*-acyl-amino sugars, which compete with *N*-acetylglucosamine for uptake (Bates & Pasternak, 1965) and the phosphate esters of which are degraded by *N*-acetylglucosamine 6-phosphate deacetylase, might be expected to induce that enzyme also. An explanation may be the fact that glucosamine, *N*-formylglucosamine and *N*-propionylglucosamine are incorporated very much more slowly than *N*-acetylglucosamine by *B. subtilis* (Table 1 and Bates & Pasternak, 1965). The basal concentration of deacetylase may thus be adequate for incorporation and in no case does the concentration of glucosamine 6-phosphate rise sufficiently to warrant removal by the degradative route.

The induction of glucosamine 6-phosphate deaminase after prolonged periods of incubation in the absence of added amino sugar may be related to the appearance of lytic enzymes (Salton, 1960) in stationary-phase cultures of *B. subtilis* (Nomura & Hosada, 1956). Enzyme degradation of *B. subtilis* cell walls releases amino sugar-containing material (Richmond, 1959; Young & Spizizen, 1963), which could cause induction of deaminase and reutilization of amino sugars. The fact that in the presence of glucose induction does not occur and that amino sugar-like material accumulates in the growth medium is in accord with the effect of glucose on these processes (Table 2).

We are indebted to Dr P. W. Kent for helpful advice and to the Medical Research Council for the award of a scholarship (to C. J. B.). The work was supported by the Rockefeller Foundation and the National Institutes of Health, U.S. Public Health Service (Grant no. A-3369).

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