The Uptake of 2-Deoxy-D-glucose by *Pseudomonas aeruginosa* and its Regulation

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(Received 27 June 1972)

The non-metabolizable glucose analogue 2-deoxy-D-glucose is taken up by *Pseudomonas aeruginosa* against a concentration gradient, in a predominantly unchanged form. D-Glucose competitively inhibits 2-deoxy-D-glucose uptake and also causes a rapid exit of intracellular 2-deoxy-D-glucose. Thus these two sugars share the same stereospecific carrier system, and glucose transport can be studied reliably with 2-deoxy-D-glucose. The transport system is inducible, and is strongly repressed by a number of organic acids such as acetate, citrate, succinate, fumarate and malate, even in the presence of adequate excess of the inducer (D-glucose). Repression by organic acids can be relieved by transferring cells to a glucose medium, but in the presence of chloramphenicol the cells fail to recover from repression, indicating that the formation of the transport system involves the synthesis of protein. The results demonstrate that the regulation of glucose metabolism effected by citric acid-cycle intermediates in *P. aeruginosa* is manifest at the level of the glucose-transport system.

The pattern of control of enzymes of glucose metabolism in *Pseudomonas aeruginosa* is in sharp contrast with that found in the coliform bacteria. Enzymes of the tricarboxylic acid cycle are constitutive in *P. aeruginosa* (Tigerstrom & Campbell, 1966) and are not subject to catabolite repression as they are in *Escherichia coli* (Tiwari & Campbell, 1969). Rather, the reverse appears to be true in that Hamilton & Dawes (1959, 1960, 1961) demonstrated that organic acids are utilized by *P. aeruginosa* in preference to glucose, and Tiwari & Campbell (1969) have shown that cells grown on succinate possess extremely low activities of glucose-oxidizing enzymes. Similarly, citrate acts as a repressor of enzymes of glucose metabolism (Hamlin *et al.*, 1967; Ng & Dawes, 1967, 1973).

In this report it is demonstrated that this control of glucose metabolism by succinate and other tricarboxylic acid-cycle intermediates is manifest at the level of the glucose permease, in that the inducible glucose-transport system as measured by the uptake of the non-metabolizable analogue, 2-deoxy-D-glucose, is strongly repressed by tricarboxylic acid-cycle intermediates. Some of our preliminary findings have already been reported (Mukkada *et al.*, 1971). In addition, parallel results have now been reported with methyl- α -glucoside used as the transport substrate (Midgley, 1972; Midgley & Dawes, 1973).

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Experimental

Organism and growth conditions

Pseudomonas aeruginosa CGH 7709, obtained from the Cincinnati General Hospital collection, was used in these studies. This organism was grown in a basal synthetic medium containing, per litre: K_2HPO_4 , 3.0 g; $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2 \cdot 2H_2O$, 0.001 g; $NaMoO_4 \cdot 2H_2O$, 0.001 g. Carbon sources were added to this basal medium as indicated in the Results section. Unless otherwise stated, 100 ml amounts of medium contained in 250 ml baffled conical flasks were inoculated with cells harvested from the medium specified, and washed twice with sterile 0.9% NaCl soln. Incubation was carried out at 37°C on a New Brunswick incubator shaker at 250 rev./min. Growth was measured turbidimetrically with a Bausch and Lomb Spectronic 20 spectrophotometer at 540 nm, and converted into mg dry wt. of cell material from a standard curve relating cell weight to extinction.

Uptake studies

Cells grown on basal medium with the carbon sources specified were harvested in mid-exponential phase or at the time specified in the Results section, washed twice with 0.9% NaCl soln. and resuspended in basal medium without carbon source at a cell density of 0.3 mg dry wt./ml. Cell suspensions (10 ml

final vol.) were incubated in a water-bath shaker at 30°C for 20 min to allow temperature equilibration; 2-deoxy-D-[U-¹⁴C]glucose (International Chemical and Nuclear Corp., Irvine, Calif., U.S.A.) was then added at a final concentration of 0.1 mM and a specific radioactivity of 0.2 μ Ci/ μ mol, unless specified otherwise. Samples of 1 ml were removed at 1 min intervals, filtered rapidly through Millipore filters (0.8 μ m porosity; Millipore Corp., New Bedford, Mass., U.S.A.) and washed immediately with 5 ml of basal medium. Washed filters with the cells thereon were transferred to vials containing 10 ml of Bray's (1960) scintillation fluid and the radioactivity was determined with a Packard Liquid Scintillation Spectrometer (Packard Instrument Co., Des Plaines, Ill., U.S.A.). Results were calculated in terms of c.p.m./mg of cells and converted into nmol of 2-deoxy-D-glucose/mg dry wt., a counting efficiency of 85% being used.

Extraction and identification of products

Cells grown in basal medium plus 25 mM-glucose were harvested, washed and suspended in basal medium without carbon source at a density of 0.3 mg dry wt./ml. Cell suspension (10 ml) was then incubated at 37°C with 0.1 mM-2-deoxy-D-[U-¹⁴C]glucose (0.4 μ Ci/ μ mol) for 3 min and filtered on a Millipore filter (0.8 μ m porosity). After washing with basal medium, the filter with the cells was placed in 5 ml of water at 95°C and extracted with stirring for 15 min. The suspension was then filtered and the filtrate analysed. For the determination of sugar phosphates, the chromatographic separation of Winkler (1966) was employed. Samples of 0.5 ml of extract were applied to columns (4 cm by 0.6 cm) of Bio-Rad AG1 anion-exchange resin (X2; 100–200 mesh), which had been converted into the formate form. Free sugars were eluted with five 0.5 ml portions of water; sugar phosphates and other anionic substances were subsequently eluted with seven 0.5 ml portions of 0.5 M-ammonium formate in 0.2 M-formic acid. The eluted materials were collected directly in scintillation vials for radioassay.

2-Deoxy-D-gluconic acid was prepared by bromine oxidation of 2-deoxy-D-glucose according to a modification of the method of Overend *et al.* (1949) by Williams & Eagon (1959). To an aq. 0.78 M-2-deoxy-D-glucose solution, bromine was added to a final concentration of 1.6 M. The solution was kept at room temperature for 2 h, after which the excess of bromine was removed by vigorous aeration. The HBr formed during the reaction was removed by adding silver oxide and filtering off the silver bromide precipitate. Any residual silver in solution was precipitated with hydrogen sulphide. The resulting solution of 2-deoxy-D-gluconic acid was further concentrated by evaporation.

Descending paper chromatography with Whatman no. 1 paper was used to identify 2-deoxy-D-glucose and 2-deoxy-D-gluconic acid. A known standard of the latter compound was not available to us, but the material prepared behaved chromatographically as described by Williams & Eagon (1959). A total of 0.1 ml of the concentrated extract was applied to the paper. The solvent was butan-1-ol–acetic acid–water (4:1:1, by vol.). Dried chromatograms were sprayed with ammoniacal AgNO₃ (0.5 M-AgNO₃ in aq. 5 M-NH₃ soln.) and heated at 105°C for 5 min to locate 2-deoxy-D-glucose and 2-deoxy-D-gluconic acid.

Results

Nature of the uptake system

The time-course of the uptake of 2-deoxy-D-glucose and the effect of sodium azide are shown in Fig. 1. The control cells accumulated this sugar rapidly; within 4–5 min the internal concentration reached 20–40 times the external concentration. The minimum estimate is based on an assumption that the cell can contain 80% water, and all of it is accessible to the substrate; thus 1 mg dry wt. of cell

Fig. 1. Time-course of 2-deoxy-D-glucose transport, and the effect of sodium azide

Cells grown on 25 mM-glucose were incubated with 0.1 mM-2-deoxy-D-[U-¹⁴C]glucose (0.2 μ Ci/ μ mol). ●, Untreated control; ○, 5 mM-sodium azide was added 1 min before addition of labelled sugar; ▲, 5 mM-sodium azide added 3 min after addition of labelled sugar.

Fig. 2. Lineweaver-Burk plot of the inhibition of 2-deoxy-D-glucose uptake by D-glucose

Cells grown on 25 mM-glucose were incubated with 2-deoxy-D-[U- 14 C]glucose ($0.2 \mu\text{Ci}/\mu\text{mol}$) with no further addition ($\bullet$) and with 0.01 mM-D-glucose ($\circ$).

Fig. 4. Ion-exchange chromatography of a hot-water extract of *P. aeruginosa* after uptake of 2-deoxy-D-glucose

Cells grown on 25 mM-glucose were exposed to 2-deoxy-D-[U- 14 C]glucose ($0.4 \mu\text{Ci}/\mu\text{mol}$) and extracted with hot water as described in the Experimental section.

Fig. 3. Exchange of intracellular 2-deoxy-D-glucose with external D-glucose

Cells grown on 25 mM-glucose were incubated with 0.1 mM-2-deoxy-D-[U- 14 C]glucose ($0.2 \mu\text{Ci}/\mu\text{mol}$) with no further addition ($\circ$) and with 0.1 mM-D-glucose added (at arrow) after 6 min ($\bullet$).

substance would correspond to $4 \mu\text{l}$ of water, and 8 nmol/mg of cells would represent a concentration of 2 nmol/ μl of cell water (2 mM). The higher estimate is based on the actual measurement of intracellular water in glucose-grown *P. aeruginosa* by Midgley & Dawes (1973), which amounted to $2 \mu\text{l}/\text{mg}$ dry wt. of cells.

That this concentrative uptake is energy-dependent is further indicated by the effect of sodium azide; this respiratory inhibitor prevented the uptake of 2-deoxy-D-glucose when added before this substrate, and brought about leakage of the accumulated sugar when added after uptake had commenced. Uptake was also inhibited by the energy-uncoupling agents 2,4-dinitrophenol and carbonylcyanide-*m*-chlorophenylhydrazone at 1 mM concentration.

The uptake of 2-deoxy-D-glucose shows saturation kinetics with respect to substrate concentration, with an apparent K_m of 0.22 mM. A Lineweaver-Burk plot of the uptake of 2-deoxy-D-glucose in the absence and the presence of D-glucose is shown in Fig. 2. It is

clear that glucose is a strong inhibitor of 2-deoxy-D-glucose uptake; moreover, the inhibition is competitive. The apparent K_i , which can be taken as a measure of the affinity of glucose for this transport system, calculated from these data is $6.7 \mu\text{M}$.

The fact that glucose competitively inhibits 2-deoxy-D-glucose uptake constitutes strong evidence that 2-deoxy-D-glucose is in fact transported by the glucose system. Further evidence that these two sugars share the same stereospecific carrier is provided by the observation that the intracellular pool of 2-deoxy-D-glucose is exchangeable with externally supplied glucose. Fig. 3 shows that a rapid exit of 2-deoxy-D-[U- ^{14}C]glucose from the cells is brought about by the addition of non-labelled glucose.

It was important to determine whether 2-deoxy-D-glucose was transported as free sugar or whether it was phosphorylated or otherwise changed either during or subsequently to transport. Accordingly, cells grown on 25mM-glucose were allowed to take up labelled 2-deoxy-D-glucose and were extracted with hot water (see the Experimental section). A portion of the hot-water extract was chromatographed, as described, on anion-exchange resin. As shown in

Fig. 5. Paper chromatogram of a hot-water extract of *P. aeruginosa* after exposure to 2-deoxy-D-glucose

Cells grown on 25mM-glucose were exposed to 2-deoxy-D-[U- ^{14}C]glucose ($0.4 \mu\text{Ci}/\mu\text{mol}$) and extracted with hot water as described in the Experimental section. A and B represent positions occupied by known 2-deoxy-D-glucose and 2-deoxy-D-gluconic acid respectively.

Fig. 4 the bulk of radioactivity appeared in the neutral fractions (fractions 1-6) eluted with water, indicating that most of the sugar existed in the free non-ionic form. A minor peak appeared in subsequent fractions eluted with 0.5M-ammonium formate in 0.2M-formic acid, which presumably contained small amounts of anionic products such as sugar phosphates or sugar acids (such as 2-deoxy-D-gluconic acid) or both. These represented a small fraction of the total counts. This is consistent with the findings of Phibbs & Eagon (1970) and of Romano *et al.* (1970) that *P. aeruginosa* lacks the phosphoenolpyruvate-phosphotransferase system (Roseman, 1969) and that sugar transport is not accompanied by phosphorylation in this organism.

That the 2-deoxy-D-glucose taken up appeared in the cell predominantly in the free unaltered form was also demonstrated by paper chromatography of hot-water extracts of cells, 2-deoxy-D-glucose and 2-deoxy-D-gluconic acid being used as known standards. As described, that portion of the chromatogram (before spraying) corresponding to the extract was cut into successive strips, each $2.2\text{cm} \times 2.5\text{cm}$ beginning with the starting line, and these were

Fig. 6. Uptake of 2-deoxy-D-glucose by *P. aeruginosa* grown on different carbon sources

Cells grown on 25mM-glucose (●), sodium acetate (○) and sodium succinate (▲) were incubated with 2-deoxy-D-[U- ^{14}C]glucose (0.1 mM , $0.2 \mu\text{Ci}/\mu\text{mol}$).

Fig. 7. Effect of growth substrate on 2-deoxy-D-glucose uptake

Cells grown on 25 mM-glucose (●), or 25 mM-glucose plus 25 mM-sodium acetate (○) or sodium succinate (▲), were incubated with 2-deoxy-D-[U-¹⁴C]glucose (0.1 mM, 0.2 μ Ci/ μ mol).

extracted with 2 ml of water each. The extracts were filtered and 0.5 ml of filtrate was added to scintillation vials and total radioactivity in each strip determined. As shown in Fig. 5, essentially all of the radioactivity (98% of the total) appeared in one peak corresponding to free 2-deoxy-D-glucose and none was detected in the region corresponding to 2-deoxy-D-gluconic acid. The R_F value (0.61) of 2-deoxy-D-gluconic acid is in agreement with that reported by Williams & Eagon (1959) under similar conditions.

Regulation of the transport system

The capacities of cells grown on glucose, acetate and succinate to transport 2-deoxy-D-glucose are compared in Fig. 6. Glucose-grown cells actively transported the sugar against a concentration gradient but cells grown on acetate and succinate did not. Cells grown on pyruvate, citrate, fumarate and malate also did not take up the sugar (Table 1). These results indicate that the transport system for glucose and its analogues is specifically induced by glucose. Moreover, Fig. 7 shows that cells grown in media con-

Table 1. Transport of 2-deoxy-D-[U-¹⁴C]glucose by *Pseudomonas aeruginosa* grown on different carbon sources

Cells in the exponential phase, grown on the different substrates (25 mM each), were incubated at 30°C with 0.1 mM-2-deoxy-D-[U-¹⁴C]glucose (0.2 μ Ci/ μ mol) and the amount of sugar accumulated in 5 min was determined as described in the Experimental section.

Growth substrate	2-Deoxy-D-glucose transported in 5 min (nmol/mg dry wt.)
Glucose	9.3
Pyruvate	2.7
Acetate	0.3
Citrate	0.3
Succinate	0.5
Fumarate	0.2
Malate	0.7
Glucose + pyruvate	8.9
Glucose + acetate	0.4
Glucose + citrate	2.8
Glucose + succinate	0.4
Glucose + fumarate	0.8
Glucose + malate	2.1

taining glucose plus an organic acid (succinate or acetate) were not able to take up 2-deoxy-D-glucose. Similar results were obtained when citrate, fumarate or malate was provided in the growth medium along with glucose, and the cells were harvested before the organic acid had been exhausted (Table 1). However, the presence of pyruvate with glucose did not affect the transport system (Table 1). All these results suggest that a number of organic acids or their products, including citrate, succinate, malate and fumarate, but not pyruvate, can repress the synthesis of the transport system and/or inhibit it once it is formed.

Addition of varying amounts of succinate or acetate (5–20 mM) to suspensions of glucose-grown cells failed to cause inhibition of 2-deoxy-D-glucose uptake, indicating that the transport system is not inhibited by these acids once it is formed. These results must be interpreted with caution, however, since the uptake systems for these acids are themselves inducible (Kogut & Podoski, 1953; Clarke & Meadow, 1959).

Recovery from repression

Growth studies cited earlier showed that, in media containing organic acids and glucose, the acids were utilized preferentially, but when the organic acid was exhausted the cells commenced using glucose. This could be interpreted as a recovery from repression of the glucose-transport system exerted by the preferred

Fig. 8. Recovery of the glucose-transport system from repression by succinate

Cells grown on 25 mM-sodium succinate were inoculated into a medium containing 5 mM-succinate and 25 mM-glucose. Portions of the culture were removed at indicated intervals during further incubation at 37°C, washed and resuspended as described in the Experimental section and incubated with 2-deoxy-D-[U-¹⁴C]glucose (0.1 mM, 0.2 μ Ci/ μ mol). ●, Succinate-grown cells (control). Cells were removed 2 h (○), 4 h (▲), 6 h (△) and 7 h (■) after transfer to the succinate-glucose medium.

Fig. 9. Recovery of the glucose-transport system from repression by acetate

Cells grown on 25 mM-sodium acetate were inoculated into a medium containing 10 mM-sodium acetate and 25 mM-glucose. Portions of the culture were removed at indicated intervals during further incubation at 37°C, washed and resuspended as described in the Experimental section and incubated with 2-deoxy-D-[U-¹⁴C]glucose (0.1 mM, 0.2 μ Ci/ μ mol). ●, Acetate-grown cells (control); cells were removed 2 h (○), 3.5 h (▲), 5 h (△) and 6 h (■) after transfer to the acetate-glucose medium.

substrate (succinate or acetate). To verify this, early exponentially growing cells from succinate or acetate medium, which were repressed for the transport system, were harvested, washed and inoculated into media containing 25 mM-glucose plus a limiting concentration of succinate or acetate (5 mM and 10 mM respectively). At different time-intervals during growth, portions of sufficient volume were withdrawn and the capacity of cells to transport 2-deoxy-D-glucose was determined. The cells grew without lag and, as shown in Figs. 8 and 9, during early growth when the cells were presumably still growing on succinate or acetate the transport system was repressed, as in the controls grown on succinate and acetate alone. However, as growth continued the cells began to recover the glucose-transport system, and in 5–6 h it was fully restored. This recovery was, in fact, a relief from repression and was dependent upon the synthesis of new protein. Cells in the ex-

ponential phase from the succinate medium inoculated into glucose medium containing an inhibitor of protein synthesis, chloramphenicol (50 μ g/ml), showed no recovery as compared with the control without the antibiotic (see Fig. 10). The presence of chloramphenicol in the uptake reaction mixture, however, did not affect uptake by glucose-grown cells (Fig. 10), establishing that chloramphenicol does not affect the system once it is induced.

Discussion

Phibbs & Eagon (1970) studied the uptake of glucose, fructose and mannitol by *P. aeruginosa*. They concluded that the uptake of these sugars is inducible and does not involve associated phosphorylation. They failed to distinguish whether active transport or facilitated diffusion is operative,

Fig. 10. Effect of chloramphenicol on the recovery of the glucose-transport system from repression by succinate

Cells grown on 25 mM-sodium succinate were transferred to medium containing 25 mM-glucose in the absence (▲) or the presence (●) of chloramphenicol (50 μ g/ml), and incubated for 6 h at 37°C. Cells were then harvested, washed and resuspended as described in the Experimental section, and uptake was measured with 2-deoxy-D-[U- 14 C]glucose (0.1 mM, 0.2 μ Ci/ μ mol). Controls included succinate-grown cells (○), and cells transferred to and incubated in glucose medium, but uptake of 2-deoxy-D-glucose (Δ) was measured in the presence of chloramphenicol (50 μ g/ml).

since they studied utilizable substrates. The use of 2-deoxy-D-glucose in our studies has avoided this ambiguity. Eagon (1971) has reported more recently that *P. aeruginosa* takes up 2-deoxy-D-glucose by passive diffusion. This, however, does not seem tenable since our results show that the sugar is transported against a concentration gradient in a predominantly unchanged state, that the process requires metabolic energy and is subject to induction, repression and competitive inhibition, all of which indicate a stereospecific active transport system.

Eagon (1971) based his conclusions that 2-deoxy-D-glucose enters the cell by passive diffusion principally on his failure to demonstrate saturation kinetics

for the uptake of this sugar, and his failure to demonstrate inhibition of uptake of [14 C]glucose by 2-deoxy-D-glucose, except at high relative concentrations of the latter sugar. With regard to the former point: the highest concentration of 2-deoxy-D-glucose used by Eagon was 0.1 mM (data were shown only up to 0.07 mM); these concentrations are considerably below that required for maximum velocity. In our experiments half-maximum velocity is attained at 0.22 mM (the measured K_m). Concerning the second point: it is not surprising that inhibition of [14 C]glucose 'uptake' by 2-deoxy-D-glucose could be demonstrated only at high 2-deoxy-D-glucose concentrations. First, the affinity of glucose for the transport system is much higher than that of 2-deoxy-D-glucose (we found the K_i for the D-glucose inhibition of 2-deoxy-D-glucose uptake to be 0.0067 mM); moreover, glucose can be metabolized and incorporated into cellular material, a factor which complicates measurement of uptake kinetics. It is of interest that Eagon did find that glucose inhibited the uptake of 2-deoxy-D-glucose as we have, but interpreted this as a competition for intracellular glucose dehydrogenase (EC 1.1.1.47). In our study, the fact that internally accumulated 2-deoxy-D-glucose is readily exchangeable with externally supplied glucose would argue strongly for the presence of a common stereospecific carrier.

Eagon's thesis is that any apparent accumulation of 2-deoxy-D-glucose is due to its oxidation to 2-deoxy-D-gluconic acid. The degree of intracellular accumulation of 2-deoxy-D-gluconic acid was minimal in the strain used in this study. This sugar acid was undetectable by paper chromatography of extracts of cells that were allowed to take up labelled 2-deoxy-D-glucose. By the more sensitive ion-exchange chromatographic procedure, the total anionic substances derived from 2-deoxy-D-[14 C]glucose accounted for little more than 10% of the total radioactivity. The possibility that 2-deoxy-D-gluconic was formed, and subsequently metabolized through action of gluconate dehydrogenase (EC 1.1.99.3), can be ruled out, since the activity of this enzyme requires the presence of a free hydroxyl group at the C-2 position of the substrate molecule. Thus the fact that 2-deoxy-D-glucose is concentrated intracellularly against a gradient in an unchanged state, and that D-glucose competitively inhibits its uptake as well as provoking its rapid exit from the intracellular pool, establishes this analogue as a useful substrate for studying glucose transport.

Strictly aerobic bacteria, such as *Pseudomonas aeruginosa*, *Arthrobacter globiformis* and *Azotobacter vinelandii*, have glucose-transport systems that are fundamentally different from that found in facultative anaerobes such as the coliform bacteria. The latter transport glucose by the phosphoenolpyruvate-phosphotransferase system (Roseman, 1969), whereas

strictly aerobic bacteria do not (Romano *et al.*, 1970). This study shows that the pattern of regulation of glucose transport in *P. aeruginosa* is also sharply divergent from that shown in the Enterobacteriaceae. The glucose-transport system in *E. coli* is constitutive (Cohen & Monod, 1957), and a repression of uptake systems for other substances is manifest in the presence of this sugar (Loomis & Magasanik, 1967). In contrast, the glucose-transport system of *P. aeruginosa* is inducible, and is subject to strong repression by a number of organic acids, including acetate, citrate, succinate, malate and fumarate. It is of interest that *Arthrobacter crystallopoietes*, also a strictly aerobic organism which like the pseudomonads occurs widely in soil and water, shares this divergence from the pattern exhibited by the coliform bacteria, in that its glucose-transport system is also repressed and inhibited by succinate (Krulwich & Ensign, 1969). Thus differences in both the mechanism and mode of regulation of glucose-transport systems in the pseudomonads and arthrobacters on the one hand, and the coliform bacteria on the other, parallel other physiological differences, and are further examples of the evolutionary divergency exhibited by these groups.

This work was supported by grants from the National Science Foundation (U.S.A.), the University of Cincinnati Research Development Fund and the University of Connecticut Research Foundation.

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