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Transport and Incorporation of *N*-Acetyl-D-Glucosamine in *Bacillus subtilis*

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Bacillus subtilis 168 has been found to possess a high-affinity transport system for *N*-acetyl-D-glucosamine (GlcNAc). The *Km* for uptake was approximately 3.7 μM GlcNAc, regardless of the nutritional background of the cells. Apparent increases in V_{max} were noted when the bacteria were grown in the presence of GlcNAc. The uptake of GlcNAc by *B. subtilis* was highly stereoselective; D-glucose, D-glucosamine, *N*-acetyl-D-galactosamine, D-galactose, D-mannose, and *N*-acetylmuramic acid did not inhibit GlcNAc uptake. In contrast, glycerol was an effective inhibitor of [^{3}H]GlcNAc transport and incorporation. Partial inhibition of GlcNAc uptake was observed with azide, fluoride, and cyanide anions, carbonyl cyanide-*m*-chlorophenyl hydrazone, methyltriphenylphosphonium bromide, *N*,*N*′-dicyclohexylcarbodiimide, gramicidin, valinomycin, monensin, and nigericin. Two anions, arsenite and iodoacetate, were potent inhibitors of the uptake of GlcNAc in *B. subtilis*. Results from paper chromatography showed that there was no intracellular pool of free GlcNAc and that the acetylamino sugar was probably phosphorylated during transport. A modification of the Park-Hancock cell fractionation scheme indicated that cells grown on glycerol or D-glucose incorporated [^{3}H]GlcNAc primarily into the cell wall fraction. When GlcNAc was used as the sole carbon source, label could be demonstrated in fractions susceptible to protease and nuclease, as well as lysozyme, showing that the *N*-acetylamino sugar was utilized in macromolecular synthesis and energy metabolism.

The availability of *N*-acetyl-D-glucosamine (GlcNAc) is a prerequisite for cell wall synthesis in bacteria. In cell wall synthesis, GlcNAc is incorporated, without modification, into glycan strands and is also converted to *N*-acetylmuramic acid (MurNAc). Both GlcNAc and MurNAc are essential for peptidoglycan biosynthesis.

In *Escherichia coli*, GlcNAc is transported by the phosphoenolpyruvate-dependent phosphotransferase systm (PTS) (26). Two independent PTSs that are coded for by two independent genes, *ptsM* and *ptsN*, have been characterized (14). Research involving the recognition and transport of amino sugars in gram-positive organisms has been somewhat limited. Simoni et al. (22) and Schäfer et al. (21) have described the transport of β-galactosides in *Staphylococcus aureus* and have found evidence for components of the classical PTS. Similarly, the PTS is involved in the transport of D-glucose (20) and lactose (4) in *Streptococcus mutans*. In *Bacillus subtilis*, Bates and Pasternak demonstrated the repression and derepression of biosynthetic and degradative enzymes related to the metabolism of GlcNAc (1, 2). They found that GlcNAc could be utilized for growth when the cells synthesized GlcNAc-6-phosphate deacetylase and D-glucosamine-6-phosphate deaminase. There are no reports describing the transport of GlcNAc in *B. subtilis*. Because we have begun to establish a relationship between the insertion of cell wall material and the segregation of DNA during growth (7, 8, 14a), it has become necessary to study the incorporation of GlcNAc in *B. subtilis*. The results show that there is a high-affinity transport system for GlcNAc in *B. subtilis*. The system is partially inducible and highly stereoselective, as no other sugars effectively competed for uptake. In addition, uptake of GlcNAc is surprisingly resistant to inhibition by metabolic poisons. A preliminary account of this work has appeared (H. L. T. Mobley, R. J. Doyle, and U. N. Streips, Abstr. Annu. Meet. Am. Soc. Microbiol. K60, p. 36).

MATERIALS AND METHODS

Organism and growth conditions. *B. subtilis* 168 (*trpC2*) was used throughout this study. The organism

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was originally obtained from D. C. Birdsell (University of Washington, Seattle) and was maintained on AK sporulation agar (Difco Laboratories, Detroit, Mich.). Cultures were grown in antibiotic medium 3 (Penassay broth, Difco Laboratories) or in the minimal salts of Spizizen (24) without sodium citrate, pH 7.2, supplemented with tryptophan (50 μg/ml) (hereafter referred to as modified minimal medium) and the carbon sources indicated. Cultures were shaken at 37°C in a gyratory shaker at 200 rpm. Growth was monitored with a Klett-Summerson colorimeter (no. 54 filter).

Uptake experiments. Cultures (100 ml) were grown in minimal salts medium, and samples of exponential culture (10 ml) were harvested by vacuum filtration through nitrocellulose filters (0.45 μm; Schleicher & Schuell Co., Keene, N.H.) and suspended in 4 ml of minimal salts at 23°C. The optical density at 450 nm was determined on a Coleman model 20 spectrophotometer and used to calculate the dry weight of the suspension. Samples of cells (0.8 or 0.9 ml) were rapidly mixed with small volumes (20 to 200 μl) of [1-^{3}H] GlcNAc. All stock solutions were prepared from a 91 μM concentration of the labeled GlcNAc (specific activity, 11 Ci/mmol; Amersham Corp. Arlington Heights, Ill). Samples (100 μl) were quickly filtered through nitrocellulose filters (0.45 μm; Millipore Corp., Bedford, Mass.) at timed intervals after mixing and washed immediately with ice-cold minimal salts. The filtration and wash procedures were completed within 2 to 3 s. Filters were added to scintillation vials, dissolved in 5 ml of Aqueous Counting Scintillant (Amersham Corp.), and counted in a liquid scintillation counter. Modifications of this general scheme are indicated in the figure legends and in the footnotes to the tables. The dry weights of cells added to the filters never exceeded 15 μg. Control samples (radioactive solution, but no cells) were run to establish background radioactivity levels. Usually, no more than 80 cpm per disk were found.

Group translocation assay. The method of Germaine and Tellefson, which uses filter paper disks to estimate the transport of carbohydrates by group translocation, was used to analyze the transport of GlcNAc by suspensions of whole cells (10). The method consists of applying a small volume (50 μl) of cell suspension to Whatman 3MM (2.3-cm) filters and immediately immersing the filters in absolute methanol. *N*-Acetylamino sugar, when phosphorylated, remains insoluble and bound to the filter.

Labeling cells for cell fractionation. Cultures (500 ml) were grown in minimal salts medium supplemented with one of four groups of carbon sources: (i) glucose (0.5%); (ii) glycerol (0.5%); (iii) glycerol (0.5%) plus GlcNAc (2 mg/ml); or (iv) GlcNAc (0.5%). When exponentially growing cultures reached 30 Klett units, the following procedures were used for labeling the cells. (i) Glucose-grown cells and (ii) glycerol-grown cells were pulsed with 200 μCi of [^{3}H]GlcNAc (Amersham Corp.; 2.94 Ci/mmol) for 30 min, harvested by centrifugation at 10,000 × *g* for 10 min, and washed twice with deionized distilled water. (iii) Glycerol plus GlcNAc-grown cells were harvested at 23°C, washed with warm glycerol-containing medium, suspended in 500 ml of glycerol-containing medium, shaken for 10 min, then pulsed with 200 μCi of [^{3}H]GlcNAc for 30 min, harvested, and washed twice with deionized distilled water. (iv) GlcNAc-grown cells were harvested at 23°C, washed with warm minimal salts, suspended in 500 ml of minimal salts, pulsed with 200 μCi of [^{3}H]GlcNAc for 10 min, chased with 2.5 g of unlabeled GlcNAc for 20 min, harvested, and washed twice with deionized distilled water. Cell pellets were subsequently frozen and lyophilized.

Fractionation of [^{3}H]GlcNAc-labeled cells. Freeze-dried [^{3}H]GlcNAc-labeled cells were suspended in 5 ml of deionized distilled water (final cell suspension, 1 mg/ml) in thick-walled glass centrifuge tubes (Ivan Sorvall, Inc., Norwalk, Conn.), and a sample (100 μl) was added to a scintillation vial with 5 ml of Aqueous Counting Scintillant. The cells were centrifuged at 12,000 × *g* for 10 min, and the pellet was suspended in 5 ml of 5% cold trichloroacetic acid (TCA) for 16 h at 4°C, centrifuged, and washed twice in 50 mM potassium phosphate buffer (pH 7.0). The cells were then suspended in 5 ml of the phosphate buffer containing RNase (Calbiochem, La Jolla, Calif.; 50 μg/ml) and DNase (Nutritional Biochemicals Corp., Cleveland, Ohio; 50 μg/ml) and incubated for 4 h at 37°C. The suspensions were then centrifuged, washed in phosphate buffer (pH 7.3), suspended in 5 ml of phosphate buffer (pH 7.3) containing pronase (50 μg/ml; Sigma Chemical Co., St. Louis, Mo.), and incubated for 4 h at 37°C. The mixtures were centrifuged and washed in phosphate buffer, pH 6.5. The remaining insoluble residue was suspended in 5 ml of the pH 6.5 phosphate buffer and digested with lysozyme (Calbiochem; final concentration, 50 μg/ml). The residual insoluble material was suspended in 5 ml of phosphate buffer (pH 6.5). At each step, duplicate samples (100 μl) were taken from the supernatants, added to scintillation vials with 5 ml of Aqueous Counting Scintillant, and counted in a scintillation counter. The supernatants were poured off and the pellets were carefully drained after each centrifugation.

Paper chromatography. Cell cultures (20 ml) of *B. subtilis* 168, growing exponentially in Penassay broth, were pulsed with 100 μCi of [^{3}H]GlcNAc (Amersham Corp.; 2.94 Ci/mmol) for 1 min, immediately poured into chilled tubes, and harvested by centrifugation. The supernatant was poured off and retained. The pellet was extracted with 10 ml of ice-cold 10% TCA, and insoluble material was removed by centrifugation. Samples (10 μl) of the TCA extract and the medium supernatant were spotted onto Whatman no. 1 filter paper (Fisher Scientific Co., Pittsburgh, Pa.) and subjected to ascending paper chromatography. Two solvent systems were employed: (A) butanol-pyridine-water (9:5:4) (3) and (B) butanol–propanol–acetone–80% formic acid–30% TCA (8:4:5:5:3) (5). Standards, D-glucose (2 mg/ml) and GlcNAc (2 mg/ml), were run in parallel. Chromatograms were cut into 0.5-cm strips, added to vials with 5 ml of scintillation fluid, and counted in a scintillation counter. Standards were developed with alkaline silver nitrate as described by Trevelyan et al. (25), and R_f values were calculated from points of peak radioactivity.

Reagents. All salts were of reagent grade. Gramicidine D, *N,N'*-dicyclohexylcarbodiimide (DCCD), sodium arsenite, sodium iodoacetate, glucose, and GlcNAc were purchased from Sigma Chemical Co. Sugars for inhibition studies were obtained from Sigma Chemical Co. or from Pfanstiehl Laboratories, Waukeegan, Ill. Carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) was obtained from Aldrich Chemical

Co., Milwaukee, Wis. Valinomycin (A grade) and monensin (A grade) were purchased from Calbiochem. The source of methyltriphenylphosphonium bromide (MTPB) was ICN Pharmaceuticals, Inc., Plainview, N.Y. Fisher Scientific Co. supplied sodium azide. Potassium cyanide and glycerol, both ACS grade, were obtained from Baker and Adamson Products, Morristown, N.J. Nigericin was provided by Eli Lilly & Co., Indianapolis, Ind.

RESULTS

Uptake of GlcNAc by intact cells. Washed cells of *B. subtilis* 168 were mixed with radioactive GlcNAc; samples were removed, filtered, and washed with cold modified minimal salts. The results showed that *B. subtilis* has a high-affinity transport system for the *N*-acetylamino sugar (Fig. 1 and Table 1). When the carbon source for growth was varied, considerable changes in the rates of uptake, but not in the affinity of the cells for the permeant, were observed. For example, when D-glucose was the carbon source, an apparent V_{max} of 25 pmol/min per mg of cells was found, whereas the K_m was 3.8 μM (Table 1). When glycerol was the carbon source, an apparent V_{max} of 10 pmol/min per mg of cells was determined, but the K_m remained at 3.8 μM. Similarly, the K_m was 3.7 μM for GlcNAc-adapted cells, but the V_{max} was 60 pmol/min per mg of cells.

Incorporation of GlcNAc after transport. To determine the fate of the transported GlcNAc, experiments involving longer times of uptake were performed. Filtered cells were washed with cold minimal salts, cold 5% TCA, or 3% sodium dodecyl sulfate (SDS). Uptake was followed for 1.5 min, and the results are shown in Fig. 2. When washed with salts, the cells demonstrated a linear uptake with time. However, when the cells were washed with TCA, there was a 30- to 45-s lag before uptake became linear. When washed with SDS, the labeled amino sugar remained SDS soluble for 60 s before it became resistant to removal by the detergent. The same kind of experiment carried out for 9 min is also shown in Fig. 2. After 1 min, the uptake was linear for both salts-washed and TCA-washed cells. After 2 min, the uptake was linear for SDS-washed cells. These results suggest that GlcNAc is taken into the cells, converted into TCA-insoluble but SDS-soluble precursors, and finally incorporated into components that are insoluble in all of the solvents.

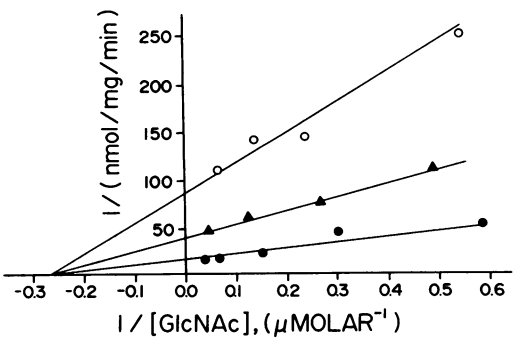

FIG. 1. Kinetics of GlcNAc uptake in *B. subtilis* 168. Cells were grown in modified minimal medium supplemented with glucose (0.5%; ▲▲), glycerol (0.5%; ●●), or glycerol (0.5%) plus GlcNAc (2 mg/ml; ○○). Final concentrations of labeled substrate ranged from 2 to 18 μM. Rates were determined from the linear portions of the uptake curves.

TABLE 1. Kinetics of [^{3}H]GlcNAc uptake in *B. subtilis* 168

Carbon source in medium[a]	K_m of uptake (μM)[b]	V_{max} (nmol/min per mg of dry wt)
Glucose (0.5%)	3.8	0.025
Glycerol (0.5%)	3.8	0.010
Glycerol (0.5%) plus GlcNAc (2 mg/ml)	3.7	0.060

[a] *B. subtilis* 168 was grown in modified minimal medium with the carbon source indicated.

[b] Exponentially growing cells were filtered, washed with minimal salts, and suspended in minimal salts. [^{3}H]GlcNAc was added to 0.9 ml of cell suspension, and uptake experiments were performed as described in the text. Final concentrations of labeled substrate ranged from 2 to 18 μM. Data were obtained from the linear regions of uptake-time curves.

It is common practice to use labeled GlcNAc as a marker for cell wall in *B. subtilis*. The results of Bates and Pasternak (2), however, suggest that the organism can grow in GlcNAc as the sole carbon source. We found that *B. subtilis* 168 could grow on the modified minimal salts medium containing 0.5% (wt/vol) GlcNAc as the carbon source with a generation time of 195 min at 37°C. *B. subtilis* was grown on several carbon sources and then pulsed-chased with [^{3}H]GlcNAc. The cells were then fractionated by a modification of the classical Park-Hancock (17) scheme to ascertain the fate of the label. The cells incorporated [^{3}H]GlcNAc in all media tested. However, the fate of the labeled molecules seemed to depend on the carbon sources on which the cells were grown (Table 2). Cells grown on glycerol as the sole carbon source channelled 89% of the label into a lysozyme-soluble fraction, but only small percentages of the label were solubilized by RNase-DNase or by protease. Similar results were obtained with cells grown on glycerol plus GlcNAc. Lysozyme solubilized 84% of the label, and less than 5% of the label was released

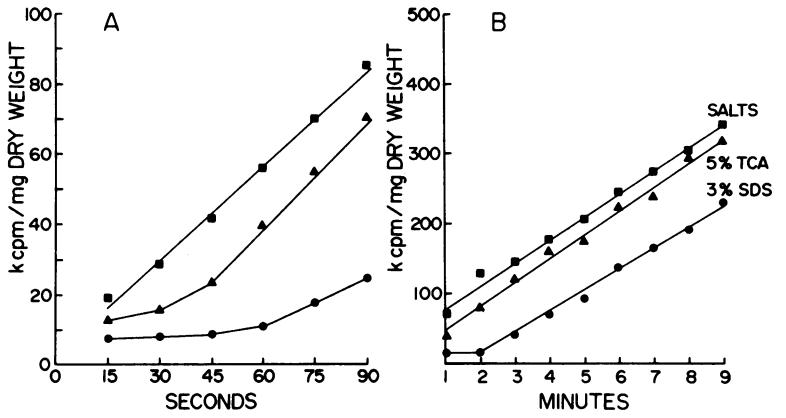

FIG. 2. Uptake and incorporation of GlcNAc by *B. subtilis*. Cells were grown in Penassay broth at 37°C. Exponentially growing cells (1 ml) were added to a tube containing 50 μl of [^{3}H]GlcNAc (0.5 μCi/ml, 4.5 μM final concentration), and samples (100 μl) were filtered and washed immediately with 2 ml of cold 5% TCA (▲▲), cold minimal salts (■), or 3% (wt/vol) SDS at room temperature (●●). Filters were added to scintillation vials with 5 ml of scintillation fluid and counted in a beta spectrometer. (A) 0 to 90 s; (B) 0 to 9 min.

upon nuclease and protease digestion. Glucose-grown cells showed 80.4% uptake in the lysozyme-soluble fraction, but considerably more label appeared in the nuclease (4.5%) and protease (4.4%) fractions. Interestingly, a high percentage (8.3%) of the label appeared in the TCA-soluble fraction, which represents the cell pool of soluble precursor molecules. However, when cells were grown on GlcNAc as the sole carbon source, the fractions that incorporated label shifted significantly. Lysozyme released 38% of the label, nucleases released approximately 15%, and protease released about 27%. In addition, 12.1% of the label remained insoluble after all enzyme treatments. Membrane lipid or protein refractory to protease may have accounted for this amount of label.

Specificity of the GlcNAc transport system in *B. subtilis*. To determine whether the transport system demonstrated specificity toward GlcNAc, inhibition studies with carbohydrates were performed. Carbohydrates were preincubated with cells before labeled GlcNAc was mixed with cell suspensions. Regular uptake experiments were then performed, with cold modified minimal salts to wash the filters. Inhibitions calculated for the various sugars are shown in Table 3. The transport system demonstrated high specificity with regard to GlcNAc. The structural analog of GlcNAc, MurNAc, did not reduce uptake at all. Similarly, D-glucose, D-glucosamine, and D-mannose were noninhibitors (less than 5% inhibition). The acetylamino sugar *N*-acetyl-D-mannosamine was a poor inhibitor.

TABLE 2. Fractionation of [^{3}H]GlcNAc-labeled *B. subtilis* 168

Treatment[a]	Cpm/mg of cell dry wt in following growth medium[b]:			
	Glucose (0.5%)	Glycerol (0.5%)	Glycerol (0.5%) plus GlcNAc (2 mg/ml)	GlcNAc (0.5%)
Whole washed cells	2,103,999	1,840,998	2,885,090	164,551
Cold 5% TCA	175,505 (8.3 ± 0.8)[c]	94,688 (5.1 ± 0.5)	162,852 (5.7 ± 0.9)	11,226 (6.8 ± 0.2)
RNase-DNase	95,334 (4.5 ± 0.6)	42,192 (2.3 ± 0.2)	91,232 (3.2 ± 0.3)	24,214 (14.7 ± 0.8)
Protease	91,942 (4.4 ± 1.9)	38,761 (2.1 ± 0.5)	51,761 (1.8 ± 0.5)	45,349 (27.6 ± 1.3)
Lysozyme	1,692,025 (80.4 ± 6.7)	1,637,261 (89.0 ± 2.3)	2,422,075 (84.0 ± 2.1)	63,014 (38.3 ± 2.3)
Insoluble residue	35,459 (1.7 ± 0.1)	30,925 (1.7 ± 0.1)	58,123 (2.0 ± 0.6)	20,847 (12.1 ± 1.6)

[a] Freeze-dried [^{3}H]GlcNAc-labeled cells were suspended in 5 ml of cold 5% TCA for 16 h, centrifuged at 12,000 × *g* for 10 min, and washed in 0.05 M potassium phosphate buffer, pH 7.0. The pellet was sequentially treated with enzymes (50 μg/ml) for 4 h each in phosphate buffers adjusted to the appropriate pH. Duplicate samples (100 μl) of the supernatants were added to scintillation vials with 5 ml of Aqueous Counting Scintillant and counted in a scintillation counter. All values are the averages of four separate runs (± standard deviations).

[b] Cells were grown in minimal salts medium (500 ml) supplemented with the carbon sources indicated. Cultures were labeled with 200 μCi of [^{3}H]GlcNAc as described in the text.

[c] Numbers within parentheses indicate percentages.

TABLE 3. Stereospecificity of GlcNAc uptake in *B. subtilis* 168[a]

Inhibitor	% Inhibition of uptake
N-Acetyl-D-galactosamine	0
N-Acetyl-D-mannosamine	20
MurNAc	5
2-Deoxy-D-glucose	0
D-Galactosamine	0
D-Galactose	0
D-Glucosamine	3
D-Glucose	0
Glycerol	65
D-Mannose	0
Ribose	0
Sucrose	0

[a] *B. subtilis* 168 was grown in modified minimal medium supplemented with glycerol (0.5%). Exponentially growing cells were harvested by rapid filtration through nitrocellulose filters (0.45-μm pore size; Scheicher & Schuell), washed quickly with cold minimal salts, and suspended in minimal medium containing the inhibitor. The tubes (0.9 ml) were shaken at 23°C for 30 s and then combined rapidly with 0.1 ml of [^{3}H]GlcNAc, and rates of uptake were determined. The final concentration of [^{3}H]GlcNAc was 8.0 μM, or approximately twice the K_m. The inhibitor concentrations were 80 μM. The results shown are the averages of two to five separate runs.

Other noninhibitors included D-ribose, sucrose, D-mannosamine, D-galactose, D-galactosamine, and *N*-acetylgalactosamine. An inhibitor of many PTSs in bacteria, 2-deoxy-D-glucose (15), was not an effective inhibitor of GlcNAc uptake in *B. subtilis*. It is interesting that glycerol, which typically enters bacteria by facilitated diffusion (16), was a more effective inhibitor than any of the sugars tested.

GlcNAc phosphorylation during transport. Germaine and Tellefson (10) noted that when the radiolabeled sugar was added to a suspension of whole cells and samples were spotted onto filter paper disks and then washed in methanol, results similar to those of the vacuum filtration method were obtained. They determined that D-glucose was washed from the disk, but that D-glucose-6-phosphate was methanol insoluble and therefore was retained. Figure 3 shows the results of similar experiments after the addition of [^{3}H]GlcNAc to a whole-cell suspension of *B. subtilis*. Radioactivity retained by the disks increased linearly with time, suggesting that GlcNAc may be phosphorylated in vivo as a result of the transport process. In addition, as expected, the deposition of cell-associated radioactivity could be inhibited by preincubation of cells with unlabeled GlcNAc (10 mM). Neither D-glucose nor D-glucosamine (each 10 mM) inhibited the incorporation of methanol-insoluble material on the filters.

To determine whether GlcNAc was altered during transport, paper chromatography was employed to compare the mobility of [^{3}H]GlcNAc with that of its transported derivative. Cultures were pulsed for 1 min with [^{3}H]GlcNAc, rapidly chilled to stop transport, harvested by centrifugation, and extracted with 10% TCA. Samples of the medium supernatant (untransported GlcNAc) and the TCA extract (intracellular derivatives) were spotted onto filter paper and subjected to ascending paper chromatography in two solvent systems. In solvent A, the intracellular derivative was immobile (R_f = 0); a peak of radioactivity from the medium supernatant corresponded with both a [^{3}H]GlcNAc standard and an unlabeled GlcNAc standard (R_f = 0.39) developed with alkaline $AgNO_3$ (25). Sugar phosphates have been found to remain at the origin in solvent A (3). Chromatography in solvent B resulted in the migration of both the intracellular radioactive product (R_f = 0.27) and the untransported GlcNAc (R_f = 0.37). Assays for glucose, by a glucose oxidase

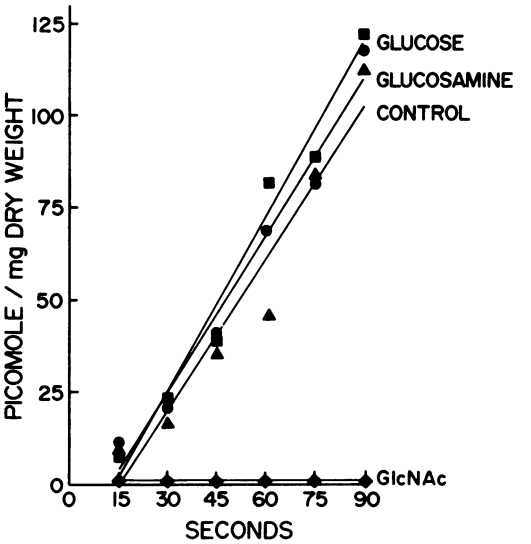

FIG. 3. GlcNAc uptake by *B. subtilis* 168 as estimated by the filter paper disk method. Cell culture (5 ml), growing exponentially in modified minimal medium supplemented with glycerol (0.5%), was filtered, washed, and suspended in minimal salts containing glucose, GlcNAc, glucosamine (all 10 mM), or no carbohydrate. Cell suspensions (0.9 ml) were added to test tubes with 0.1 ml of [^{3}H]GlcNAc (10 μCi). Samples (50 μl) were taken every 15 s, applied to filter paper disks (Whatman 3MM), and washed in three changes of methanol. Dried disks were placed in vials with 5 ml of a toluene-based scintillant and counted by liquid scintillation.

method, revealed the absence of the hexose in the cell extracts. The results support the premise that GlcNAc is converted into GlcNAc-6-phosphate during transport. In addition, a small, slowly migrating peak (R_f = 0.05) which may correspond to the higher-molecular-weight cell wall precursors formed during early peptidoglycan synthesis was detected.

Efforts to monitor PTS activity by standard spectrophotometric assays (23) were unsuccessful when suspensions of *B. subtilis* were decryptified with various concentrations of toluene (9) or toluene-acetone mixtures (23).

Effects of metabolic inhibitors on GlcNAc transport in *B. subtilis*. It is frequently possible to characterize the energy-coupling mechanism of substrate transport by the use of specific inhibitors (11, 12). We have studied the uptake of GlcNAc in *B. subtilis* after the addition of several classes of metabolic inhibitors. The results (Table 4) reveal that most of the inhibitors were not totally effective in preventing GlcNAc uptake. Classical uncoupling agents, such as the azide anion and CCCP, did not completely abolish transport. Monensin and nigericin were also poor inhibitors of GlcNAc transport. Valinomycin, an ionophore which conducts potassium across membranes without significant modification of ΔpH (13), was a poor inhibitor of GlcNAc uptake. Other nonspecific metabolic poisons, such as arsenate, fluoride, and gramicidin, were also not highly effective inhibitors of transport.

TABLE 4. Inhibition of [^{3}H]GlcNAc uptake in *B. subtilis* by metabolic inhibitors[a]

Metabolic inhibitor	Concn	% Inhibition of [^{3}H]GlcNAc uptake
Sodium azide	10 mM	10
Sodium cyanide	10 mM	15
Sodium arsenate	10 mM	37
Sodium fluoride	10 mM	24
Sodium arsenite	10 mM	67
Iodoacetate	10 mM	98
MTPB	100 μM	9
CCCP	40 μM	30
DCCD	100 μg/ml	63
Gramicidin D	1 μg/ml	34
Monensin	10 μg/ml	48
Valinomycin	10 μg/ml	25
Nigericin	1 μM	16

[a] *B. subtilis* 168 was grown in modified minimal medium with glycerol (0.5%). Exponentially growing cells were harvested by vacuum filtration through nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell), washed with minimal salts, and suspended in minimal salts containing the metabolic inhibitor. The tubes were shaken at 23°C for 60 s, 0.9 ml of cells was combined with 0.1 ml of [^{3}H]GlcNAc, and rates of uptake were determined. The [^{3}H]GlcNAc final concentration was 7.4 μM.

One inhibitor, MTPB, which causes a transient elevation in ATP in *B. subtilis* (13) but destroys both ΔpH and Δψ, was not an efficient inhibitor of transport of GlcNAc. Only iodoacetate, DCCD, and arsenite were potent inhibitors of uptake of the *N*-acetylamino sugar.

DISCUSSION

The uptake and incorporation of GlcNAc in *B. subtilis* appears to involve a complex and presently incompletely characterized series of reactions. Some of the observations and conclusions that we have made in this research are summarized below. (i) The uptake of GlcNAc is highly specific. Other carbohydrates, such as D-glucose, D-glucosamine, and MurNAc, do not compete with GlcNAc for transport. (ii) The uptake of GlcNAc is not readily inhibited by membrane-soluble proton-conducting agents, by ionophores, or by an ATPase inhibitor. Furthermore, the uptake is not directly related to the ATP content of the cells, as we have previously shown that many of the inhibitors completely dissipate the intracellular pool of the nucleotide (13). In contrast, sulfhydryl group-modifying agents markedly inhibit uptake. (iii) The uptake of GlcNAc is probably accompanied by phosphorylation of the sugar. It does not appear that the cell contains an intracellular pool of unmodified GlcNAc. (iv) The uptake system for GlcNAc is at least partially inducible. Adaptation of the cells to GlcNAc results in a several-fold increase in the apparent V_{max} for uptake of the sugar, compared with the rates of uptake for cells grown in the absence of GlcNAc. (v) When cells are fully adapted to GlcNAc, the sugar can be used as a sole carbon source for growth. When cells are grown in media containing other carbon sources, such as glycerol or D-glucose, GlcNAc is used almost exclusively for cell wall synthesis.

The stereospecificity of the transport of GlcNAc in *B. subtilis* was demonstrated by the observation that none of the sugars tested was an effective inhibitor of uptake. Only *N*-acetyl-D-mannosamine, which is a close structural analog of GlcNAc, afforded any readily measurable inhibition of uptake. Saier and Feucht (19) found that any natural sugar substrate of the PTS could inhibit the uptake of any other PTS substrate in *E. coli ptsI17*, provided that the enzyme II specific for the inhibitory sugar was present in sufficient amounts. In our studies, glucose and 2-deoxy-D-glucose did not significantly inhibit the uptake of GlcNAc. If it is assumed that the transport of GlcNAc and D-glucose (as well as other sugars, such as 2-deoxy-D-glucose) enter the cell via a PTS-mediated uptake, it would be expected that competition for the common com-

ponents of the PTS would occur. The lack of competition may reveal the high selectivity of PTS components for GlcNAc. To our knowledge, the K_m for D-glucose uptake in *B. subtilis* has not been reported. The lack of competition by D-glucosamine is probably due to the basic character of the sugar.

This work does not provide direct proof for a PTS involvement in the uptake of GlcNAc by *B. subtilis*. The results, however, provide evidence that GlcNAc is modified during transport. When a cell extract was subjected to paper chromatography, a spot was located which had an R_f value identical to a value reported in the literature for GlcNAc-6-phosphate (5). This same R_f has also been reported for D-glucose (5). Because we could not find evidence for D-glucose in the extract, it is suggested that GlcNAc is in fact phosphorylated. The inability to monitor GlcNAc conversion spectrophotometrically is perplexing. Several reports (15, 18) describe the difficulty in the coupling of spectral methods to PTS assays in gram-positive bacteria. A selective permeability problem in the decryptified cells may result in a lack of substrate or cofactor to the membrane proteins. Further work is in progress in an effort to more clearly define the components of the PTS in *B. subtilis*.

Previous work in our laboratory (13) has established that reagents such as azide, cyanide, CCCP, gramicidin, and MTPB rapidly abolish both $\Delta\Psi$ and ΔpH in *B. subtilis*. Furthermore, we observed that although the proton motive force was dissipated by MTPB, there was an increase in the cellular ATP content (13). In addition, it was shown that monensin completely abolished ΔpH but had little effect on $\Delta\Psi$ (13). Another agent, DCCD, abolished the cellular ATP content and the ΔpH, but reduced the $\Delta\Psi$ component by one-half. On the basis of these results and the transport rates of GlcNAc in *B. subtilis* (Table 2), we conclude that there is no direct coupling of GlcNAc uptake to cellular ATP levels, $\Delta\Psi$, or ΔpH. None of the inhibitors completely abolished the uptake of GlcNAc. The inhibition of the uptake of GlcNAc in *B. subtilis* by arsenite and by iodoacetate (Table 4) probably reflects sulfhydryl group modification of transport proteins.

The use of GlcNAc as a marker for cell wall synthesis is valid even though the cells have been grown in the presence of GlcNAc (Table 2). The GlcNAc is incorporated primarily into the wall when cells have been grown in glycerol, glucose, or glucose-GlcNAc. In contrast, cells adapted only to GlcNAc can use the sugar as a carbon source for all growth processes. It is interesting that cells grown on glucose plus GlcNAc incorporate the *N*-acetylamino sugar primarily in the wall fraction. D-Glucose may repress the synthesis of GlcNAc-6-phosphate deacetylase and D-glucosamine-6-phosphate deaminase (1, 2).

The utilization of GlcNAc by *B. subtilis* follows reasonably predictable paths. The GlcNAc is modified as it is transported across the plasma membrane. The putative GlcNAc-6-phosphate is then converted into cell wall precursors. The precursors become insoluble and incorporated into the wall within approximately 2 min. The newly incorporated wall polymers are then cross-linked into the wall matrix. Wall growth then occurs in an inside-to-outside direction (8) for 1 to 1.5 generations, when the wall polymers become susceptible to autolysins (13) and are lost into the medium as growth continues.

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