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Control of Amino Sugar Metabolism in *Escherichia coli* and Isolation of Mutants Unable to Degrade Amino Sugars

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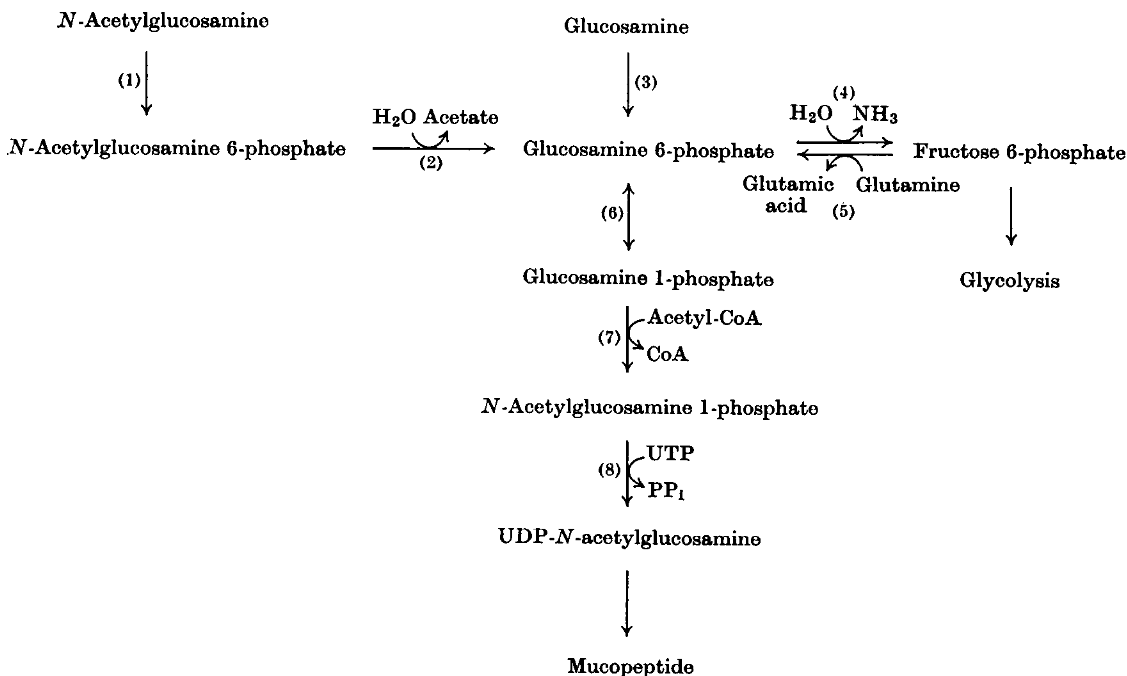
1. Growth of *Escherichia coli* on glucosamine results in an induction of glucosamine 6-phosphate deaminase [2-amino-2-deoxy-D-glucose 6-phosphate ketol-isomerase (deaminating), EC 5.3.1.10] and a repression of glucosamine 6-phosphate synthetase (L-glutamine–D-fructose 6-phosphate aminotransferase, EC 2.6.1.16); glucose abolishes these control effects. 2. Growth of *E. coli* on *N*-acetylglucosamine results in an induction of *N*-acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase, and in a repression of glucosamine 6-phosphate synthetase; glucose diminishes these control effects. 3. The synthesis of amino sugar kinases (EC 2.7.1.8 and 2.7.1.9) is unaffected by growth on amino sugars. 4. Glucosamine 6-phosphate synthetase is inhibited by glucosamine 6-phosphate. 5. Mutants of *E. coli* that are unable to grow on *N*-acetylglucosamine have been isolated, and lack either *N*-acetylglucosamine 6-phosphate deacetylase (deacetylaseless) or glucosamine 6-phosphate deaminase (deaminaseless). Deacetylaseless mutants can grow on glucosamine but deaminaseless mutants cannot. 6. After growth on glucose, deacetylaseless mutants have a repressed glucosamine 6-phosphate synthetase and a super-induced glucosamine 6-phosphate deaminase; this may be related to an intracellular accumulation of acetylamino sugar that also occurs under these conditions. In one mutant the acetylamino sugar was shown to be partly as *N*-acetylglucosamine 6-phosphate. Deaminaseless mutants have no abnormal control effects after growth on glucose. 7. Addition of *N*-acetylglucosamine or glucosamine to cultures of a deaminaseless mutant caused inhibition of growth. Addition of *N*-acetylglucosamine to cultures of a deacetylaseless mutant caused lysis, and secondary mutants were isolated that did not lyse; most of these secondary mutants had lost glucosamine 6-phosphate deaminase and an uptake mechanism for *N*-acetylglucosamine. 8. Similar amounts of ^{14}C were incorporated from [1-^{14}C]-glucosamine by cells of mutants and wild-type growing on broth. Cells of wild-type and a deaminaseless mutant incorporated ^{14}C from *N*-acetyl[1-^{14}C]glucosamine more efficiently than from *N*[1-^{14}C]-acetylglucosamine, incorporation from the latter being further decreased by acetate; cells of a deacetylaseless mutant showed a poor incorporation of both types of labelled *N*-acetylglucosamine.

The large variety of carbon sources that *Escherichia coli* can utilize includes glucosamine and *N*-acetylglucosamine. Preliminary work (Soodak, 1955; Comb & Roseman, 1958; Bates, 1964) has suggested that these amino sugars are degraded by the pathways shown in Scheme 1. Roseman (1957) has studied an enzyme in extracts of *E. coli* that degrades *N*-acetylglucosamine to glucosamine and acetate, but the present work indicates that *N*-acetylglucosamine 6-phosphate deacetylase is the physiologically important means of deacetylation; the purification of this enzyme from *E. coli* has been reported by White & Pasternak (1967). Bates & Pasternak (1965*a*) have proposed a similar scheme for glucosamine and *N*-acetylglucosamine degradation in *Bacillus subtilis* and have shown that growth in the presence of these amino sugars caused an induction of the enzymes necessary for their catabolism; exogenous amino sugar also caused a repression of the enzyme responsible for amino sugar synthesis [L-glutamine–D-fructose 6-phosphate aminotransferase (EC 2.6.1.16)].

In the present work adaptation of *E. coli* to growth on glucosamine and *N*-acetylglucosamine



Scheme 1. Amino sugar metabolism in *E. coli*: (1) *N*-acetylglucosamine kinase (EC 2.7.1.9); (2) *N*-acetylglucosamine 6-phosphate deacetylase (White & Pasternak, 1967); (3) glucosamine kinase (EC 2.7.1.8); (4) glucosamine 6-phosphate deaminase (EC 5.3.1.10); (5) glucosamine 6-phosphate synthetase (EC 2.6.1.16); (6) phosphoglucosamine mutase; (7) glucosamine 1-phosphate acetylase; (8) UDP-*N*-acetyglucosamine pyrophosphorylase.

was studied and the catabolic routes involved were verified by the isolation of mutants unable to grow on these amino sugars.

A preliminary report has been presented (White & Pasternak, 1965).

MATERIALS

Amino sugars, L-glutamine, eosin and methylene blue were obtained from British Drug Houses Ltd. (Poole, Dorset), fructose 6-phosphate (sodium salt) was from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and phosphoglucose isomerase (EC 5.3.1.9) were from Boehringer Corp. (London, W. 5). Glucosamine 6-phosphate and *N*-acetylglucosamine 6-phosphate were prepared as described by White & Pasternak (1967). [1-^{14}C]Glucosamine, [1-^{14}C]-acetic anhydride and [U-^{14}C]glucose 6-phosphate were obtained from The Radiochemical Centre (Amersham, Bucks.). *N*[1-^{14}C]-Acetylglucosamine and *N*-acetyl[1-^{14}C]-glucosamine were prepared by the method of Bates & Pasternak (1965*b*). *N*[1-^{14}C]-Acetylglucosamine 6-phosphate was prepared from [1-^{14}C]acetic anhydride and a fivefold excess of glucosamine 6-phosphate (Kuhn & Bister, 1958); the reaction products were adsorbed on a column (1cm. × 5cm.) of Dowex 1 (X4; Cl^- form), excess of glucosamine 6-phosphate and [1-^{14}C]acetate were eluted with 0·05N-acetic acid, and the *N*[1-^{14}C]-acetylglucosamine 6-phosphate was eluted with 0·1N-HCl. The acid eluate was neutralized with NaOH and stored frozen.

METHODS

Growth of organisms and preparation of extracts

E. coli K_{12} 701 was maintained on slopes of Oxoid nutrient agar CM4 at 4°, and grown in Oxoid nutrient broth CM1, Difco heart infusion broth or minimal medium (Davis & Mingioli, 1950). Glucose, glycerol and media were autoclaved separately (15lb./in.^{2} for 15min.); amino sugars were sterilized by membrane filtration. Where indicated, carbon sources were added to the medium to give a final concentration of 0·01M. Medium (500ml. in 2l. flasks) was inoculated with 2–5ml. of a starter culture in exponential phase and shaken at 37° for 10–14hr. in a Gyrotary shaker (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.). Growth was measured with a nephelometer (EEL Unigalvo type 20). Cells were harvested towards the end of exponential phase, washed once in 200ml. of 0·01M-sodium phosphate buffer, pH7·0, containing 0·01M-2-mercaptoethanol, resuspended in 5ml. of buffer and disrupted by passage through a French pressure cell (American Instrument Co., Silver Springs, Md., U.S.A.). The disrupted-cell suspension was centrifuged at 105000*g* for 1hr. in a refrigerated ultracentrifuge, and the resultant supernatant (8–15mg. of protein/ml.) was retained for enzyme assays.

Assay of enzymes

Glucosamine 6-phosphate synthetase. Assay of this enzyme in crude extracts proved difficult owing to interference by glucosamine 6-phosphate deaminase; this problem was overcome by using protamine sulphate fractionation (Clarke & Pasternak, 1962) and by performing the assay at pH5·5 in sodium citrate buffer. Incubation mixtures contained 10μmoles of fructose 6-phosphate, 10μmoles of L-glutamine, 25μmoles of sodium citrate buffer, pH 5·5, and fractionated extract (0·2–1·0mg. of protein) in a total volume of 0·45ml. Reactions were carried out at 37° for 10min. and stopped by the addition of 0·05ml. of 50% (w/v) trichloroacetic acid. Protein was removed by centrifugation and samples from the supernatant were analysed for amino sugar. In certain experiments a radioactive assay was used. Incubation mixtures contained 0·5μmole of [U-^{14}C]-glucose 6-phosphate (1·5μc), 10μmoles of L-glutamine, 20μmoles of sodium phosphate buffer, pH 7·0, 8 units of glucose phosphate isomerase and fractionated enzyme in a total volume of 0·5ml. Reactions were carried out at 37° for 10min. and terminated by boiling for 1min. Protein was removed by centrifugation and samples of the supernatant were analysed by radioactive assay after paper electrophoresis in 0·1M-ammonium acetate, pH 8·0, for 4hr. (13·5v/cm.) to separate [U-^{14}C]glucose 6-phosphate from [U-^{14}C]glucosamine 6-phosphate.

Glucosamine 6-phosphate deaminase and N-*acetylglucosamine 6-phosphate deacetylase.* These were assayed as described by White & Pasternak (1967).

Amino sugar kinases. These were assayed by the method of Bates & Pasternak (1965*a*).

Amino sugar phosphate acetylase. This was assayed by the method of Bates & Pasternak (1965*a*) except that 1·0μmole of amino sugar phosphate was used in each incubation.

Analytical procedures

Radioactive determinations. Radioactivity on dried electrophoresis strips was measured in a radioactive chromatogram counter (Baird and Tatlock Ltd., Chadwell Heath, Essex) with a counting efficiency of 3%. Radioactivity in other samples was measured on aluminium planchets in an automatic sample-changing assembly (Lobetamat, type 6051LA; Isotope Developments Ltd., Beenham, Berks.) at infinite thinness; counting efficiency was 5%.

Chemical analyses. Amino sugar and acetylamino sugar were determined by the method of Levvy & McAllan (1959), phosphate by the method of Chen, Toribara & Warner (1956), reducing sugar by the method of Park & Johnson (1957) and protein by the method of Gornall, Bardawill & David (1957), with bovine plasma albumin as standard.

Paper chromatography. Descending chromatography on Whatman no. 1 paper was carried out with the following solvents: butan-1-ol–pyridine–water (6:4:3, by vol.); butan-1-ol–acetic acid–water (4:1:5, by vol.). The spray reagents described by Partridge (1948) and Trevelyan, Procter & Harrison (1950) were used to detect amino sugars and reducing compounds respectively.

Isolation of primary mutants. Mutants of *E. coli* were obtained by ultraviolet irradiation or by treatment with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine (Adelberg, Mandel & Chen, 1965), and enriched by penicillin selection (Davis, 1949) in the presence of *N*-acetylglucosamine, followed by replication from glucose-containing to *N*-acetylglucosamine-containing agar plates. Initially glucose was used as a carbon source for 'phenotypic expression', but subsequently glucosamine was used to prevent the isolation of glucosamine-negative mutants. Colonies with a glucose-positive *N*-acetylglucosamine-negative phenotype were picked off, grown in nutrient broth and maintained on Oxoid nutrient agar CM4 at 4°.

Agar for isolation of secondary mutants. The agar used was a modification of that originally described by Levine (1921), and contained 10g. of peptone, 10g. of *N*-acetylglucosamine, 2g. of K_2HPO_4, 0·4g. of eosin Y, 0·065g. of methylene blue and 15g. of agar in 1l. of water. Colonies utilizing the *N*-acetylglucosamine in this agar are purple, whereas those not doing so are colourless.

RESULTS

Growth rate on amino sugar. The generation time of *E. coli* grown in minimal medium containing glucose, glycerol, glucosamine or *N*-acetylglucosamine as carbon source was 53, 80, 75 or 62min. respectively. A washed glucose-grown inoculum had difficulty in adapting to glucosamine, but this could be overcome by the addition of a trace of glucose (0·5mM). During the change-over from glucose to glucosamine (Fig. 1) there was a diauxic

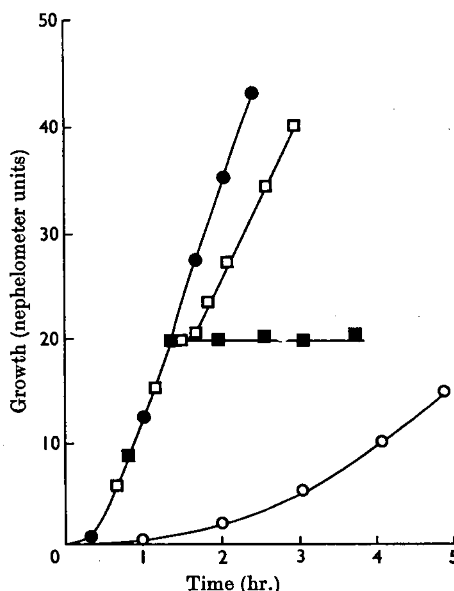


Fig. 1. Growth of *E. coli* on glucose + glucosamine. Minimal medium was inoculated with a washed glucose-grown suspension of *E. coli* and shaken at 37°, and growth was measured in a nephelometer. ●, 10mM-Glucose; ○, 10mM-glucosamine; ■, 1·4mM-glucose; □, 1·4mM-glucose + 10mM-glucosamine.

lag (Monod, 1941), which could be overcome by pre-growth on N-acetylglucosamine (Fig. 2). Growth on a mixture of limiting glucose and excess of N-acetylglucosamine was not diauxic.

Enzyme adaptation to growth on amino sugar. The induction and repression of several enzymes involved in amino sugar metabolism are shown in Table 1. Specific activities of enzymes after growth on glucose and glycerol were similar. Glucosamine and N-acetylglucosamine caused repression of glucosamine 6-phosphate synthetase and induction of glucosamine 6-phosphate deaminase. Growth on glucosamine did not alter the specific activity of N-acetylglucosamine 6-phosphate deacetylase, whereas growth on N-acetylglucosamine caused an eightfold induction of this enzyme. Amino sugar kinases appeared to be synthesized constitutively. Glucose abolished the control effects exerted by glucosamine, but only diminished those of N-acetylglucosamine.

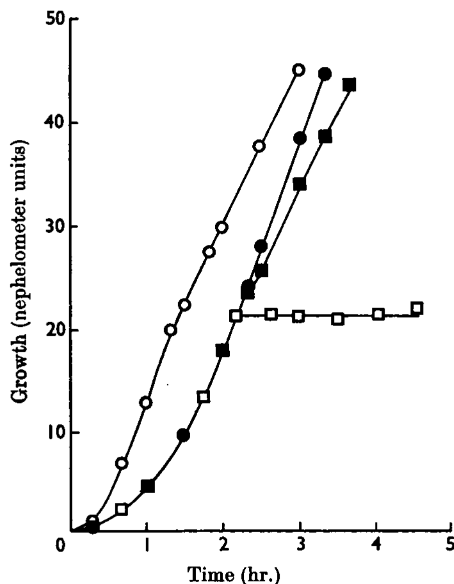


Fig. 2. Growth of *E. coli* on glucose + N-acetylglucosamine. Minimal medium was inoculated with a washed glucose-grown suspension of *E. coli* and shaken at 37°, and growth was measured in a nephelometer. ○, 1·4mM-Glucose + 10mM-N-acetylglucosamine; ●, 10mM-N-acetylglucosamine; □, 1·13mM-N-acetylglucosamine; ■, 1·13mM-N-acetylglucosamine + 0·01M-glucosamine.

Control of amino sugar synthesis by product inhibition. Although UDP-N-acetylglucosamine was a feedback inhibitor of glucosamine 6-phosphate synthetase from rat liver, it had no effect on the enzyme from *E. coli* (Kornfeld, Kornfeld, Neufeld & O'Brien, 1965). A radioactive assay for glucosamine 6-phosphate synthetase was used to test the effect of several possible inhibitors (Table 2). Glucosamine 6-phosphate was the most effective; inhibition by N-acetylglucosamine 6-phosphate might have been the result of its degradation to glucosamine 6-phosphate by a deacetylase. This possibility was supported by the observation that N-acetylglucosamine 6-phosphate did not inhibit when a mutant lacking deacetylase was used. Because Wolfe & Nakada (1956) have shown that glucosamine 6-phosphate is a potent inhibitor of glucose phosphate isomerase, an excess of glucose phosphate isomerase was always added to the incubation mixtures; even at the highest concentration of glucosamine 6-phosphate used, glucose 6-phosphate and fructose 6-phosphate had equilibrated in $1\frac{1}{2}$ min.

Isolation and characterization of N*-acetylglucosamine-negative mutants.* Initially all the N-acetylglucosamine-negative mutants isolated (negative and positive denote ability or inability to grow on a particular carbon source) were also glucosamine-

Table 1. *Induction and repression of enzymes involved in amino sugar metabolism*

Cultures grown in minimal medium containing the carbon sources indicated (all 10mM) were harvested in exponential phase (0·2–0·3mg. dry wt. of cells/ml.) and a cell-free extract was prepared. Enzymes were assayed as described in the Methods section. Enzyme activities are expressed as mμmoles of substrate metabolized/mg. of protein/min.; N.D., not determined.

Carbon source	Glucosamine 6-phosphate synthetase	Glucosamine 6-phosphate deaminase	N-Acetyl-glucosamine 6-phosphate deacetylase	Glucosamine kinase	N-Acetyl-glucosamine kinase
Glucose	36·5	6·0	18·5	2·5	5·0
Glycerol	41·2	8·0	20·3	N.D.	N.D.
Glucosamine	16·0	25·6	20·0	2·5	5·5
N-Acetylglucosamine	9·3	54·9	162·4	3·0	6·0
Glucosamine + glucose	33·4	7·3	17·5	N.D.	N.D.
N-Acetylglucosamine + glucose	9·1	31·2	72·0	N.D.	N.D.
N-Acetylglucosamine + glycerol	9·5	45·0	141·1	N.D.	N.D.

Table 2. *Inhibition of glucosamine 6-phosphate synthetase*

A radioactive technique (see the Methods section) was used to assay glucosamine 6-phosphate synthetase activity in a protamine sulphate-fractionated extract of *E. coli* (10mg. of protein/ml.). [U-^{14}C]Glucose 6-phosphate and L-glutamine were present at concentrations of 1·0mM and 20·0mM respectively.

Addition	Time (min.)	Vol. of enzyme soln. (ml.)	Conversion (%)	Inhibition (%)
None	5	0·05	11·0	—
None	5	0·10	20·1	—
None	10	0·05	18·3	—
Glucosamine (10mM)	10	0·05	19·2	<5·0
N-Acetylglucosamine (10mM)	10	0·05	17·4	<5·0
N-Acetylglucosamine 6-phosphate (2mM)	10	0·05	16·5	10·0
Glucosamine 6-phosphate (1mM)	10	0·05	11·0	40·0
Glucosamine 6-phosphate (2mM)	10	0·05	8·0	56·0
Glucosamine 6-phosphate (3mM)	10	0·05	5·8	68·0

Class 1

Deaminaseless

Glucose +, glucosamine −, *N*-acetylglucosamine −

↑

Wild-type *E. coli*

Glucose +, glucosamine +, *N*-acetylglucosamine +

↓

Class 2

Deacetylaseless

Glucose +, glucosamine +, *N*-acetylglucosamine −

Lysed by *N*-acetylglucosamine

Class 3

Deacetylaseless and deaminaseless

Glucose +, glucosamine −, *N*-acetylglucosamine −

Not lysed by *N*-acetylglucosamine

Class 4

Deacetylaseless and diminished deaminase

Glucose +, glucosamine +, *N*-acetylglucosamine −

Not lysed by *N*-acetylglucosamine

Scheme 2. Derivation and growth characteristics of four classes of mutants unable to grow on *N*-acetylglucosamine. +, Growth; −, no growth; deaminaseless, lacks glucosamine 6-phosphate deaminase; deacetylaseless, lacks *N*-acetylglucosamine 6-phosphate deacetylase.

negative (class 1 mutants); in a subsequent attempt glucosamine was used as carbon source during phenotypic expression (see the Methods section), and as a result of this a further type of mutant that was glucosamine-positive and *N*-acetylglucosamine-negative (class 2 mutant) was isolated. Several types of class 1 and 2 mutants were isolated, of which mutants 1-1 and 2-1 were used for most of the further work. The growth characteristics of class 1 and 2 mutants are shown in Scheme 2.

Table 3. *Enzyme activities in* N*-acetylglucosamine-negative mutants*

Bacteria were grown in minimal medium containing the compounds indicated (all 10mM) and harvested in exponential phase (0·2–0·3mg. dry wt. of cells/ml.), and a cell-free extract was prepared. Enzymes were assayed as described in the Methods section. Specific activity of enzymes is expressed relative to glucose-grown wild-type in Table 1; N.D., not determined.

Strain	Carbon source	Glucosamine 6-phosphate synthetase	Glucosamine 6-phosphate deaminase	*N*-Acetyl-glucosamine 6-phosphate deacetylase	Glucosamine kinase	*N*-Acetyl-glucosamine kinase
Wild-type	Glucose	1·00	1·00	1·00	N.D.	N.D.
	Glucosamine	0·40	4·00	1·10	N.D.	N.D.
	N-Acetylglucosamine + glucose	0·25	5·0	4·0	1·0	1·0
Mutant 1·1	Glucose	1·00	<0·02	0·90	N.D.	N.D.
	N-Acetylglucosamine + glucose	0·25	<0·02	15·00	0·8	1·1
Mutant 2·1	Glucose	0·25	20·00	<0·02	N.D.	N.D.
	Glucosamine	0·20	25·60	<0·02	N.D.	N.D.
	N-Acetylglucosamine + glucose	0·20	23·20	<0·02	1·2	0·9

Enzymology of mutants after growth in the presence of N*-acetylglucosamine.* Table 3 lists the specific activities of several enzymes after growth of mutants 1-1 and 2-1 on various carbon sources. Mutant 1-1 lacked glucosamine 6-phosphate deaminase (deaminaseless), whereas mutant 2-1 lacked *N*-acetylglucosamine 6-phosphate deacetylase (deacetylaseless). The activity of synthetase and deacetylase in the mutant 1-1 were normal after growth on glucose, but growth in the presence of *N*-acetylglucosamine caused a super-induction of deacetylase; repression of synthetase was normal. Mutant 2-1 had a repressed synthetase and a super-induced deaminase irrespective of the conditions of growth. Attempts to isolate mutants deficient in amino sugar kinases were not successful.

Intracellular accumulation of amino sugar by mutants. After growth on glucose plus *N*-acetylglucosamine, extracts of class 1 mutants contained amino sugar, less than 2% of it being *N*-acetylated, and 75% of the amino sugar accumulated by mutant 1-1 could be adsorbed by Dowex 1 (X4; acetate form) and eluted by 0·05N-acetic acid. Paper chromatography suggested that the material adsorbed by the anion-exchange resin was glucosamine 6-phosphate, the remainder being glucosamine.

During growth on glucose class 2 mutants accumulated *N*-acetylamino sugar; in mutant 2-1 the intracellular concentration was shown to be 3·2mM. After growth under the same conditions the concentration of *N*-acetylamino sugar was 0·1mM in wild-type and 0·22mM in mutant 1-1. Glucose-grown cells of mutant 2-1 were extracted with cold trichloroacetic acid and the extract was chromatographed on a column of Dowex 1 (X4; Cl^- form). The column was eluted with a hydrochloric acid gradient, and fractions were collected and analysed for amino sugar before and after acid hydrolysis; the results are shown in Fig. 3. Before acid hydrolysis only one peak (*B*) was detectable and this was coincident with radioactive marker *N*[1-^{14}C]-acetylglucosamine 6-phosphate. Acetylamino sugar, reducing sugar and organic phosphate contents of peak *B* were in the molar proportions 1·0:1·08:1·67. In an enzyme assay for *N*-acetyl-

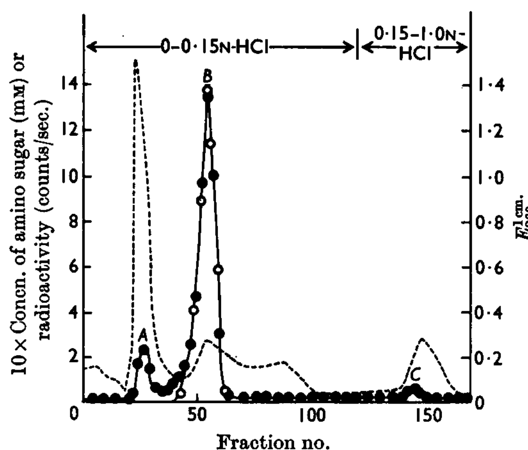


Fig. 3. Ion-exchange chromatography of acetylamino sugar accumulated by mutant 2-1. Glucose-grown cells of mutant 2-1 were extracted with 5% (w/v) trichloroacetic acid at 0° for 1hr., and the insoluble residue was discarded. Trichloroacetic acid was removed from the supernatant by ether extraction. *N*[1-^{14}C]-Acetylglucosamine 6-phosphate (0·2μmole, 2μc; preparation described in the Materials section) was added to the neutral extract, and the mixture was applied to a column (50cm. × 1·5cm. diam.) of Dowex 1 (Cl^- form). The column was eluted with a linear gradient of HCl and 4ml. fractions were collected. Fractions were analysed for u.v.-absorbing material at 260mμ (---), radioactivity (○) and amino sugar (●).

glucosamine 6-phosphate (see the Methods section) the *N*-acetylamino sugar in peak *B* gave the same rate and total of NADPH formation as did *N*-acetylglucosamine 6-phosphate. Analysis of peak *B* by paper chromatography and electrophoresis also suggested that *N*-acetylglucosamine 6-phosphate was a major component, though the high organic phosphate content and u.v. absorption showed that the peak was not homogeneous. Acid hydrolysis of the column fractions revealed two further peaks (*A* and *C*), of which *A* yielded amino sugar only after strong acid hydrolysis (1·0N-hydrochloric acid at 100° for 30min.) whereas peak *C* liberated acetylamino sugar on mild acid hydrolysis (0·1N-hydrochloric acid at 100° for 10min.). The amino sugar in peaks *A* and *C* was coincident with the u.v.-absorbing material; the extinction ratios (*A*, E_{250}/E_{260} 0·84 and E_{280}/E_{260} 0·38; *C*, E_{250}/E_{260} 0·77 and E_{280}/E_{260} 0·51) were consistent with the suggestion that the base was uridine in both cases. However, peaks *A* and *C* contained more than one component and were not further characterized.

Table 4. *Incorporation of* [1-^{14}C]*glucosamine by wild-type and mutants*

Cells in nutrient broth (0·25mg. dry wt. of cells/ml.) were incubated at 37° with [1-^{14}C]glucosamine (0·1mM, 0·4μc/ml.). Whole cells and material insoluble in cold 5% trichloroacetic acid were collected on membrane filters and washed, and their radioactive contents were determined as described in the Methods section. Results are expressed in arbitrary units; 100=60% incorporation.

		Incorporation	
Strain	Time (min.)	Whole cells	Trichloroacetic acid-insoluble material
Wild-type	30	82	65
	60	100	92
Mutant 1-1	30	79	17
	60	102	35
Mutant 2-1	30	76	70
	60	95	84

Uptake and incorporation of labelled amino sugars by mutants and wild-type. Incorporation of [1-^{14}C]-glucosamine by cells of wild-type, mutant 1-1 and mutant 2-1 is summarized in Table 4. The uptake of [1-^{14}C]glucosamine into whole cells was the same by mutants and by wild-type, but incorporation into trichloroacetic acid-insoluble material by the deaminaseless mutant (1-1) was considerably less, the difference representing the cytoplasmic pool. Bates & Pasternak (1965*b*) studied the uptake and incorporation of ^{14}C from *N*[1-^{14}C]-acetylglucosamine and *N*-acetyl[1-^{14}C]glucosamine by *B. subtilis*, and found that deacetylation occurred before incorporation. Thus after incorporation from *N*[1-^{14}C]-acetylglucosamine the distribution of label in cell fractions resembled that for [^{14}C]acetate, whereas for *N*-acetyl[1-^{14}C]glucosamine the distribution resembled that for [1-^{14}C]glucosamine. Addition of unlabelled acetate diminished incorporation from *N*[1-^{14}C]-acetylglucosamine but

Table 5. *Uptake and incorporation of labelled* N-*acetylglucosamine by wild-type and mutants*

Cells in nutrient broth (0·25mg. dry wt. of cells/ml.) were incubated for 30min. at 37° with *N*[1-^{14}C]-acetylglucosamine or *N*-acetyl[1-^{14}C]glucosamine (each 0·6μc; each 0·01mM). Acetate (10mM) was added to incubation mixtures where indicated. Samples of whole cells and material insoluble in cold 5% trichloroacetic acid were collected on membrane filters and washed, and their radioactive contents were determined as described in the Methods section. Incorporation of ^{14}C into whole cells and trichloroacetic acid-insoluble material is given in arbitrary units; 100=55% incorporation.

			Incorporation	
Strain	Addition to incubation	Acetate	Whole cells	Trichloroacetic acid-insoluble material
Wild-type	*N*-Acetyl[^{14}C]glucosamine	−	100	70
		+	91	50
	N[^{14}C]-Acetylglucosamine	−	8	8
		+	1	1
Mutant 1-1	*N*-Acetyl[^{14}C]glucosamine	−	71	28
		+	70	24
	N[^{14}C]-Acetylglucosamine	−	2	2
		+	1	1
Mutant 2-1	*N*-Acetyl[^{14}C]glucosamine	−	46	3
		+	48	2
	N[^{14}C]-Acetylglucosamine	−	58	2
		+	38	1

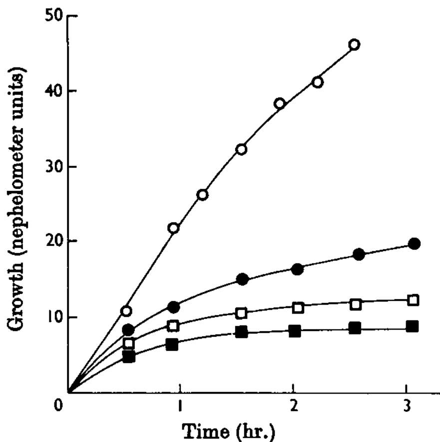


Fig. 4. Inhibition of growth of mutant 1-1 by amino sugar. Nutrient broth was inoculated with washed broth-grown cells of mutant 1-1 and shaken at 37°, and growth was measured in a nephelometer. Where indicated, compounds were added to give 10mM solutions. ○, No additions, + glucose, or + glucose + glucosamine; ●, + glucosamine; □, + glucose + *N*-acetylglucosamine; ■, + *N*-acetylglucosamine.

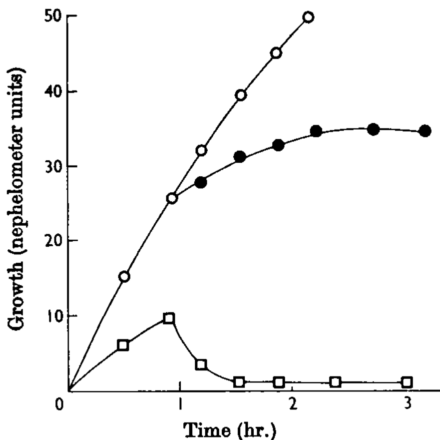


Fig. 5. Lysis of mutant 2-1 by *N*-acetylglucosamine. Nutrient broth was inoculated with washed broth-grown cells of mutant 2-1 and shaken at 37°, and growth was measured in a nephelometer. Where indicated, compounds were added to give 10mM solutions. ○, No additions, + glucosamine, + glucose, or + glucose + *N*-acetylglucosamine; ●, + *N*-acetylglucosamine at 1 hr.; □, + *N*-acetylglucosamine at zero time.

not from *N*-acetyl[1-^{14}C]glucosamine. Similar experiments are now reported for the uptake and incorporation of labelled *N*-acetylglucosamine by wild-type *E. coli*, mutant 1-1 and mutant 2-1 (Table 5). Incorporation of label from *N*[1-^{14}C]-acetylglucosamine was poor in all cases. Since radioactivity present as [^{14}C]acetate was lost in the counting procedure used the results for *N*[1-^{14}C]-acetylglucosamine uptake by mutant 1-1 and wild-type did not mean that there had been no uptake, but that deacetylation had occurred immediately after uptake and that the label was subsequently lost as [^{14}C]acetate. This suggestion is supported by the result for mutant 2-1, where uptake of ^{14}C occurred with little incorporation; this mutant could not deacetylate the *N*[1-^{14}C]-acetylglucosamine and consequently did not lose the label as [^{14}C]acetate. Incorporation of *N*-acetyl-[1-^{14}C]glucosamine by wild-type and mutant 1-1 was probably identical with that for [1-^{14}C]-glucosamine (Table 4); mutant 1-1 had a large cytoplasmic pool of label in both cases. Presumably the small amount of *N*-acetylglucosamine added (about 2μg. for each 250μg. of cells) was immediately deacetylated. Mutant 2-1 had a poor incorporation of ^{14}C from both types of labelled *N*-acetylglucosamine. The small amount of incorporation by this mutant could have been due to 'leakiness' (residual deacetylase) or could have occurred without deacetylation. Unlabelled acetate caused some diminution of uptake and incorporation in all cases but the most marked effect was on incorporation of ^{14}C from *N*[1-^{14}C]-acetylglucosamine by wild-type.

Inhibition of growth of mutants by amino sugars. Class 1 mutants grew markedly more slowly on a mixture of glucose and *N*-acetylglucosamine than on glucose alone, whereas class 2 mutants grew equally well in the presence of *N*-acetylglucosamine. To investigate this effect more fully, amino sugars were added to broth cultures of mutants and wild-type (Figs. 4 and 5). Growth of mutant 1-1 was severely retarded by glucosamine and *N*-acetylglucosamine; glucose abolished inhibition by glucosamine, but only diminished that by *N*-acetylglucosamine. Glucosamine had no effect on the growth of mutant 2-1, but *N*-acetylglucosamine caused lysis after a short period of growth. The type of medium used influenced the effect of *N*-acetylglucosamine, and on occasions there was no lysis. Lysis occurred invariably in heart infusion broth and this medium was used for further work. Addition of glucose and fructose to cultures prevented lysis, but glycerol did not. Lysis occurred over a wide range of *N*-acetylglucosamine concentrations (0·001–1·0%). Addition of sucrose (0·25M) to cultures permitted normal growth in the presence of *N*-acetylglucosamine; phase-contrast microscopy showed that such cells resembled spheroplasts, and if the sucrose-protected cells were resuspended in water they lysed. Chloramphenicol (25μg./ml.) and glucose (0·01M) could be added up

to 45min. after *N*-acetylglucosamine and still prevented lysis; under these conditions glucose permitted normal growth whereas chloramphenicol caused growth stasis.

Amino sugar content of cell walls. Lysis of mutant 2-1 by *N*-acetylglucosamine resembled that of galactose-negative mutants by galactose (Fukasawa & Nikaido, 1961), which were shown to have an abnormal cell wall, devoid of galactose. To see whether the present mutants had any similar defect, the amino sugar content of their cell walls was determined. The yield of cell walls and amino sugar content was very similar in wild-type and both mutants (Table 6).

Isolation of secondary mutants resistant to lysis by N-*acetylglucosamine.* Lysis of mutant 2-1 by *N*-acetylglucosamine in liquid cultures (Fig. 6) could be reproduced on solid media, in that growth on nutrient agar containing *N*-acetylglucosamine (see the Methods section) produced small colonies with an irregular morphology and a pitted surface, probably due to localized lysis. Although the small irregular colony type predominated, a few large regular colonies were also produced. These always bred true and produced only large colonies on the same medium, whereas small colonies always produced a mixture of small and large colonies. Of 45 large colonies isolated, 42 were glucosamine-negative (class 3 mutants) and three were glucosamine-positive (class 4 mutants); the parent, mutant 2-1, was glucosamine-positive. All class 3 and class 4 mutants were still *N*-acetylglucosamine-negative (i.e. they had not reverted), but were resistant to lysis in liquid media. The derivation and growth characteristics of the two classes of mutants obtained is shown in Scheme 2.

Activity of enzymes in secondary mutants. Table 7 gives the specific activity of several enzymes after growth of mutants 3-1 and 4-1 (which were both typical mutants of their respective classes) on minimal medium. As with the class 1 mutants, loss of ability to grow on glucosamine in mutant 3-1 was associated with the loss of deaminase. Mutant 4-1 had a decreased deaminase activity and a synthetase activity approximately that of wild-type grown under these conditions.

In addition to the altered enzyme activities shown in Table 7 mutants 3-1 and 4-1 had a defective uptake mechanism for *N*-acetylglucosamine (Table 8);

Table 6. *Amino sugar contents and yield of cell walls prepared from wild-type and mutants*

Cells were grown in heart infusion broth, washed, disrupted in a French pressure cell and centrifuged at 15000*g* for 15min. The sediment was washed twice in M-NaCl and twice with water; intact cells were removed by centrifuging at 4000*g* for 10min. Cell walls were sedimented by centrifuging at 15000*g* for 15min. and stored as a concentrated suspension in water.

Strain	Amino sugar* (% of cell wall)	Yield† (mg.)
Wild-type	1·8	69
Mutant 1-1	2·3	65
Mutant 2-1	1·9	68

* Determined after hydrolysis in 2N-HCl for 2hr. at 100°, expressed as glucosamine equivalents.

† Yield of purified cell walls from 1g. dry wt. of whole cells.

Table 8. *Uptake of* N-*acetyl*[1-^{14}C]*glucosamine by secondary mutants*

Cells in nutrient broth (0·25mg. dry wt. of cells/ml.) were incubated for 15min. at 37° with *N*-acetyl[1-^{14}C]glucosamine (0·02mM, 0·08μc/ml.), and glucose was added (+) where indicated. Whole cells were collected by membrane filtration and washed, and their radioactive contents were determined as described in the Methods section. Uptakes are given in arbitrary units; 100=63% uptake.

Strain	Glucose	Uptake
Mutant 2-1	−	100
	+	75
Mutant 3-1	−	78
	+	0·3
Mutant 4-1	−	88
	+	21

Table 7. *Enzyme activities in secondary mutants*

Cells were grown on minimal medium containing 10mM-glucose and harvested in exponential phase (0·2–0·3mg. dry wt. of cells/ml.), and a cell-free extract was prepared. Enzymes were assayed as described in the Methods section. Activities are given relative to the specific activity for mutant 2-1 (see Table 3).

Strain	Glucosamine 6-phosphate synthetase	Glucosamine 6-phosphate deaminase	*N*-Acetylglucosamine 6-phosphate deacetylase	*N*-Acetylglucosamine kinase
Mutant 2-1	1·0	1·0	<0·01	1·0
Mutant 3-1	1·7	<0·01	<0·01	1·1
Mutant 4-1	3·9	0·13	<0·01	0·8

this defect became apparent only in the presence of glucose. This suggests that there were two mechanisms for the entry of *N*-acetylglucosamine into the cell, one specific (absent in class 3 mutants) and the other shared with glucose. The difference between class 3 and class 4 mutants seemed to be quantitative rather than qualitative, since uptake and deaminase activities were reduced or absent in both cases (i.e. the site of the mutation may be the same).

Acetylation of amino sugar phosphates. Kornfeld & Glaser (1962) studied the specificity of amino sugar phosphate acetylation in *E. coli*, and showed that glucosamine 1-phosphate and UDP-glucosamine were good substrates, but that glucosamine 6-phosphate was a poor one. The poor acetylation of glucosamine 6-phosphate might have been due to degradation of product since the crude extracts used probably contained *N*-acetylglucosamine 6-phosphate deacetylase. Degradation of product and substrate was avoided in the present work by the use of mutant 3-1, which lacked deacetylase and deaminase. An enzyme extract of glucose-grown mutant 3-1 was assayed for acetylase activity with glucosamine 6-phosphate and glucosamine 1-phosphate as substrates. The activity with glucosamine 6-phosphate was 0·9 mμmole of substrate metabolized/min./mg. of protein and with glucosamine 1-phosphate was 25·0 mμmoles of substrate metabolized/min./mg. of protein.

DISCUSSION

Growth of wild-type on amino sugar. Growth of *E. coli* on glucosamine and *N*-acetylglucosamine causes a pattern of induction and repression resembling that obtained for *B. subtilis* (Bates & Pasternak, 1965*a*). One significant difference is that both amino sugar kinases are inducible in *B. subtilis* whereas they are constitutive in *E. coli*. However, it is not certain that the amino sugar kinases assayed in the present work are the only ones operative *in vivo*. In this connexion, Kundig, Ghosh & Roseman (1964) have reported the presence of an inducible phosphotransferase system in *E. coli* that phosphorylates glucose, glucosamine and *N*-acetylglucosamine; further, a mutant of *E. coli* that lacks this phosphotransferase activity (Tanaka, Fraenkel & Lin, 1967) grows poorly on glucosamine and *N*-acetylglucosamine (White, 1966), indicating its importance in amino sugar phosphorylation.

Glucosamine 6-phosphate deaminase and *N*-acetylglucosamine 6-phosphate deacetylase are not synthesized co-ordinately, as growth on glucosamine induces only the deaminase (Table 1); this is also true for *B. subtilis* (Bates & Pasternak, 1965*a*).

The effect of glucose on induction and repression by glucosamine and *N*-acetylglucosamine is paralleled by the growth curves obtained on a mixture of glucose and amino sugar (Figs. 1 and 2). Diauxic growth on a mixture of glucose and glucosamine could be due to catabolite repression (Magasanik, 1961) or to competition for an enzyme (permease or kinase); Loomis & Magasanik (1967) have suggested that decreased uptake and catabolite repression play a role in diauxie on glucose and lactose. Glucose has little effect on induction or repression by *N*-acetylglucosamine, and both carbon sources can be utilized simultaneously, and consequently there is no diauxie.

Significance of repression. The isolation of mutants with permanently repressed synthetase (Table 3, class 2 mutants) that can grow at normal rates on glucose, have a cell wall with the same amino sugar content as wild-type (Table 6) and accumulate an excess of intracellular *N*-acetylglucosamine 6-phosphate implies a considerable excess of synthetic capacity in the wild-type. *Aerobacter aerogenes* has been shown to have an excess of capacity for histidine synthesis, as mutants that wastefully accumulate urocanate (derived from histidine) grow at normal rates in histidine-free media (Schlessinger, Scotto & Magasanik, 1965).

Pathways of amino sugar catabolism. The growth characteristics of deaminaseless and deacetylaseless mutants confirm the catabolic routes for glucosamine and *N*-acetylglucosamine shown in Scheme 1. Since deacetylaseless and deaminaseless mutants are unable to grow on *N*-acetylglucosamine, whereas only deaminaseless mutants are unable to grow on glucosamine, clearly deacetylase is not involved in glucosamine degradation. Although Roseman (1957) has found an inducible *N*-acetylglucosamine deacetylase in *E. coli*, the present work shows that this enzyme does not play an important role in *N*-acetylglucosamine utilization.

Although *N*-acetylglucosamine 6-phosphate is a degradative intermediate, the observation that [1-^{14}C]glucosamine incorporation by a deacetylaseless mutant is normal (Table 4) suggests that it is not a synthetic intermediate (this mutant has a large *N*-acetylglucosamine 6-phosphate pool). This is consistent with the results obtained for acetylation of amino sugar phosphates by extracts of mutant 3-1; it is not known whether glucosamine 1-phosphate or UDP-glucosamine is the acetylase substrate *in vivo*. The precise route for glucosamine 6-phosphate into mucopeptide remains to be established (reactions 6, 7 and 8 in Scheme 1), but if glucosamine 6-phosphate is not the acetylase substrate a phosphoglucosamino mutase must be involved; little data are available on such an enzyme.

If *N*-acetylglucosamine 6-phosphate is not a

synthetic intermediate, its origin in deacetylaseless mutants is obscure. The accumulation of urocanate by mutants of *A. aerogenes* lacking urocanase poses a similar problem (Schlessinger *et al.* 1965). Chaloupka, Rihova & Kreckova (1964) have reported a widespread occurrence of mucopeptide turnover in growing bacteria. For *Bacillus megaterium* it was shown that turnover involved the release of diaminopimelic acid, this being reincorporated into mucopeptide or decarboxylated and incorporated into protein as lysine. If mucopeptide turnover involves the release of amino sugars as well as amino acids, the *N*-acetylglucosamine 6-phosphate in mutant 2-1 may be derived from *N*-acetylglucosamine. Normally any *N*-acetylglucosamine produced by mucopeptide turnover would be salvaged via reactions 1 and 2 in Scheme 1, but loss of deacetylase would prevent reutilization and cause *N*-acetylglucosamine 6-phosphate to accumulate.

Several workers have suggested that the reversible glucosamine 6-phosphate deaminase participates in amino sugar synthesis (Comb & Roseman, 1958; Pattabiraman & Bachhawat, 1961). However, since all deaminaseless mutants grow at normal rates on glucose, the irreversible synthetase must be capable of synthesizing all the amino sugar required.

Secondary effects in deacetylaseless mutants. All the deacetylaseless mutants isolated have abnormal specific activities for deaminase and synthetase, in mutant 2-1 the deaminase activity being about 20-fold that of wild-type grown on glucose, whereas the synthetase activity is fourfold lower. These altered enzyme activities are probably secondary effects resulting from the loss of deacetylase (which is presumably the genetic defect). This gives rise to elevated concentrations of *N*-acetylglucosamine 6-phosphate and amino sugar nucleotides, which may super-induce deaminase and repress synthetase. Similar secondary effects have been described by other workers; thus mutants lacking galactokinase have elevated activities of two other enzymes involved in galactose metabolism (Jordan, Yarmolinsky & Kalckar, 1962) and mutants lacking urocanase have an elevated histidine lyase (Schlessinger *et al.* 1965). Jordan *et al.* (1962) have suggested that the secondary effects in galactokinaseless mutants are a result of endogenous induction, and a similar explanation has been proposed for the urocanaseless mutants (Schlessinger *et al.* 1965).

Incorporation of labelled amino sugars. The poor incorporation from [1-^{14}C]glucosamine by mutant 1-1 (Table 4) could indicate that the labelling in wild-type is into protein rather than mucopeptide; alternatively the ^{14}C may be trapped in an abnormally large pool of some intermediate (the intracellular concentration of acetylamino sugar in mutant 1-1 is twice that in the wild-type). The poor incorporation of ^{14}C from *N*[1-^{14}C]-acetylglucosamine and its dilution by unlabelled acetate (Table 5) indicate that deacetylation occurs before incorporation, and the results obtained with the deacetylaseless mutant demonstrate that *N*-acetylglucosamine 6-phosphate deacetylase is responsible for this. Ingram & Salton (1957) have estimated free amino groups in cell walls and shown that most of the amino sugar present is *N*-substituted (probably *N*-acetylated). It is therefore rather surprising that exogenous acetylamino sugar is deacetylated before incorporation, but similar results have been obtained with streptococci (Dorfman, 1955), *Lactobacillus bifidus* (Lambert, Saito & Veerkamp, 1965) and *B. subtilis* (Bates & Pasternak, 1956*b*).

Lysis and inhibition caused by N*-acetylglucosamine.* Lysis of deacetylaseless mutants by *N*-acetylglucosamine resembles that of certain galactose-negative mutants by galactose (Fukasawa & Nikaido, 1961). A further similarity between the galactose-negative mutants and the present ones is that not all types are lysed, some being only inhibited by galactose (as deaminaseless mutants are by *N*-acetylglucosamine). It seems likely from the present work that lysis is the result of inhibited cell-wall synthesis, rather than synthesis of defective walls or degradation by enzymes. The nature of the mutations in classes 3 and 4 reveals little about the mechanism of lysis. Loss of deaminase (class 3 mutant) may prevent lysis by sparing the supply of essential precursors for mucopeptide synthesis.

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