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The Role of the Phosphoenolpyruvate Phosphotransferase System in the Transport of *N*-Acetyl-D-glucosamine by *Escherichia coli*

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The properties of an *N*-acetyl-D-glucosamine-transport system have been studied by following the intracellular accumulation of methyl 2-acetamido-2-deoxy-α-D-[1-^{14}C]glucoside by *Escherichia coli*. The same analogue was used to assay phosphoenolpyruvate phosphotransferase activity of toluene-treated cells. Transport and phosphorylation are induced by growth on D-glucosamine or *N*-acetyl-D-glucosamine. Mutants resistant to *N*-iodoacetyl-D-glucosamine are defective in the uptake and phosphorylation of the labelled glycoside.

In the preceding paper (White & Kent, 1970) it was shown that mutations to INAG†-resistance affected the ability of *Escherichia coli* to utilize *N*-acetylglucosamine as carbon source. However, this defect could not be correlated with the loss of any enzyme known to be involved in the catabolism of *N*-acetylglucosamine. Previously no satisfactory method has been available for assaying uptake of *N*-acetylglucosamine by intact cells. This has now been investigated by studies of the uptake of αMNAG as a non-metabolizable substrate. The resistant mutants are shown to be defective in the uptake and phosphorylation of this glycoside.

MATERIALS AND METHODS

N-Acetyl-D-[1-^{14}C]glucosamine and *N*[U-^{3}H]-acetyl-D-glucosamine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of substrate. [^{14}C]αMNAG was prepared by the method of Zilliken, Rose, Braun & György (1955). *N*-Acetyl[1-^{14}C]glucosamine (20μCi/mg) (2.5mg) and 3mg of dry Amberlite IR-120 (H^+ form) were suspended in dry methanol (0.5ml) and incubated in a sealed tube, under nitrogen, at 80°C for 16h. Ion-exchange resin was removed by filtration and the reaction products were purified by descending paper chromatography with butan-1-ol–ethanol–water (4:1:5, by vol.) as solvent, or by fractionation on a column of Dowex 1 (X8; OH^- form) as described by Matsushima, Miyazaki & Park (1963). Two major radioactive peaks were obtained by both methods, corresponding to the α- (25%) and β- (75%) anomers of αMNAG. [^{3}H]αMNAG was made by the same method by using *N*[U-^{3}H]-acetyl-D-glucosamine as the starting material.

Assay of phosphotransferase system. The phosphoenolpyruvate phosphotransferase system was assayed by the method of Gachelin (1969) with [^{3}H]αMNAG as substrate. Before assay, cells (250μg cell dry wt./ml) were treated with toluene (1μl/ml) by gentle agitation at 37°C for 30min in minimal medium.

Chromatography, electrophoresis and other techniques were as described previously (White & Kent, 1970).

RESULTS

The success of methyl α-D-glucoside as a transport analogue for glucose (Cohen & Monod, 1957) prompted the use of αMNAG as a non-metabolizable transport analogue for *N*-acetylglucosamine. Incubation of *Escherichia coli* cells with labelled glycoside ([^{14}C]αMNAG) resulted in an initial rapid uptake that levelled off after about 10min (Fig. 1). Conditions of pre-growth had marked effects on the initial rate of uptake (Table 1). Fractionation of *N*-acetylglucosamine-grown cells after incubation with its [^{14}C]glycoside ([^{14}C]αMNAG) showed that 99.1% of the ^{14}C was soluble in cold 5% (w/v) trichloroacetic acid. Electrophoresis separated the acid-soluble radioactive material into two peaks, a major anionic peak and a minor neutral one. After treatment of the anionic peak with alkaline phosphatase for 2h at 37°C (2.5 units of enzyme in 0.1M-tris–HCl buffer, pH 8.0) the substance no longer migrated under electrophoresis. Both the minor neutral and major anionic peak after phosphatase treatment had the same chromatographic properties as the glycoside (αMNAG) on paper chromatography with butan-1-ol–ethanol–water (4:1:5, by vol.) as solvent. The amount of radioactivity present in the

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† Abbreviations: INAG, *N*-iodoacetyl-D-glucosamine; αMNAG, methyl 2-acetamido-2-deoxy-α-D-glucoside; [^{14}C]αMNAG, methyl 2-acetamido-2-deoxy-α-D-[1-^{14}C]-glucoside; [^{3}H]αMNAG, methyl 2[U-^{3}H]acetamido-2-deoxy-α-D-glucoside.

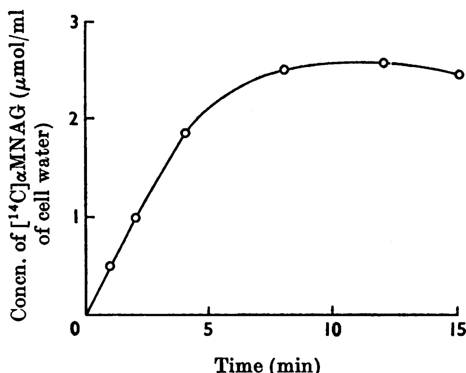


Fig. 1. Uptake of [^{14}C]αMNAG by *E. coli*. *N*-Acetylglucosamine-grown cells (500μg dry wt./ml) were incubated at 25°C in minimal medium containing 50μg of chloramphenicol/ml and 0.01mM-[^{14}C]αMNAG (14μCi/mg). Samples (0.2ml) were collected on membrane filters and washed with 5ml of cold minimal medium. The filters were dried and radioactivity was determined by scintillation counting.

Table 1. *Induction of* [^{14}C]*αMNAG uptake by growth of* E. coli *on amino sugars*

After growth of *E. coli* on the appropriate carbon source (10mM in minimal medium), the initial rate of uptake of [^{14}C]αMNAG was assayed as described for Fig. 1. Results are expressed in arbitrary units, taking the value for glucose-grown cells as 1.0 (actual value for glucose-grown cells was 0.067μmol of substrate accumulated/min per ml of cell water).

Carbon source for growth	Initial rate of uptake
Glucose	1.0
Glucosamine	4.0
N-Acetylglucosamine + glucose	4.5
N-Acetylglucosamine	7.3

Table 2. *Inhibition of uptake of* [^{14}C]*αMNAG by various sugars in* E. coli

Initial rate of uptake (1min sample) of [^{14}C]αMNAG (0.01mM) was assayed as described for Fig. 1. Sugars were added to give 10mM solutions as indicated. The results are expressed as percentages of control value (no additions) (100% = 0.3μmol of substrate accumulated/min per ml of cell water).

Addition	Uptake (%)
αMNAG	0.9
N-Acetylglucosamine	0.5
Glucosamine	80.0
Methyl α-glucoside	23.1
Glucose	32.2

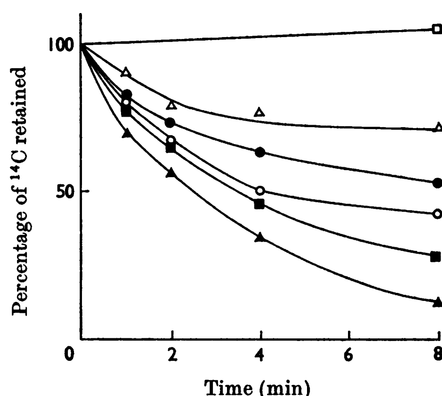


Fig. 2. Stimulation of efflux from cells pre-loaded with [^{14}C]αMNAG. *E. coli* was pre-loaded by incubation with [^{14}C]αMNAG for 12min at 37°C (conditions as in Fig. 1). The appropriate sugars were added to give a final concentration of 10mM, and the radioactivity retained by the cells was assayed by collecting 0.2ml samples on membrane filters and washing with 5ml of cold medium. Filters were dried and radioactivity was determined by scintillation counting. Results are expressed as the percentage radioactivity retained by whole cells after addition of unlabelled sugar, taking the 12min pre-loading sample as 100. □, Control cells, no additions; ■, αMNAG; ○, glucose; ●, methyl α-glucoside; △, glucosamine; ▲, *N*-acetylglucosamine.

major peak varied in the range 71–92% of the total in different experiments.

A number of sugars were tested as possible inhibitors of [^{14}C]αMNAG uptake (Table 2); of the compounds tested *N*-acetylglucosamine and the unlabelled related glycoside (αMNAG) had the most marked effect. If the cells were pre-loaded with the radioactive substrate ([^{14}C]MNAG) and the same sugars were tested for their ability to accelerate loss of label, rather different results were obtained (Fig. 2). Although *N*-acetylglucosamine and its methyl glycoside (αMNAG) caused the largest stimulation of efflux, the differences were not as marked as those obtained for inhibition of uptake (Table 2). Analysis of the medium after efflux of label from pre-loaded cells failed to detect any phosphorylated substrate; at least 92% of the ^{14}C was present as αMNAG. The addition of 2,4-dinitrophenol to incubations caused a slight stimulation of uptake, but the addition of a mixture of fluoride and azide was inhibitory (Table 3). Lower concentrations of fluoride and azide gave variable results, occasionally causing a stimulation. Preincubation of cells with iodoacetate and INAG

Table 3. *Effect of energy poisons and alkylating agents on uptake of* [^{14}C]α*MNAG by* E. coli

Cells (500 μg dry wt./ml) were preincubated with the compounds indicated for 30 min at 25°C and the initial rate of [^{14}C]αMNAG uptake was assayed at 25°C as described for Fig. 1. With INAG, the inhibitor was removed by centrifuging bacteria and resuspending them in fresh medium before measurement of uptake; other additions as shown were present during the assay. Results are expressed in arbitrary units, taking the control value as 100 (100 is equivalent to 0.3 μmol of substrate accumulated/min per ml of cell water).

Additions	Uptake
None	100
2,4-Dinitrophenol (1 mM)	140
Sodium azide (0.3 M) + sodium fluoride (0.3 M)	22
Iodoacetate (1 mM)	5
INAG (1 mM)	6

Table 4. *Uptake of* [^{14}C]α*MNAG by* E. coli *mutants*

Initial rate of [^{14}C]αMNAG (0.01 mM) uptake was determined as described for Fig. 1. Cells were grown on glucose + *N*-acetylglucosamine.

	Initial rate of uptake (μmol/min per ml of cell water)
Wild-type	0.823
Mutant 26	0.037
Mutant 34	0.030
Mutant 41	0.036
Double mutant 26L	0.015
Double mutant 34M	0.005

under the same conditions showed both compounds to be inhibitory (Table 3).

Uptake and phosphorylation of αMNAG by mutants. All INAG-resistant mutants had an impaired ability to take up (Table 4) and phosphorylate (Table 5) αMNAG; in every case defective uptake was accompanied by defective phosphorylation. In this instance phosphorylation was by means of a phosphoenolpyruvate-dependent phosphotransferase system, which involved several proteins (Kundig, Ghosh & Roseman, 1964) and was assayed in toluene-treated cell suspensions; this system is different from the ATP-dependent kinase that is assayed in cell-free extracts and has the same activity in resistant mutants as in the wild-type organism (see White & Kent, 1970). Residual uptake by resistant mutants was increased, relative to the value for the wild-type by raising the substrate concentration to 0.1 mM. It is possible that another permease, which transports glucosamine and *N*-acetylglucosamine (White & Kent, 1970), will also take up αMNAG at high concentrations.

Table 5. *Phosphoenolpyruvate-dependent phosphorylation of* [^{3}H]α*MNAG by* E. coli *mutants*

Before assay of phosphotransferase activity, cell suspensions (310 μg dry wt./ml in minimal medium containing 50 μg of chloramphenicol/ml) were shaken with 1 μl of toluene/ml at 37°C for 30 min. Phosphotransferase incubations contained 0.8 ml of toluene-treated cell suspension, 0.05 ml of 50 mM-phosphoenolpyruvate (sodium salt) and 0.25 mM-[^{3}H]αMNAG (267 μCi/mg) in a total volume of 1 ml. After 20 min at 37°C 0.2 ml samples from incubations were ejected into 1.8 ml of ice-cold 90% ethanol containing 11 mg of $BaBr_2$/ml. Precipitated sugar phosphate was collected on membrane filters and washed with 80% ethanol (20 ml), and radioactivity was determined by scintillation counting.

Bacteria	Carbon source for growth	Phosphotransferase activity (nmol formed/min per g dry wt.)
Wild-type	Glucose	26.2
Wild-type	Glucosamine	145.0
Wild-type	*N*-Acetylglucosamine	106.2
Wild-type	*N*-Acetylglucosamine + glucose	69.4
Mutant 26	*N*-Acetylglucosamine + glucose	1.8
Double mutant 26L	*N*-Acetylglucosamine + glucose	1.1
Mutant 34	*N*-Acetylglucosamine + glucose	0.5
Double mutant 34M	*N*-Acetylglucosamine + glucose	0.5
Mutant 41	*N*-Acetylglucosamine + glucose	0.5

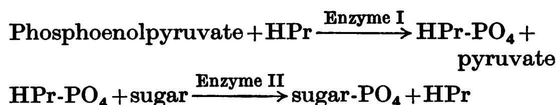
Conditions of growth affected phosphorylation and uptake in a similar manner (Tables 1 and 5).

DISCUSSION

In the preceding papers (Kent, Ackers & White, 1970; White & Kent, 1970) it was shown that there are at least two permeases capable of transporting *N*-acetylglucosamine and INAG. The present results demonstrate that only one of these transports αMNAG at low concentrations. A severe inhibition of [^{14}C]αMNAG uptake by *N*-acetylglucosamine and unlabelled αMNAG, coupled with the poor inhibition by glucosamine, suggested that this glycoside is transported by the specific *N*-acetylglucosamine permease (White & Kent, 1970). Although the best stimulation of efflux from preloaded cells was given by the most potent inhibitors of uptake, glucose and methyl α-glucoside had a more marked effect on efflux than uptake. As glucose and methyl α-glucoside are transported via a different permease their effect on efflux is paradoxical, and implies that different specificities are

involved in entrance and exit. Previously it has been shown that the stimulation of methyl α-glucoside efflux by unrelated sugars depends on ability to phosphorylate the sugar in question. Thus glycerol has no effect in a mutant lacking glycerol kinase (Hagihara, Wilson & Lin, 1963). This may be true in the present case, for although methyl α-glucoside cannot be completely degraded it can be phosphorylated (Kaback, 1968).

All the INAG-resistant mutants were defective in their ability to take up and phosphorylate αMNAG and had presumably lost enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system described by Kundig, Ghosh & Roseman (1964). This system consists of two enzymes (I and II) and a small heat-stable protein (HPr). Enzyme I is responsible for catalysing the transfer of phosphate from phosphoenolpyruvate to HPr, and enzyme II (which is membrane-bound) effects the phosphorylation of a specific sugar:



$$\text{Phosphoenolpyruvate} + \text{HPr} \xrightarrow{\text{Enzyme I}} \text{HPr-PO}_4 + \text{pyruvate}$$

$$\text{HPr-PO}_4 + \text{sugar} \xrightarrow{\text{Enzyme II}} \text{sugar-PO}_4 + \text{HPr}$$

The results quoted in Table 2 refer to the overall activity of the phosphotransferase system, and cannot be taken as giving an accurately quantitative indication of the amount of enzyme II present. Nevertheless it is safe to conclude that enzyme II is induced by growth on glucosamine and *N*-acetylglucosamine but that it phosphorylates only the latter. Other bacterial mutants lacking enzyme II are similarly defective in their ability to grow on a single sugar (Tanaka & Lin, 1967), in contrast with the pleiotropic mutants lacking enzyme I, which are unable to grow on as many as nine different carbohydrates (Egan & Morse, 1965; Tanaka, Fraenkel & Lin, 1967; Simoni *et al.* 1967). A pleiotropic mutant of *Escherichia coli* (MM6) has a severely diminished growth rate on glucosamine and *N*-acetylglucosamine (R. J. White, unpublished work), which suggests that both transport systems for *N*-acetylglucosamine are phosphoenolpyruvate-dependent; the pleiotropic mutant of *Salmonella typhimurium* isolated by Simoni *et al.* (1967) is also unable to grow on *N*-acetylglucosamine. All mutants so far described that lack enzymes I or II are defective in uptake and phosphorylation of the sugars concerned.

The effects of energy poisons on αMNAG transport parallel those on methyl α-glucoside (Hoffee & Englesberg, 1962; Hoffee, Englesberg & Lamy, 1964; Kennedy & Scarborough, 1967), where it has been established that the phosphotransferase system is operating. In other systems where compounds have been shown to accumulate in an unaltered form against a concentration gradient energy poisons have a different effect: 2,4-dinitrophenol can abolish uptake and cause a rapid loss of label from pre-loaded cells (Osborn, McLellan & Horecker, 1961; Winkler & Wilson, 1966). There is now some doubt that the effect of 2,4-dinitrophenol is simply due to uncoupling of phosphorylation, as it gives the same results anaerobically, when oxidative phosphorylation would not be expected to occur (Pavlasova & Harold, 1969). Mutant 41 has a defective glucosamine 6-phosphate deaminase as well as lacking the phosphotransferase system (White & Kent, 1970); it is possible that the structural genes for these proteins are genetically linked, since both are induced under the same conditions. Earlier work with other mutants of *E. coli* also indicated a linkage between deaminase and an uptake system for *N*-acetylglucosamine (White & Pasternak, 1967; White, 1968).

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