The Incorporation of Labelled Amino Sugars by *Bacillus subtilis*

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1. *N*-Acetyl[1-¹⁴C]glucosamine (10 μ M–5 mM) is incorporated by cells of *Bacillus subtilis* at a constant rate (0.2–2 m μ moles/mg. dry wt./hr.). The rate of [1-¹⁴C]-glucosamine (2.5 μ M–5 mM) incorporation is proportional to the concentration; it approaches that of *N*-acetyl[1-¹⁴C]glucosamine at 5 mM. 2. Label from *N*-acetyl[1-¹⁴C]glucosamine and [1-¹⁴C]glucosamine is incorporated predominantly into the 'hot-trichloroacetic acid-soluble' and 'residue' fractions of cells. Acid hydrolysis of the hot-trichloroacetic acid-soluble fraction yields mainly [1-¹⁴C]glucosamine; hydrolysis of the residue fraction yields [1-¹⁴C]glucosamine and [1-¹⁴C]muramic acid. The label from *N*-[1-¹⁴C]acetylglucosamine and sodium [1-¹⁴C]acetate enters most cell fractions. Incorporation of *N*-[1-¹⁴C]acetylglucosamine is inhibited by the addition of unlabelled acetate. 3. Glucose competes with [1-¹⁴C]glucosamine for incorporation. *N*-Propionylglucosamine and *N*-formylglucosamine compete with *N*-acetyl[1-¹⁴C]glucosamine. 4. Cells pregrown on *N*-acetylglucosamine or glucosamine incorporate up to ten times as much *N*-acetyl[1-¹⁴C]glucosamine or [1-¹⁴C]-glucosamine in a given time as cells pregrown on glucose.

In the course of studies on the regulation of amino sugar metabolism in *Bacillus subtilis* it was noted that exogenous amino sugar was removed during growth (Bates & Pasternak, 1965). In the cell, amino sugars can either be degraded to hexose phosphates and ammonia or be incorporated into polymers. The repression of glucosamine 6-phosphate synthetase (L-glutamine-D-fructose 6-phosphate aminotransferase, EC 2.6.1.16) that occurs during growth in the presence of amino sugars (Clarke & Pasternak, 1962; Bates & Pasternak, 1965) suggested that incorporation might take place. We therefore investigated the incorporation of ¹⁴C-labelled amino sugars into various cell fractions. The effect of glucose on incorporation was studied because certain sugars had been found to alleviate the enzymic changes caused by growth in the presence of amino sugars (Clarke & Pasternak, 1962; Bates & Pasternak, 1965). Some preliminary results have been presented (Bates & Pasternak, 1962).

MATERIALS AND METHODS

Chemicals. [1-¹⁴C]Glucosamine hydrochloride was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. *N*-Acetyl[1-¹⁴C]glucosamine was prepared from it by the method of Kuhn & Bister (1958), by using a tenfold excess of acetic anhydride. Paper chromatography of the products in solvent A (see below) gave a spot, coincident

with unlabelled *N*-acetylglucosamine, that contained 90% of the radioactivity; the other 10% was present in spots moving near the solvent front. These did not appear if the products were treated for 12 hr. before chromatography with methanol saturated with ammonia, and may therefore be *O*-acetyl derivatives. *N*-[1-¹⁴C]Acetylglucosamine was prepared (Kuhn & Bister, 1958) from [1-¹⁴C]acetic anhydride (The Radiochemical Centre, Amersham, Bucks.) and a tenfold excess of glucosamine hydrochloride. The products were diluted tenfold with water, heated for 2 min. at 100° and passed through a column of Amberlite IR-120 (H⁺ form) and then through one of Dowex 1 (Cl⁻ form). All the radioactivity in the effluent (corresponding to a 91% yield in terms of [1-¹⁴C]acetic anhydride) was in the form of *N*-[1-¹⁴C]acetylglucosamine, as judged by chromatography in solvent A. Sodium [1-¹⁴C]acetate was obtained from The Radiochemical Centre. *N*-Formylglucosamine and *N*-propionylglucosamine were prepared as described by Bates & Pasternak (1965). Muramic acid was a gift from Dr R. E. Strange. Glucosamine and all labelled amino sugars were sterilized by filtration through Oxoid membrane filters.

Incorporation of labelled compounds. These were added to cultures of *B. subtilis* N.C.T.C. 1379 in 100 ml. of broth containing 0.5% of glutamate (Bates & Pasternak, 1965) at the beginning of, or during, the exponential phase of growth and the cultures were then shaken at 37° for various periods of time. The cells were harvested by centrifugation at 4°, washed twice with 100 ml. of 0.85% NaCl and suspended in 5 ml. of water. Samples were removed for measurement of total protein and radioactivity. The remainder was fractionated by the method of Roberts, Abelson, Cowie, Bolton & Britten (1955). The 'cold-trichloroacetic acid-soluble' fraction was prepared by adding 0.5 ml. of 50%

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(w/v) trichloroacetic acid to 5 ml. of the cell suspension and centrifuging after 30 min. below 4°. The pellet was extracted with 5 ml. of ethanol-water (3:1, v/v) for 15 min. at 40–50° to give the 'alcohol 1' fraction. The pellet was next extracted with ethanol-ether (1:1, v/v) for 15 min. at 40–50° to give the 'alcohol-ether' fraction. The pellet was extracted with 5 ml. of 5% (w/v) trichloroacetic acid for 15 min. at 100° to give the 'hot-trichloroacetic acid-soluble' fraction. The pellet was finally extracted with ethanol-water (3:1, v/v) for 5 min. at room temperature to give the 'alcohol 2' fraction. The pellet was suspended in 5 ml. of water ('residue' fraction) and a sample removed for the measurement of protein.

Hydrolysis and investigation of labelled cell fractions. The hot-trichloroacetic acid-soluble fraction was extracted with ether until neutral (no radioactivity was extracted), evaporated to dryness and hydrolysed at 100° with *N*-HCl for 6½ hr. The residue fraction was hydrolysed at 100° with *N*-HCl for 30 hr. Some solid material remaining in the latter case was discarded as it was not radioactive. Most of the HCl was removed by repeated evaporation to dryness and addition of water. Samples of the resulting solutions (total vol. 2 ml.) were removed for measurement of amino sugar content and radioactivity. Unlabelled glucosamine and galactosamine (5 μmoles of each) were added to the remainder, which was adsorbed on a column (59 cm. × 0.8 cm. diam.) of Dowex 50 (H⁺ form) and eluted (4 ml./hr.) with 240 ml. of 0.3 *N*-HCl (Gardell, 1953), followed by 200 ml. of 6 *N*-HCl. Amino sugar and radioactivity in the effluent fractions (2 ml.) were measured. In a separate experiment it was found that this method did not separate galactosamine and muramic acid satisfactorily. The effluent fractions corresponding to the galactosamine peak were therefore pooled, HCl was removed by repeated evaporation and the products were examined by paper chromatography in solvent B, which separates galactosamine (*R*_{glucosamine} 0.97) and muramic acid (*R*_{glucosamine} 1.78), and in solvent C (*R*_{glucosamine} of galactosamine 0.86 and of muramic acid 1.16). Acetylation (Kuhn & Bister, 1958) of the effluent fractions after the removal of HCl gave products moving with the *R*_{*N*-acetylglucosamine} of *N*-acetylgalactosamine (0.93) and of acetylated muramic acid (1.71) (but note Carroll, 1963).

Paper chromatography. Descending chromatography on Whatman no. 1 paper was carried out in the following solvents: solvent A, butan-1-ol-pyridine-water (6:4:3, by vol.); solvent B, butan-1-ol-acetic acid-water (4:1:5, by vol.); solvent C, pyridine-ethyl acetate-acetic acid-water (5:3:5:1, by vol.). The papers were sprayed with reagents to detect amino sugars (Partridge, 1948) and other reducing compounds (Trevelyan, Procter & Harrison, 1950; Partridge, 1949). Radioactivity on dried papers was measured in a Radioactive Chromatogram Counter (Baird and Tatlock Ltd.) and the coincidence of label and stain checked by radioautography on Kodirex X-ray film.

Other analytical methods. Amino sugar, protein and radioactivity were measured as described by Bates & Pasternak (1965).

RESULTS

Kinetics of uptake. *N*-Acetyl[¹⁴C]glucosamine was incorporated into whole cells much more rapidly than [¹⁴C]glucosamine. At 0.1 mM, for example, 17% of added *N*-acetyl[¹⁴C]glucosamine was taken up within 1 hr., compared with 7.2% of [¹⁴C]glucosamine in 10 hr. The rate of *N*-acetyl[¹⁴C]glucosamine incorporation (0.2–2.0 μmoles/mg. of protein/min. in various experiments) did not vary appreciably between 10 μM- and 5 mM-*N*-acetyl[¹⁴C]glucosamine, which indicates that the rate-limiting process, be it permease, kinase or other enzyme, was saturated at rather low concentrations. In contrast, the rate of [¹⁴C]glucosamine incorporation was proportional to the concentration, approx. 0.014% of the material added being incorporated/mg. of protein/hr. between 2.5 μM- and 5 mM-[¹⁴C]glucosamine. Hence the rate-limiting step has a relatively high *K_m*.

Distribution of label in the cell. When cells were grown for 10 hr. in the presence of 5–10 μM-*N*-acetyl[¹⁴C]glucosamine, -*N*-[¹⁴C]acetylglucosamine, -[¹⁴C]glucosamine or -[¹⁴C]acetate, 80% of the label from *N*-acetyl[¹⁴C]glucosamine was incorporated

Table 1. *Distribution of radioactivity in cells grown with labelled compounds*

B. subtilis cells were grown for 10 hr. in broth containing the labelled compound indicated (1.3–10 μCi/l.). The cells were harvested and the radioactivity in the various fractions was determined as described in the Materials and Methods section. The values quoted are percentages of the amount present in whole cells.

Cell fraction	Radioactivity after growth with:			
	<i>N</i> -Acetyl[¹⁻¹⁴ C]- glucosamine (5 μM)	[¹⁻¹⁴ C]Glucosamine (10 μM)	<i>N</i> -[¹⁻¹⁴ C]Acetyl- glucosamine (7 μM)	Sodium [¹⁻¹⁴ C]- acetate (5 μM)
Whole cells	100	100	100	100
Cold-trichloroacetic acid-soluble	10	12	3	5
Alcohol 1	5	3	38	35
Alcohol-ether	< 1	< 1	14	12
Hot-trichloroacetic acid-soluble	65	60	14	10
Alcohol 2	< 1	< 1	13	11
Residue	19	25	18	27

and 10% from the other substance present. The difference between *N*-[^{14}C]acetylglucosamine and *N*-acetyl[^{14}C]glucosamine is explicable by the fact that *N*-[^{14}C]acetylglucosamine loses most of its label as [^{14}C]acetate during incorporation. This was demonstrated by treating the medium after growth in the presence of *N*-[^{14}C]acetylglucosamine with Dowex 1: more than 90% of the radioactivity was removed. Further, the distribution of label in the cells resembled that of [^{14}C]acetate, rather than that of *N*-acetyl[^{14}C]glucosamine or [^{14}C]glucosamine (Table 1). The addition of unlabelled acetate (50mM) together with *N*-[^{14}C]acetylglucosamine decreased the incorporation by more than 70%, but did not decrease the incorporation of *N*-acetyl[^{14}C]glucosamine or [^{14}C]glucosamine. The pattern of incorporation of *N*-acetyl[^{14}C]glucosamine and [^{14}C]glucosamine is similar in that 60% of the label enters the hot-trichloroacetic acid-soluble fraction, with about 20% in the residue fraction (Table 1). The latter is presumably composed of residual protein and mucopeptide (Strange & Powell, 1954), whereas the hot-trichloroacetic acid-soluble fraction would contain compounds such as teichuronic acid (Janczura, Perkins & Rogers, 1961). To examine this further, cells that had been pregrown with 5mM-glucosamine (see below) were incubated for two further generations in the presence of 10 μM -[^{14}C]glucosamine, the hot-trichloroacetic acid-soluble and residue fractions were isolated and hydrolysed, and the constituent amino sugars were analysed by chromatography on Dowex 50 followed by paper chromatography. Although the total recovery was poor, perhaps

owing to incomplete hydrolysis (cf. Young, Spizizen & Crawford, 1963), the results presented in Table 2 show that most of the label in the hot-trichloroacetic acid-soluble fraction is in glucosamine, whereas that in the residue fraction is in glucosamine and muramic acid.

Competition with sugars. The uptake into whole cells of 5 μM -[^{14}C]glucosamine was inhibited more than 95% by 5mM-glucose and 80–90% by 5mM-fructose, but *N*-acetylglucosamine, *N*-propionylglucosamine and *N*-formylglucosamine were without effect. The uptake of *N*-acetyl[^{14}C]glucosamine, on the other hand, was most strongly inhibited by *N*-propionylglucosamine and *N*-formylglucosamine (Table 3). The uptake of 0.01mM-*N*-acetyl[^{14}C]glucosamine was inhibited 35% by 0.01mM- and 50% by 0.02mM-*N*-propionylglucosamine. Increasing the concentration of *N*-acetylglucosamine restored the incorporation. The inhibition by glucose (1mM) varied little (40–60%) between 10 μM - and 2.5mM-*N*-acetylglucosamine. It thus seems that glucose and glucosamine are taken up by one pathway and *N*-acetylglucosamine, *N*-propionylglucosamine and *N*-formylglucosamine by another.

Uptake by 'repressed' cells. Table 4 shows that cells pregrown on glucosamine incorporate more labelled amino sugar than do cells pregrown on glucose. Pregrowth on *N*-acetylglucosamine gave similar results. The distribution of label within the various cell fractions (Table 1) was the same with repressed as with non-repressed cells.

Table 2. *Composition of labelled cell fractions*

Hot-trichloroacetic acid-soluble and residue fractions of *B. subtilis* cells grown on 10 μM -[^{14}C]glucosamine were hydrolysed and adsorbed on Dowex 50 as described in the Materials and Methods section. Of the radioactivity in the hot-trichloroacetic acid-soluble fraction 19% was eluted by 0.3N-HCl; a further 17% was eluted by 6N-HCl; 64% remained on the column. Of the radioactivity in the residue fraction 26% was eluted by 0.3N-HCl, 17% by 6N-HCl and 57% remained on the column. The compounds eluted by 0.3N-HCl were identified as described in the Materials and Methods section. The values quoted are percentages of the total radioactivity eluted by 0.3N-HCl.

Fraction	Glucos- amine (%)	Galactos- amine (%)	Muramic acid (%)
Hot-trichloroacetic acid-soluble (0.3N-HCl eluate)	57	5	6
Residue (0.3N-HCl eluate)	54	1	35

Table 3. *Effect of sugars on the incorporation of N-acetyl[1- ^{14}C]glucosamine*

B. subtilis cells (0.25mg. dry wt./ml.) in broth were incubated with *N*-acetyl[1- ^{14}C]glucosamine (5 μM , 2.5 μC /l.) and the compounds indicated (each at 5mM) for 15–20 min. and harvested, and the radioactivity in whole cells was measured as described in the Materials and Methods section. The incorporation is expressed as a percentage of that by a control culture without added sugar (which incorporated 10–40% of *N*-acetyl[1- ^{14}C]glucosamine).

Addition to medium	Radioactivity incorporated (%)
—	100
<i>N</i> -Propionylglucosamine	1
<i>N</i> -Formylglucosamine	3
Glucose	40
Fructose	40
Sucrose	50
Glucosamine	60
Mannose	70
Galactose	70
Lactose, ribose, arabinose or sodium acetate	> 90

Table 4. *Effect of pregrowth conditions on the incorporation of labelled amino sugars*

B. subtilis cells (0.09 mg. dry wt./ml.) that had been grown for 10 hr. in broth containing D-glucose (5 mM) or D-glucosamine (5 mM) were incubated with [^{14}C]glucosamine (1 mM, 4 $\mu\text{C}/\text{l.}$) or *N*-acetyl[^{14}C]glucosamine (1 mM, 1.3 $\mu\text{C}/\text{l.}$) for 1 hr. and harvested, and the radioactivity in whole cells was measured as described in the Materials and Methods section.

Labelled amino sugar	Sugar in medium during pregrowth	Radioactivity incorporated (%)
[^{14}C]Glucosamine	Glucose	0.7
[^{14}C]Glucosamine	Glucosamine	4.8
<i>N</i> -Acetyl[^{14}C]glucosamine	Glucose	0.5
<i>N</i> -Acetyl[^{14}C]glucosamine	Glucosamine	5.0

DISCUSSION

The present results confirm and extend the observations on the regulation of amino sugar metabolism by exogenous amino sugars (Clarke & Pasternak, 1962; Bates & Pasternak, 1965). For example, glucosamine is indeed a 'poorer' source of intracellular amino sugars than is *N*-acetylglucosamine, since the uptake of [^{14}C]glucosamine is much slower below 5 mM than that of *N*-acetyl[^{14}C]glucosamine. Likewise, the inhibitory effect of glucose on induction and repression (Bates & Pasternak, 1965) is explicable in terms of the decreased uptake of *N*-acetyl[^{14}C]glucosamine in the presence of glucose (Table 3), though other effects of glucose (see Magasanik, 1961) are not ruled out. Again, the pathway of amino sugar metabolism suggested by Bates & Pasternak (1965) receives support from the present studies. Thus *N*-propionylglucosamine and *N*-formylglucosamine appear to enter by the same route as *N*-acetylglucosamine (Table 3), whereas glucosamine and glucose share a common route. This is compatible with the observed deacylation of *N*-propionylglucosamine 6-phosphate by *N*-acetylglucosamine 6-phosphate deacetylase and the conclusion that glucose and glucosamine are phosphorylated by one kinase, and *N*-acetylglucosamine by another (Bates & Pasternak, 1965). Pattabiraman & Bachhawat (1961) have shown that *N*-propionylglucosamine, but not glucosamine, is a substrate of an *N*-acetylglucosamine kinase from sheep brain. The removal of the [^{14}C]acetyl group of *N*-[^{14}C]acetylglucosamine during incorporation (Table 1) and the similar distribution of label from *N*-acetyl[^{14}C]glucosamine and [^{14}C]glucosamine (Table 1) support the view (Bates & Pasternak, 1965) that *N*-acetylglucosamine 6-phosphate is deacetylated before anabolism to polymers. A similar situation has been observed with streptococci (Dorfman, 1955).

The low [^{14}C]galactosamine content of the hot-trichloroacetic acid-soluble fraction (Table 2) is surprising in view of the reported presence of teichuronic acid (Janczura *et al.* 1961) and other galactosamine-containing polymers (Sharon, 1957) in *B. subtilis*, an organism that contains the enzyme UDP-*N*-acetylglucosamine epimerase, converting glucosamine into galactosamine (Glaser, 1959). Roberts & Johnson (1962) found no galactosamine at all in *B. subtilis* (Marburg), but their analytical technique might not have separated galactosamine satisfactorily in one operation (see the Materials and Methods section). It is unlikely that the label from [^{14}C]glucosamine was 'diluted out' by high concentrations of endogenous galactosamine compounds because the cell mass had increased more than fourfold during the incorporation period. The difference in strain used by Sharon (1957), Janczura *et al.* (1961) [the organism used by Janczura *et al.* (1961) has recently been identified as *B. licheniformis* (Dr H. J. Rogers, personal communication)] and us may be the reason for the discrepancy, since Young *et al.* (1963) have shown that sub-strains of *B. subtilis* 168 vary in content of galactosamine.

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