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The Regulation of Amino Sugar Metabolism in *Bacillus subtilis*

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A primary stage in the biosynthesis of amino sugars is the amination of hexose 6-phosphate by L-glutamine to yield glucosamine 6-phosphate, a reaction demonstrated in extracts of *Neurospora crassa* (Leloir & Cardini, 1953), *Streptococcus haemolyticus* (Lowther & Rogers, 1956) and mammalian tissues (Castellani & Zambotti, 1956; Pogell & Gryder, 1957; Kizer & McCoy, 1959):

This investigation is concerned with the control of reaction (1) in bacteria. The organism chosen for study was *Bacillus subtilis*, since it is known to synthesize a polysaccharide rich in glucosamine and galactosamine (Sharon, 1957) and contains the enzyme uridine diphosphate N-acetylglucosamine epimerase (Glaser, 1959). Reaction (1) was demonstrated in extracts of *B. subtilis*, glucose 6-phosphate or fructose 6-phosphate serving as substrate. While the work was in progress, Ghosh, Blumenthal, Davidson & Roseman (1960) showed fructose 6-phosphate to be the substrate for purified enzymes from *Escherichia coli*, *N. crassa* and rat liver. The properties of the *E. coli* enzyme were so close to those of the crude *B. subtilis* enzyme that further purification seemed superfluous. Gram-positive organisms have been shown to contain higher amounts of aminotransferase (reaction 1) than Gram-negative ones (Strange & Dark, 1960).

When *B. subtilis* was grown in the presence of exogenous amino sugar the capacity to synthesize glucosamine 6-phosphate was decreased (Pasternak & Clarke, 1961; Clarke & Pasternak, 1961) owing to a reduced synthesis of aminotransferase. There was a concomitant induced synthesis of glucosamine 6-phosphate deaminase [2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating), EC 5.3.1.10] which destroys the product of the aminotransferase reaction (Roseman, 1959; Strange & Dark, 1960):

These two effects have now been separated and their significance clarified.

MATERIALS AND METHODS

Growth of organisms. A strain of *B. subtilis* derived from NCTC 1379 was maintained on slopes of Oxoid nutrient agar CM4 at 4° and used for all experiments. The growth medium was 1.3% (w/v) Oxoid nutrient broth CM1 sterilized by autoclaving at 15 lb./in.² for 20 min. Glucose and sodium L-glutamate were sterilized in the same way and added afterwards to give final concentrations of 0.1 and 0.5% respectively. Amino sugars were sterilized by filtration through Seitz filter pads or Oxoid membrane filters. The medium (500 ml. in 1 l. flasks) was inoculated with 5 ml. of a starter culture in logarithmic phase and shaken for 10–14 hr. on a reciprocal shaker at 37°. Growth

was measured on 5 ml. samples with an EEL Unigalvo type 20 nephelometer with the red filter OR2 and the instrument adjusted to give a reading of 25 with the Perspex standard.

Chemicals. Amino sugars and L-glutamine were commercial samples (British Drug Houses Ltd.). The barium salts of sugar phosphates (British Drug Houses) were converted into potassium salts before use by addition of a slight excess of K_2SO_4 and removal of $BaSO_4$ by centrifuging. Coenzyme A, NADP and glucose 6-phosphate dehydrogenase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Glucosamine 6-phosphate was prepared from glucosamine hydrochloride and ATP by the hexokinase method of Brown (1957). The other reaction products were removed by precipitation of the barium salts with ethanol, the water-soluble supernatant being passed through Dowex 50 X 8 (H^+ form) and chromatographed on paper in 2-methylpropionic acid-aq. NH_3 (sp.gr. 0.88) (5:3, v/v). The yield after elution with water was 40%. N-Acetylglucosamine 6-phosphate was prepared by acetylation of glucosamine 6-phosphate by the method of Kuhn & Bister (1958). An aqueous solution of glucosamine 6-phosphate (60 μ moles in 5 ml.) was freeze-dried and dissolved in 2 ml. of 50% (v/v) methanol. Triethylamine (35 μ l.) was added, followed by acetic anhydride (32 μ l.). The solution was kept at room temperature for 1 hr. and excess of triethylamine removed by extraction with ether. The resulting solution contained 48 μ moles of N-acetyl amino sugar phosphate (80% yield). Chromatography in butan-1-ol-propan-1-ol-acetone-80% (w/v) formic acid-30% (w/v) trichloroacetic acid (8:4:5:5:3, by vol.) (Gerlach, Weber & Doring, 1955) gave one spot with R_F of glucose when sprayed with phosphate (Hanes & Isherwood, 1949) or reducing sugar (Partridge, 1949) reagents. Acetyl-coenzyme A was prepared by the method of Stadtman (1957) except that the initial pH was adjusted to 7.0 and that 5 μ l. of undiluted acetic anhydride was added. It was kindly assayed by Professor H. L. Kornberg by the method of Dixon & Kornberg (1959). O-Diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine were donated by Parke, Davis and Co., Detroit 32, Mich., U.S.A. Amino-acetonitrile and β -aminopropionitrile were gifts of Abbott Laboratories Ltd., Jarrow, Co. Durham. Methionine sulphoxide and glutamyl hydrazide were synthesized and given by Dr D. E. Hughes.

Preparation and fractionation of enzyme extracts. Cells (0.25–0.5 g. dry wt.) in 500 ml. of medium were harvested by centrifuging at 0° , washed twice with 5–10 ml. of ice-cold 0.01 M-cysteine–0.01 M-EDTA buffer, pH 7.5, resuspended in 3 ml. of the same buffer and disrupted by sonic oscillation for 2–5 min. in a Mullard 600 W ultrasonic generator at a frequency of 25 kcyc./sec. For certain preparations the suspension was then centrifuged at 105 000g for 1 hr. in a refrigerated Spinco centrifuge and the supernatant fraction used. This fraction (10–20 mg. of protein/ml.) could be further purified by addition of an equal volume (1 ml.) of 2% (w/v) protamine sulphate solution (pH 6.7), keeping it at 0° for 10 min. and centrifuging it to give a clear supernatant. This contained glucosamine 6-phosphate deaminase. The precipitate was washed with 1 ml. of 15 mM-KCl–8 mM-potassium phosphate buffer, pH 6.9, and extracted with 1.1 ml. of a solution of 5 mM-EDTA in 0.14 M-sodium pyrophosphate buffer, pH 6.7. This contains L-glutamine-D-hexose 6-phosphate amino-

transferase (L-glutamine-D-fructose 6-phosphate aminotransferase, EC 2.6.1.16).

Assay of enzyme extracts. Glutamine-hexose 6-phosphate aminotransferase was assayed by measuring the formation of amino sugar from 2 μ moles of D-glucose 6-phosphate, 1 μ mole of L-glutamine, 50 μ moles of sodium phosphate buffer, pH 7.6, and 0.1 ml. of supernatant fraction (1–2 mg. of protein) in a total volume of 0.5 ml. Glucosamine 6-phosphate deaminase was assayed by measuring the disappearance of amino sugar or the appearance of glucose 6-phosphate from 0.25 μ mole of glucosamine 6-phosphate, tris-HCl buffer (50 μ moles of tris), pH 7.2, and 0.1 ml. of supernatant fraction in 0.5 ml. All reactions were carried out at 37° and were terminated by heating at 100° for 2 min. or by addition of 0.1 ml. of ice-cold 20% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifuging. Amino sugar and N-acetyl amino sugar were estimated by the Levvy & McAllan (1959) method with and without acetic anhydride respectively, glucose by the method of Huggett & Nixon (1957), glucose 6-phosphate by the method of Horecker & Wood (1957) and protein by the biuret method with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) as standard (Gornall, Bardawill & David, 1957).

RESULTS

L-Glutamine-D-hexose 6-phosphate aminotransferase. Ultrasonic extracts of *B. subtilis* contained the enzyme catalysing reaction (1). The product was an amino sugar phosphate which on enzymic deamination yielded hexose 6-phosphate. The aminotransferase was present in the supernatant fraction of the cell obtained after centrifuging for 1 hr. at 105 000g. Such extracts also contained glucose phosphate isomerase, causing glucose 6-phosphate and fructose 6-phosphate to appear equally effective as substrates (Table 1). Mannose 6-phosphate had some activity but other sugar phosphates were inactive. L-Asparagine or ammonia did not replace L-glutamine as amino group donor (Table 1).

The pH optimum of the reaction was between 7 and 8 (Fig. 1). The K_m for glutamine was 0.7 mM (Fig. 2) and that for glucose 6-phosphate was 2.3 mM (Fig. 3). Cysteine and EDTA activated the enzyme and were therefore used to wash and suspend the cells during harvesting (see Materials and Methods section). Inhibitors of other aminotransferase reactions involving glutamine such as O-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (Handschumacher & Welch, 1960) had a pronounced effect on L-glutamine-hexose 6-phosphate aminotransferase also. Thus azaserine caused a 50% inhibition of the enzyme at a concentration of 2 mM and 6-diazo-5-oxo-L-norleucine inhibited 50% at a concentration of 10 μ M (Fig. 4). Similar results were obtained by Ghosh *et al.* (1960). Other analogues of L-glutamine, such as methionine

Table 1. *Synthesis of amino sugar by ultrasonic extract of B. subtilis*

Substrates and tris-HCl buffer (50 μ moles of tris), pH 7.5, were incubated with the supernatant fraction (0.1 ml., 1.3 mg. of protein) of an ultrasonic extract in a final volume of 0.5 ml. for 20 min. The reaction was terminated by heating, precipitated protein removed by centrifuging and the amino sugar content of the supernatant solution measured. P, Phosphate.

Amino group donor	Amount added (μ moles)	Sugar donor	Amount added (μ moles)	Amino sugar formed (μ moles)
L-Glutamine	1	D-Glucose 6-P	2	< 0.04*
L-Glutamine	1	D-Glucose 6-P	2	0.45
L-Glutamine	1	D-Fructose 6-P	2	0.34
L-Glutamine	1	D-Mannose 6-P	5	0.20
L-Glutamine	1	D-Galactose 6-P	5	< 0.04
L-Glutamine	1	D-Ribose 5-P	5	< 0.04
L-Glutamine	1	D-Glucose 1-P	5	< 0.04
L-Glutamine	1	D-Glucose	5	< 0.04
L-Asparagine	10	D-Glucose 6-P	2	< 0.04
Ammonium chloride	1	D-Glucose 6-P	2	< 0.04
Ammonium chloride	10	D-Glucose 6-P	2	0.11

* Heated at 0 min.

Fig. 1. Effect of pH on amino sugar formation. Glucose 6-phosphate (2 μ moles), glutamine (1 μ mole) and sodium phosphate buffer (50 μ moles) (●) or tris-HCl buffer (50 μ moles of tris) (○) were incubated with the supernatant fraction (0.1 ml., 1.2 mg. of protein) of an ultrasonic extract of *B. subtilis* in a final volume of 0.5 ml. for 30 min. The reaction was terminated by heating and the amino sugar content measured.

Fig. 2. Effect of glutamine concentration on amino sugar formation. Glucose 6-phosphate (40 μ moles) and glutamine were incubated with tris-HCl buffer (200 μ moles of tris), pH 7.5, and the supernatant fraction (0.4 ml., 5.9 mg. of protein) of an ultrasonic extract of *B. subtilis* in a final volume of 2 ml. Samples (0.5 ml.) were removed at 10, 20 and 30 min. and added to 0.5 ml. of ice-cold 10% (w/v) trichloroacetic acid, and the amino sugar content measured. The initial velocity was calculated and its reciprocal plotted as shown. The K_m was calculated from the value of $1/s$ for which $1/v = 2$ (broken lines).

sulphoxide and γ -glutamyl hydrazide, were without effect at concentrations below 2 mM; nor did β -aminopropionitrile or aminoacetoneitrile, which appear to alter amino sugar metabolism in whole animals (Kulonen, 1961), have significant action. This confirms the results of work with cell-free extracts of mammalian cartilage (Pedrini & Pedrini-Mille, 1959) and colonic mucosa (C. A. Pasternak, unpublished work).

Effect of exogenous amino sugar on enzymic activity of extracts of B. subtilis. Cultures of *B. subtilis* were grown in the presence of 0.1%

N-acetylglucosamine. No difference in growth rate was observed. Ultrasonic extracts were prepared as before, centrifuged at 105 000*g* for 1 hr. and the supernatant was assayed for aminotransferase activity. Fig. 5 shows that the synthesis of glucosamine 6-phosphate was severely depressed. This was not due to inhibition by residual *N*-acetylglucosamine as the cells had been well washed during harvesting and the extracts contained only negligible amounts of amino sugar. Moreover, *N*-acetylglucosamine added to extracts prepared from control cultures did not depress glucosamine 6-phosphate formation. Two explanations may be offered for the reduced synthesis of glucosamine 6-phosphate. First, synthesis of the aminotransferase enzyme may be repressed by *N*-acetylglucosamine in the growth medium. Secondly, induction of a degradative enzyme such as glucosamine 6-phosphate deaminase [reaction (2)] may occur. Fig. 5 shows that the induction of glucosamine 6-phosphate deaminase indeed takes place and makes investigation of the aminotransferase, which may be repressed simultaneously, difficult. By separating the two enzymes with protamine sul-

phate (see Materials and Methods section) less aminotransferase could be recovered from the protamine precipitate of induced cultures (Scheme 1). It therefore appears that addition of *N*-acetylglucosamine to the growth medium causes both effects to take place: repression of aminotransferase [reaction (1)] and induction of deaminase [reaction (2)]. The latter effect is due to increased synthesis of enzyme since *N*-acetylglucosamine added to control extracts of *B. subtilis* did not activate any 'latent' glucosamine 6-phosphate deaminase. Glucosamine hydrochloride likewise induced glucosamine 6-phosphate deaminase.

Glucosamine 6-phosphate deaminase. The fact that cultures of *B. subtilis* grown in the presence of *N*-acetylglucosamine contain relatively high amounts of glucosamine 6-phosphate deaminase enables one to study some of the properties of this enzyme in greater detail. It is specific for glucosamine 6-phosphate and will not act on glucosamine. The pH optimum is near 7.5 (Fig. 6). The supernatant fraction of *B. subtilis* extracts contains glucose phosphate isomerase and hence the deaminase may be assayed spectrophotometrically by coupling with glucose 6-phosphate dehydrogenase and measuring the rate of formation of NADPH₂ from NADP at 340 m μ . The amount of glucose 6-phosphate formed from glucosamine 6-phosphate is 70% of that deaminated. Whole washed cells of

Fig. 3. Effect of glucose 6-phosphate concentration on amino sugar formation. Glutamine (20 μ moles) and glucose 6-phosphate were incubated with tris-HCl buffer (100 μ -moles of tris), pH 7.5, and the supernatant fraction (0.2 ml., 1.6 mg. of protein) of an ultrasonic extract of *B. subtilis* in a final volume of 1 ml. for 10 min. Trichloroacetic acid [1 ml. of a 10% (w/v) solution] was added, the amino sugar content measured and the initial velocity calculated and plotted as described in Fig. 2. The K_m was calculated from the value of $1/s$ for which $1/v = 2$ (broken lines).

Fig. 4. Effect of 6-diazo-5-oxo-L-norleucine (●) and O-diazoacetyl-L-serine (azaserine) (○) on amino sugar formation. Glucose 6-phosphate (4 μ moles), glutamine (2 μ moles), inhibitor and tris-HCl buffer (100 μ moles of tris), pH 7.5, were incubated with the supernatant fraction (0.2 ml., 1.5 mg. of protein) of an ultrasonic extract of *B. subtilis* in a final volume of 1.1 ml. for 30 min. The reaction was terminated by addition of 1 ml. of 10% (w/v) trichloroacetic acid and the amino sugar content measured. The amount formed in the absence of inhibitor was 0.17 μ mole.

B. subtilis also deaminate glucosamine 6-phosphate, but at a lower rate than ultrasonic extracts.

Induction of glucosamine 6-phosphate deaminase could be demonstrated with resting-cell suspensions incubated in a defined medium (Table 2). Low concentrations of glucose (0.01–0.35 %) are required, presumably as an energy source for protein

synthesis. High concentrations of glucose (1 %), on the other hand, inhibit the synthesis of deaminase (Table 2). In growing cultures also the concentration of glucose appeared to exert a controlling effect on the synthesis of deaminase, since increased amounts of enzyme were produced only late in the growth phase, by which time most of the glucose had been used up. Repression of aminotransferase, however, is operative throughout the growth period.

Acetylation of glucosamine 6-phosphate. A further stage in the biosynthesis of amino sugars is the acetylation of glucosamine 6-phosphate by acetyl-coenzyme A to yield *N*-acetylglucosamine 6-phosphate (Chou & Soodak, 1952; Leloir & Cardini, 1953; Brown, 1955; Davidson, Blumenthal & Roseman, 1957). Attempts to demonstrate this reaction in ultrasonic extracts of *B. subtilis* or the supernatant fraction derived therefrom by spinning at 105 000*g* for 1 hr. were unsuccessful. Substitution of glucosamine 6-phosphate by glucosamine or of acetyl-coenzyme A by ATP, coenzyme A and sodium acetate was without effect. Addition of an extract of mammalian colonic mucosa known to carry out the acetylation reaction (Pasternak, 1961) showed that the lack of demonstrable acetylase activity in *B. subtilis* is not due to enzymic degradation of substrates or product. Synthetic *N*-acetylglucosamine 6-phosphate was not degraded significantly by bacterial extract.

DISCUSSION

Bacillus subtilis resembles other micro-organisms (Leloir & Cardini, 1953; Lowther & Rogers, 1956; Ghosh *et al.* 1960; Lovett & Cantino, 1960; Strange & Dark, 1960) in possessing the ability to synthesize glucosamine 6-phosphate from D-hexose 6-phosphate and L-glutamine [reaction (1)]. The properties of the enzyme (Table 1 and Figs. 1–4) closely resemble those of L-glutamine-D-fructose 6-phosphate aminotransferase of *Escherichia coli* (Ghosh *et al.* 1960).

When *N*-acetylglucosamine is added to growing cultures of *B. subtilis* two effects occur: one, repression of L-glutamine-D-hexose 6-phosphate aminotransferase (Fig. 5 and Scheme 1), and the other, induction of glucosamine 6-phosphate deaminase [reaction (2) (Fig. 5 and Scheme 1)]. The induced synthesis of glucosamine 6-phosphate deaminase appears to be controlled by glucose (Table 2) in a manner similar to that described for other inducible enzymes by Neidhardt (1960). Repression of aminotransferase may therefore be observed with little simultaneous induction of deaminase by growing cultures in the presence of amino sugar and high amounts of glucose. Omission of glutamate from the growth medium appears to yield a similar result. This may be due

Fig. 5. Time course of: (a) amino sugar formation; (b) amino sugar destruction. (a) Glucose 6-phosphate (2 μmoles), glutamine (1 μmole), sodium phosphate buffer (50 μmoles), pH 6.4, and 0.1 ml. of supernatant fraction in a final volume of 0.5 ml. were incubated for various times, the reaction was stopped by addition of 0.1 ml. of 20% (w/v) trichloroacetic acid and the amino sugar content was measured. (b) Glucosamine 6-phosphate (0.25 μmole), sodium phosphate buffer (50 μmoles), pH 7.2, and 0.1 ml. of supernatant fraction in a final volume of 0.5 ml. were incubated and assayed as described for (a). The supernatant fractions were prepared from ultrasonic extracts of *B. subtilis* grown without amino sugar (○) or in the presence of 0.1% *N*-acetylglucosamine (●) and contained 2.3 and 2.0 mg. of protein/0.1 ml. respectively.

Scheme 1. Fractionation of ultrasonic extracts of *B. subtilis*. Cultures were grown with and without 0.1% *N*-acetylglucosamine, harvested, disrupted by sonic oscillation and spun at 105 000g. Aminotransferase was assayed at pH 6.4. Each mixture was incubated for 20 min., the reaction stopped by addition of 0.1 ml. of 20% (w/v) trichloroacetic acid and the amino sugar content measured. Enzyme activities are expressed as μ m-moles of amino sugar appearing or disappearing/mg. of protein before fractionation/20 min.

Fig. 6. Effect of pH on glucosamine 6-phosphate deaminase. Glucosamine 6-phosphate (2 μ moles) and sodium phosphate buffer (50 μ moles) (●) or tris-HCl buffer (50 μ moles of tris) (○) were incubated with the supernatant fraction (0.1 ml., 1–2 mg. of protein) of an ultrasonic extract of *B. subtilis* (grown in the presence of 0.1% *N*-acetylglucosamine) in a final volume of 0.5 ml. for 20 min. The reaction was terminated by heating and the amino sugar content measured.

to an inability to utilize glucose and hence to remove a repressor of deaminase synthesis in the absence of glutamate (cf. Mandelstam, 1962).

The physiological implication of these experiments is that exogenous *N*-acetylglucosamine or

Table 2. Induction of glucosamine 6-phosphate deaminase by washed suspensions of *B. subtilis*

A culture of *B. subtilis* was grown, harvested and washed as described in the Materials and Methods section. The cells (100 mg. dry wt.) were suspended in 10 ml. of the inorganic medium of Knight & Proom (1950) and portions (1.5 ml.) added to 150 ml. conical flasks containing 8 ml. of the same medium and *N*-acetylglucosamine and glucose as indicated in a final volume of 10 ml. All procedures were carried out aseptically. The flasks were shaken at 37° for 2 hr. The cells were harvested, washed and disrupted ultrasonically and the glucosamine 6-phosphate-deaminase activity was measured as described in the Materials and Methods section.

Additions to medium		Enzymic activity (μ m-moles of amino sugar disappearing/mg. of protein/20 min.)
<i>N</i> -Acetyl- glucosamine (%)	Glucose (%)	
0.25	0	62
0.25	0.01	96
0.25	0.035	126
0.25	0.1	106
0.25	0.35	114
0.25	1.0	66
0	0.35	< 16

glucosamine is a preferential source of intracellular amino sugars to glucose. If during growth in the presence of amino sugar the glucose concentration simultaneously falls to low levels, then the cells respond by adaptively metabolizing the amino sugar of the medium to sugar and other sources of available energy. A primary stage in the assimilation of amino sugars, whether as sources of energy or as precursors of amino sugar-containing compounds, would seem to be the phosphorylation of

glucosamine or *N*-acetylglucosamine by ATP. Preliminary experiments have demonstrated glucokinase activity in ultrasonic extracts of *B. subtilis* (J. S. Clarke & C. A. Pasternak, unpublished work).

SUMMARY

1. Ultrasonic extracts of *Bacillus subtilis* contain the enzyme L-glutamine-D-hexose 6-phosphate aminotransferase (L-glutamine-D-fructose 6-phosphate aminotransferase, EC 2.6.1.16). The K_m for L-glutamine is 0.7 mM and that for D-glucose 6-phosphate is 2.3 mM. It is inhibited 50% by 10 μ M-6-diazo-5-oxo-L-norleucine. In these and other respects it closely resembles the L-glutamine-D-fructose 6-phosphate aminotransferase of *Escherichia coli*.

2. Ultrasonic extracts also contain low amounts of glucosamine 6-phosphate deaminase (2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.10) which may be separated from L-glutamine-D-hexose 6-phosphate aminotransferase by precipitation with protamine sulphate.

3. Growth of *B. subtilis* in the presence of amino sugars causes repression of L-glutamine-D-hexose 6-phosphate aminotransferase and concomitant induction of glucosamine 6-phosphate deaminase.

4. The induction of glucosamine 6-phosphate deaminase is repressible by glucose.

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