The Regulation of Transport of Glucose and Methyl α -Glucoside in *Pseudomonas aeruginosa*

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The methyl α -glucoside-transport system of *Pseudomonas aeruginosa* has been characterized with respect to its specificity, energy-dependence, kinetics and regulation. The uptake of glucose involved two components, one of which transported glucose ($K_m = 8 \mu\text{M}$) and methyl α -glucoside ($K_m = 2.8 \text{ mM}$) whereas the other was more complex, involving the extracellular activity of glucose dehydrogenase. Mutants defective in this enzyme have been isolated and characterized. The methyl α -glucoside-glucose-transport system was repressed when the organism was grown in the absence of glucose; the induction of this transport system by glucose, and its activity once induced, were inhibited by products of citrate metabolism.

Previous studies have demonstrated that when citrate-grown *Pseudomonas aeruginosa* is exposed simultaneously to glucose and citrate there is a preferential utilization of citrate, and that citrate inhibits the induction of the major glucose-catabolizing enzymes (Hamilton & Dawes, 1959, 1960, 1961; Hamlin *et al.*, 1967; Ng & Dawes, 1967, 1973). Evidence for the existence of a glucose-transport system was obtained and we have therefore investigated the role of such a system to assess control at this level by organic acids and derived metabolites. Similar observations with other systems, for example the glucose-lactose diauxie of *Escherichia coli* (Loomis & Magasanik, 1967) and the succinate-glucose diauxie of *Arthrobacter crystallopoietes* (Krulwich & Ensign, 1969), have shown that the process of substrate entry into the cell can be a major control site.

We have found that although methyl α -glucoside is a substrate for an active transport system for glucose, it is not metabolized by *P. aeruginosa* and consequently permits the transport system to be studied uncomplicated by further metabolism of the accumulated compounds. Methyl α -glucoside transport has therefore been characterized. We also present evidence that glucose can enter the organism by a second process involving its extracellular conversion into gluconate by glucose dehydrogenase (EC 1.1.99.a), which Campbell *et al.* (1962) have shown to be a membrane-bound enzyme. A preliminary account of part of this work has been presented to The Biochemical Society (Midgley, 1972).

Experimental

Organisms and growth of bacteria

Organisms. *Pseudomonas aeruginosa* 2F32 was originally obtained from Dr. R. W. Kinney of Iowa

State College, and was maintained on nutrient-agar slants. For experiments it was fully adapted to the appropriate synthetic media by daily subculture.

Escherichia coli N.C.T.C. 5928 was used for comparative studies.

Growth media. The basal medium was prepared from two solutions of the following compositions (g/litre). Solution A: KH_2PO_4 , 15; $(\text{NH}_4)_2\text{SO}_4$, 3.3; nitrilotriacetic acid, 0.8; pH adjusted to 7.1 with 5M-NaOH. Solution B: carbon and energy source (glucose, gluconate, glycerol, trisodium citrate or sodium succinate, 20); 5 ml of each of trace elements solution 1 and 2 (Ng & Dawes, 1973) except succinate media, which received only solution 1. The solutions were autoclaved separately at 15 lb/in^2 ($103186 \text{ N}\cdot\text{m}^{-2}$) for 20 min and, after cooling, the trace elements were added and solutions A and B combined in the ratio of 3:2 (v/v).

Growth conditions. The organisms were grown at 37°C in 250 ml of medium contained in 1-litre Erlenmeyer flasks on a Luckham rotary shaker (Luckham Ltd., Burgess Hill, Sussex, U.K.). Inocula (0.08%) were taken from cultures trained by at least six subcultures through the growth medium to be used, except where experimentation dictated otherwise, and the bacteria were harvested after 16 h (in the late exponential phase) at a density of approx. 1 mg dry wt./ml .

Transport studies

Uptake. Bacteria were harvested by centrifugation at $23000g$ for 5 min and washed twice at 37°C with 0.067 M -sodium-potassium phosphate buffer, pH 7.1, containing 0.1% (w/v) $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, referred to as Mg^{2+} -phosphate buffer. The cells were resuspended to the desired concentration in this same buffer and

held at 37°C on a Gallenkamp shaking water bath for 10 min before the addition of the radioactive substrate, which had been pre-warmed to 37°C. The incubation mixture comprised 0.3 ml of water or other specified component, 0.2 ml of chloramphenicol (2.5 mg/ml), 1 ml of Mg^{2+} -phosphate buffer, 0.5 ml of bacterial suspension and 0.5 ml of substrate. The minimum volume used was 2.5 ml in a 25 ml Erlenmeyer flask. When larger volumes were required the incubation mixture and flask were proportionately increased. The final cell density was between 2 and 450 μ g dry wt./ml., depending upon experimental requirements. Samples (0.5 ml) were removed at desired intervals with an Eppendorf pipette (V. A. Howe and Co. Ltd., London S.W.6, U.K.) and transferred into 2 ml of Mg^{2+} -phosphate buffer, pH 7.1, supplemented with 1% (w/v) NaCl, referred to henceforth as NaCl buffer, which was at ambient temperature and contained in the filter assembly. The filters were held in a Swinnex filter holder (25 mm diam.; Millipore Corp., Bedford, Mass., U.S.A.) fitted with Perspex castles (65 mm $\times$ 25 mm int. diam.). The vacuum was immediately applied. When all liquid had disappeared from the filter surface, two 1 ml portions of NaCl buffer were applied. The filtration time was generally 10–15 s. The filters were then transferred to scintillation vials containing scintillation fluid of the following composition: 2,5-diphenyloxazole, 4 g; naphthalene, 60 g; methanol, 100 ml; ethylene glycol, 20 ml; dioxan to 1 litre [modified from that of Bray (1960) by omission of 1,4-bis-(5-phenyloxazol-2-yl)benzene]. The filters were dissolved by vigorous shaking. Samples were counted with a Beckman LS 233 scintillation spectrometer (Beckman Ltd., Glenrothes, Fife, U.K.). Uptake rates are expressed as μ mol/min per g dry wt. of bacteria and are corrected for radioactivity counts retained by the filter when cells were preincubated (10 min) with formaldehyde (25 mM final concentration) before the addition of the radiochemical.

Exit. A dilution technique was used. Bacteria were loaded with substrate by incubation under the conditions described previously except that the cell density was increased to 2 mg dry wt./ml (final concentration). Samples (0.4 ml) were removed between 7 and 30 min after the addition of substrate and pipetted into 39.6 ml of incubation mixture (identical except that bacteria and substrate were absent) at 37°C. At timed intervals samples (5 ml) were removed and pipetted on to membrane filters, to which a vacuum was already applied. The filter was washed with two 1 ml portions of NaCl buffer at ambient temperature. Radioactivity counting was carried out as for entry experiments. To permit correction for extracellular radiochemical retained on the filter, the bacterial suspensions were incubated for 10 min with 25 mM-formaldehyde solution before the addition of methyl α -glucoside. Samples from such incubations

were diluted and processed as for the test experiments. Since treatment with formaldehyde prevents entrance of methyl α -glucoside into the bacterial cell, radioactivity remaining on the filter was taken to be of extracellular origin and deducted from the values obtained in the test experiments.

Extraction and identification of accumulated material. Intracellular material was isolated by the cold-shock technique. Samples from incubation mixtures were washed in the manner previously described and the filter was then rapidly transferred to a clean filter-holder and subjected to a further wash with 5 ml of ice-cold water. This wash was collected and suitably concentrated, by rotary evaporation under reduced pressure, for chromatography. The cold shock resulted in a greater than 95% extraction of the accumulated material, as demonstrated by removing duplicate samples from a washed suspension of bacteria accumulating methyl α -[^{14}C]glucoside, subjecting both to the washing procedure at 21°C with NaCl buffer (see the Experimental section, 'Transport studies: uptake') and then washing one of them with 5 ml of ice-cold water.

Determination of intracellular cell water. Intracellular cell water was determined from the ratio of wet weight to dry weight of a packed pellet of cells, with a correction for the extracellular pellet water. The correction was determined as described by Winkler & Wilson (1966) except that [carboxy- ^{14}C]inulin was employed.

Mutagenic procedure

Mid-exponential phase glucose-grown cells (10 ml) were harvested and washed at 37°C with Mg^{2+} -phosphate buffer under aseptic conditions. The cells (approx. 5 mg dry wt.) were resuspended in sterile citrate buffer (75 mM, pH 5.5) and sterile *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (final concentration 20 μ g/ml) was added. Incubation was for 30 min on a Gallenkamp shaking water bath at 37°C. The cells were harvested and washed twice with Mg^{2+} -phosphate buffer at 37°C before resuspension in nutrient broth (8 g/litre) and incubation at 37°C for 4 h. Suitable dilutions were made in Mg^{2+} -phosphate buffer and 0.1 ml portions were plated on to the Bromothymol Blue indicator medium described by Korman & Berman (1958). Plates were incubated for 32 h at 37°C before visual examination for non-acid-producing mutants. Only isolated organisms that produced both pyocyanine and fluorescein, as did the parent strain, on the media described by King *et al.* (1954) were retained.

Measurement of metabolism of glucose and analogues by whole cells

Bacteria (0.3–3 mg dry wt./ml) in Mg^{2+} -phosphate buffer were incubated with glucose analogues at the

final concentrations specified in the appropriate experiments. Samples (2ml) were centrifuged at 26000g for 5 min to remove the bacteria. The supernatants were assayed by the methods described below. A technique used in some experiments was to pipette samples (0.5 ml) into 10% (w/v) trichloroacetic acid (0.5 ml) and then centrifuge.

Isolation of membrane fractions

These were isolated from extracts prepared as described by Ng & Dawes (1973) by ultracentrifuging at 105000g for 90 min at 0°C on an MSE Superspeed 50 preparative ultracentrifuge. The pellet was re-suspended in glycylglycine buffer (0.12M, pH 7.1) to give a protein concentration between 1 and 4 mg/ml.

Analytical methods

Chromatography. The following separation systems, all used with Whatman no. 1 paper, were employed: (1) chloroform-methanol-water (48:56:21, by vol.), ascending; (2) butanol-ethanol-water (4:3:2, by vol.), ascending.

Standards on chromatograms were visualized by spraying with the reagent described by Dawson *et al.* (1969), which detects polyols and related compounds. Radioactivity was detected by cutting up the sample lane into sections (0.5 cm × 4 cm) and counting the radioactivity of these immersed in Bray's (1960) fluid.

Chemical analyses. The following carbohydrates were assayed by the anthrone procedure of Dische (1962): 2-deoxyglucose (535); galactose (630); 6-deoxyglucose (566); glucose (692). The figures in parentheses are the wavelengths (nm) at which the sugars were determined. Glucosamine was assayed by the indole method after deamination, and xylose

was assayed by the Molisch method, both as described by Dische (1962). Glucose was also assayed in two ways with the enzymic reagents based on glucose oxidase and hexokinase supplied by the Boehringer Corp. (London).

Protein was estimated by the biuret reaction with correction for the biuret-positive material in the glycylglycine buffer (Gornall *et al.*, 1949).

Chemicals. The chemicals used throughout this work were, where possible, of AnalaR standard. All sugars were the D-form. 2-Deoxyglucose ('glucose-free' A grade) and 3-O-methyl glucose were obtained from Calbiochem (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.). 6-Deoxyglucose, methyl α -glucoside and methyl β -glucoside were obtained from Koch-Light Co. Ltd., Colnbrook, Bucks., U.K. Methyl α -glucoside was purified by recrystallization from aq. 90% (v/v) ethanol, passage (twice) through a column of Dowex I (OH⁻ form), and further recrystallization (twice) from aq. 90% (v/v) ethanol. The final glucose content was less than 0.01% (w/w) as determined by the glucose oxidase method. 2-Deoxygalactose, methyl α -mannoside, fucose and glucose 6-phosphate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Gluconate, sorbitol, glucosamine, *N*-acetylglucosamine, xylose and galactose were obtained from Hopkin and Williams Ltd., Essex, U.K. Galactose and glucosamine were recrystallized (twice) from aq. 80% (v/v) ethanol before use. 1,5-Anhydromannitol was a generous gift from Dr. S. G. Wilkinson.

Radiochemicals. Methyl α -D-[U-¹⁴C]glucopyranoside, [1-¹⁴C]glucosamine hydrochloride, [U-¹⁴C]galactose and [U-¹⁴C]glucose were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. 2-[1-¹⁴C]Deoxyglucose was supplied by New England Nuclear Corp., Boston, Mass., U.S.A., and [¹⁴C]inulin by Calbiochem.

Table 1. *Comparison of specific wash procedures on the retention of methyl α -glucoside by glucose-grown *P. aeruginosa* 2F32*

Reactions were carried out in duplicate and four samples were removed from each flask, 6–9 min after addition of methyl α -glucoside (final concn. 2.5 mM, specific radioactivity 0.08 μ Ci/ μ mol), and washed as indicated in the Experimental section.

Composition of wash fluid	Wash temperature (°C)	Retained methyl α -glucoside (μ mol/g dry wt.)
Basal*	37	29.2
Basal	21	13.8
Basal	0	0.0
Basal + 1% (w/v) NaCl	21	29.4
Basal + 5% (w/v) NaCl	0	22.6
Basal + 0.12M-formaldehyde	37	29.2

* Basal wash fluid: 0.067 M-sodium-potassium phosphate (pH 7.1)–0.1% (w/v) MgSO₄·7H₂O.

Results

This investigation fell into two distinct parts, each with essential supporting studies. Initially methyl α -glucoside was shown to be a gratuitous substrate for the glucose-transport system of *P. aeruginosa* and this enabled transport to be divorced from further metabolism. The washing procedure was found to be critical for demonstrating uptake of methyl α -glucoside. Studies of the specificity of this transport system and its occurrence in response to the carbon source in the growth medium were made but experiments with [U- 14 C]glucose then revealed the existence of a second transport system for this hexose, possessing a much higher K_m than that of the glucose-methyl α -glucoside system. The second part of the investigation was therefore concerned with the high- K_m system, which, since it appeared to be associated with glucose dehydrogenase activity, necessitated a study of this enzyme and the isolation of mutants defective for it, to assess its role in overall glucose uptake.

Studies with methyl α -glucoside

Measurement of uptake of methyl α -glucoside. In preliminary experiments we were unable to demonstrate any significant accumulation of methyl α -glucoside by glucose-grown organisms. This was traced to the wash procedure employed to remove extracellular radiochemical from the filter and such methodological considerations are presented here because Phibbs & Eagon (1970), with a different wash procedure, have claimed that methyl α -glucoside is not accumulated by glucose-grown *P. aeruginosa*. Table 1 records the effects of varying the temperature and composition of the wash fluid. The efflux of methyl α -glucoside due to a rapid fall in temperature is reversed by the inclusion of NaCl in the wash fluid. The concentrations of NaCl employed in Table 1 (1%, w/v, at 21°C and 5%, w/v, at 0°C) were found to be the optimum for these respective temperatures. The inclusion of formaldehyde in the wash fluid, to inhibit any efflux of accumulated material during the wash procedure, resulted in no increase in apparent accumulation. The wash finally adopted for routine use at 21°C was that employing the basal wash fluid plus 1% (w/v) NaCl. Fig. 1 shows the time-course of methyl α -glucoside uptake by glucose-grown cells and Fig. 2 the chromatogram of the intracellular contents of such cells when isolated by the cold-shock procedure described in the Experimental section. For comparison, similar material isolated by the cold-shock technique from glucose-grown *E. coli* that had been exposed to methyl α -glucoside was also chromatographed. *P. aeruginosa* contained only unchanged methyl α -glucoside whereas *E. coli* also contained a derivative of this compound, probably

methyl α -glucoside 6-phosphate (Rogers & Yu, 1962). The intracellular contents chromatographed as unchanged methyl α -glucoside when either solvent system 1 or 2 was used.

Determination of the intracellular cell water by the inulin method gave a value of 1.8–2 ml/g dry wt. for glucose-grown cells. Disregarding the contribution of the periplasmic space the value for the internal concentration can be calculated and, under the conditions cited in Fig. 1, is approximately fivefold the extracellular concentration.

The accumulation process is sensitive to sodium azide (Fig. 3). Preincubation of glucose-grown cells with 30 mM-azide inhibited the initial rate of methyl α -glucoside uptake. These observations, and the accumulation of unchanged methyl α -glucoside within the cell to a concentration exceeding that in the medium, demonstrate that an active transport system is operating.

Kinetics of methyl α -glucoside transport

The response of the initial rates of methyl α -glucoside transport to increasing extracellular concentration is shown in Fig. 4. A system capable of saturation with methyl α -glucoside and with a K_m approx. 2.5 mM is indicated. The dependence of the accumulation process on the external methyl α -glucoside concentration is shown in Fig. 5. The amount of substrate accumulated follows a Langmuir adsorption isotherm with a K_m^{app} (the methyl α -glucoside concentration required to achieve half maximum capacity) of 2.8 mM and a maximum capacity of 50 μ mol/g dry wt. Independent experiments with different batches of

Fig. 1. Time-course of methyl α -glucoside transport by glucose-grown *P. aeruginosa* 2F32

The concentration of methyl α -glucoside was 2.5 mM. Transport was measured as described in the Experimental section. The bacterial density was 400 μ g dry wt./ml.

Fig. 2. Chromatogram of the material accumulated by *P. aeruginosa* 2F32 and *E. coli* N.C.T.C. 5928 after exposure to methyl α-glucoside

Intracellular methyl α-glucoside was isolated as described in the Experimental section. Solvent system no. 2 was used.

Fig. 3. Inhibition by azide of the accumulation of methyl α-glucoside by glucose-grown *P. aeruginosa* 2F32

Transport was measured as described in the Experimental section. The methyl α-glucoside concentration was 2.5mM and the final azide concentration was 30mM. The bacterial density was 390μg dry wt./ml.

glucose-grown cells gave K_m values, in initial rate studies, between 2 and 3.8mM while the V_{max} for uptake varied between 11 and 28 μmol/min per g dry

wt. Similar determinations of K_m^{app} gave values between 2.2 and 3.8mM, and the maximum capacity varied between 30 and 60 μmol/g dry wt. Because of this variation in activity appropriate controls were always included to allow comparison of different batches of cells on different days.

As seen in Fig. 1, the amount of methyl α-glucoside accumulated reaches an apparent constant value within 4–6min after addition of substrate. That this accumulation represents a dynamic state is shown by experiments in which cells were allowed to accumulate unlabelled methyl α-glucoside until, as indicated in parallel experiments employing tracer, the plateau region was established. To such pre-loaded cells radioactive methyl α-glucoside was added, in such a small amount that the pre-existing steady state was not disturbed, and the initial rate of entry into the intracellular pool was measured (Table 2). Clearly methyl α-glucoside still enters the cell at this apparent plateau. Since, in the steady state, the rate of entrance must equal the rate of exit, and since the rate of entry of methyl α-glucoside into pre-loaded cells does not differ significantly from entry into empty cells,

Fig. 4. Lineweaver-Burk plot of the initial rate of methyl α -glucoside transport by glucose-grown *P. aeruginosa* 2F32

Transport was measured as described in the Experimental section. Initial rates of transport were measured in duplicate from three samples taken during the initial 90 s after the addition of methyl α -glucoside. The bacterial density was 380 μg dry wt./ml.

initial-rate data can be used to obtain some information concerning exit, the second component of the steady state. Fig. 6 shows that the exit rate is directly proportional to the internal methyl α -glucoside concentration, i.e. a first-order process such that the accumulated methyl α -glucoside has a half-life of approx. 2 min. A series of independent experiments gave values between 1.7 and 2.1 min.

A direct measure of the exit reaction was possible by employing the dilution technique described in the Experimental section and Fig. 7 shows a time-course of exit of accumulated material. The loss of pre-accumulated material follows an exponential function that indicates a half-life of approx. 3 min. The internal concentration range covered in this experiment was 7–22 $\mu\text{mol/g}$ dry wt. Independent experiments gave values for $t_{1/2}$ of the accumulated methyl α -glucoside between 2.8 and 3.1 min. These were significantly different from the values obtained by entry measurements and this is probably due to the periplasmic space acting as a partial diffusion barrier (Robbie & Wilson, 1969), thereby providing an external compartment from which effluxing methyl α -glucoside could be recaptured by the cell. The presence of external, unlabelled methyl α -glucoside in the dilution medium converts the net flux into a unidirectional flux, such that $t_{1/2}$ for exit of the intracellular methyl α -glucoside approaches that

Fig. 5. Effect of external methyl α -glucoside concentration on the accumulation of methyl α -glucoside by glucose-grown *P. aeruginosa* 2F32

Accumulation was measured in duplicate 8 min after methyl α -glucoside addition, by the procedure described in the Experimental section. The bacterial density was 390 μg dry wt./ml.

measured in entry experiments (Table 3). The addition of azide (Table 3) has a similar effect, preventing recapture by inhibiting the entry mechanism.

Specificity of methyl α -glucoside transport

The inhibition of methyl α -glucoside accumulation by structurally related unlabelled carbohydrates was measured (Table 4) in order that the relationship between glucose and methyl α -glucoside transport might be assessed. All successful competitors were assayed enzymically for glucose content and were tested at a concentration range in which the inhibition of methyl α -glucoside uptake could not be explained by glucose contamination. Glucose and closely related compounds such as 2-deoxyglucose and 6-deoxyglucose inhibit methyl α -glucoside transport and are thus presumed substrates for the same transport system.

Inhibition of methyl α -glucoside transport by metabolizable compounds

The possibility that methyl α -glucoside transport was subject to metabolic regulation was investigated

Table 2. Comparison of the initial rate of entry of methyl α -glucoside with the rate of entry into cells pre-loaded with methyl α -glucoside

The final specific radioactivity was 0.05 $\mu\text{Ci}/\mu\text{mol}$ in all cases. The values shown are the means of triplicate assays performed over the first 90s after addition of either substrate (initial rate determination) or radiochemical to pre-loaded cells. The concentration increases on addition of radiochemical were 1.4 and 7% at 10 and 2 mM respectively.

External methyl α -glucoside concn. (mM)	Uptake of methyl α -glucoside ($\mu\text{mol}/\text{min}$ per g dry wt.)	
	Initial rate	Rate for cells pre-loaded for 7 min
10	17.9 $\pm$ 2.0	18.1 $\pm$ 1.0
2	7.7 $\pm$ 0.4	8.0 $\pm$ 1.6

Fig. 6. Proportionality of the initial rate of entry of methyl α -glucoside to the amount of methyl α -glucoside accumulated at the steady state

The values were obtained by combining measurements from experiments similar to those described in Figs. 4 and 5.

by preincubating cells with metabolizable substrates and then measuring the uptake of methyl α -glucoside in the continuing presence of the compound (Table 5). Clearly several compounds inhibit and this is accentuated by a preincubation period, as shown in experiments in which the inhibition of the initial rate of uptake was studied (Table 6). Inhibition of methyl α -glucoside transport by acetate and succinate was not constant from batch to batch of glucose-grown cells despite standardized growth conditions; with some cells no inhibition was seen. Inhibition produced by preincubation with gluconate was always observed (presumably because the glucose-grown organism metabolized at least part of the total glucose utilized via gluconate, and was thus always permeable to this compound) and in cells which showed inhibition of methyl α -glucoside transport by preincubation with either gluconate or succinate the effect of the

Fig. 7. Exit of methyl α -glucoside from glucose-grown *P. aeruginosa* 2F32

Exit was measured by the dilution technique described in the Experimental section. The methyl α -glucoside concentration during the loading phase was 5 mM.

simultaneous presence of these compounds was not additive. When cells were loaded with methyl α -glucoside in the presence of a metabolizable compound and exit was studied by using the dilution technique (the metabolizable compound being present during both the loading and unloading phase), then no change in $t_{1/2}$ of the accumulated material occurred other than that accounted for by the inhibition of recapture from the periplasmic space (Table 7). Attempts to obtain cells permeable to citrate and methyl α -glucoside by inducing washed suspensions of citrate-grown cells with glucose were unsuccessful

Table 3. *Exit of methyl α -glucoside from glucose-grown P. aeruginosa 2F32*

Values for $t_{\frac{1}{2}}$ were calculated from $\log[\text{intracellular methyl } \alpha\text{-glucoside}]$ as in Fig. 7.

Concn. of methyl α -glucoside in loading phase (mM)	Addition to dilution medium		$t_{\frac{1}{2}}$ (exit) of accumulated methyl α -glucoside (min)
	Compound	Concn. (mM)	
5.0	None		2.8
5.0	Methyl α -glucoside	5.0	1.9
2.5	None		2.9
2.5	Methyl α -glucoside	2.5	2.1
2.5	Sodium azide	30	2.5
5.0	Sodium azide	30	2.4

Table 4. *Specificity of methyl α -glucoside transport by glucose-grown P. aeruginosa 2F32*

Competitors, at the final concentrations indicated, were added 10s before methyl α -glucoside (final concn. 1.25 mM). Accumulation was calculated from four samples taken between 6 and 9 min after methyl α -glucoside addition. 100% accumulation = 18 $\mu\text{mol/g}$ dry wt. Chloramphenicol (final concn. 200 $\mu\text{g/ml}$) was present. [The following compounds, at 12 mM final concentration, except where indicated, did not depress methyl α -glucoside accumulation: 2-oxoglucuronate, fructose, ribose, mannitol, methyl α -mannoside, sorbitol, 3-O-methylglucose, N-acetylglucosamine, glucose 6-phosphate (8 mM), 1,5-anhydromannitol (2.4 mM) and 2-deoxygalactose.]

Competitor	Concentration (mM)	Relative accumulation (%)
None	—	100
Glucose	0.24	13
	0.12	75
	0.06	92
	0.06	92
2-Deoxyglucose	3.00	22
	0.12	42
	0.06	59
	0.06	59
Glucosamine	12.00	24
	2.40	33
	1.20	50
	1.20	50
Mannose	3.00	13
	1.20	16
Galactose	3.00	27
	1.20	56
6-Deoxyglucose	1.20	12
	0.48	12
Xylose	2.40	21
	1.20	24
Fucose	12.00	30
Methyl β -glucoside	12.00	18

Table 5. *Inhibition of methyl α -glucoside accumulation by organic acids*

Cells were preincubated at 37°C for 10 min with the indicated compound. Accumulation was measured between 6 and 9 min; each value is an average of four samples which varied by less than 10%. The methyl α -glucoside concentration was 1.25 mM and 100% accumulation = 21 $\mu\text{mol/g}$ dry wt.

Addition to system		
Compound	Concn. (mM)	Accumulation (% of control)
None	—	100
Sodium citrate	30	104
Sodium succinate	30	49
Sodium acetate	30	47
Sodium pyruvate	30	60
Sodium gluconate	1	40

Table 6. *Effect of preincubation time with sodium succinate or sodium gluconate on the initial rate of methyl α -glucoside transport*

Initial rates were measured in duplicate over the first 90s of transport. The methyl α -glucoside concentration was 1.25 mM. The rate corresponding to 100% (10 min preincubation with water) was 7.9 $\mu\text{mol/min}$ per g dry wt.

Period of preincubation with 1 mM organic acid (min)	Initial rate (% of control)	
	Succinate	Gluconate
10s	95	77
3	85	—
6	66	—
10	48	61
15	46	—
20	48	50

and account for the lack of inhibition recorded in Table 5. However, cells permeable to these compounds could be obtained by incubating washed suspensions of glucose-grown cells with 30 mM-citrate for 3 h at 37°C. With such cells 35% inhibition of methyl α -glucoside accumulation (20.2 μ mol/g dry wt. versus 13 μ mol/g dry wt. with external methyl α -glucoside concentration of 2.5 mM) could be detected when they were preincubated and assayed in the continuing presence of 30 mM-citrate. Alternatively, cells permeable to both compounds could be obtained by inducing cells growing on citrate with glucose (Table 8). Also recorded is the inhibitory effect of citrate on the induction process. When glucose transport was

studied directly, with glucose as the substrate, similar inhibitions of the induction process and of the activity of the transport system by citrate were observed.

Distribution of methyl α -glucoside transport in cells grown on different media

Cells were grown on different media and their ability to transport methyl α -glucoside was tested after harvesting and washing. Citrate- and succinate-grown cells contain repressed activities of the glucose-metabolizing enzymes (Hamilton & Dawes, 1960) whereas glycerol- and gluconate-grown cells contain similar enzyme activities to glucose-grown cells, and manometric studies indicate that they oxidize glucose without a lag. However, methyl α -glucoside uptake in these cells could not be demonstrated despite variation of the methyl α -glucoside concentration between 1 and 10 mM, preincubation with metabolizable substrates, or with energy poisons or starvation of cells for 4 h. The fact that there is a lack of correlation between the presence of the methyl α -glucoside-transport system and glucose transport led to experiments with labelled glucose as the substrate.

Glucose transport measured with [U- 14 C]glucose

Since it is difficult to determine the rate-limiting factor in oxygen-uptake experiments with extensively metabolized substrates, direct measurements of glucose transport were attempted with [U- 14 C]-glucose. Under all the experimental conditions used, the initial rate of 14 C uptake was linear and passed through an experimentally defined zero-time origin that was obtained with cells that had been preincubated for 10 min with 25 mM-formaldehyde.

Table 7. *Effect of preincubation with sodium succinate or sodium gluconate on the exit of methyl α -glucoside*

Cells were preincubated with the appropriate addition for 10 min. The methyl α -glucoside concentration in the loading phase was 2.5 mM. The dilution method described in the Experimental section was used except that, as noted, the pre-warmed incubation mixture also contained the organic acid. Assays were performed in quadruplicate and the values derived for the $t_{\frac{1}{2}}$ are recorded.

Addition to loading and exit media		$t_{\frac{1}{2}}$ (exit) of accumulated methyl α -glucoside (min)
Compound	Concn. (mM)	
None	—	3.0, 2.9, 3.2, 2.9
Sodium gluconate	30	1.9, 2.0, 1.8, 1.8
Sodium succinate	30	2.0, 2.2, 1.9, 2.1

Table 8. *Induction by glucose of a glucose-methyl α -glucoside-transport system in growing cultures of citrate-trained *P. aeruginosa* 2F32: interaction of the induction process and the induced transport system with citrate*

In Expt. 1 methyl α -glucoside transport was measured with a concentration of 2.5 mM. In Expt. 2 glucose transport was measured with 5 μ M-glucose. The figures in parentheses are the results of an independent experiment. Different batches of citrate-grown cells were used for the two experiments. The initial cell density in the induction medium was approx. 60 μ g dry wt./ml, which during growth increased to 200 μ g dry wt./ml for glucose alone and to 600 μ g dry wt./ml for the glucose+citrate addition.

Expt. no.	Addition to growth medium during 3 h induction period		Addition to transport assay during 10 min preincubation		Initial rate of transport (μ mol/min per g dry wt.)
	Substrate	Concn. (mM)	Compound	Concn. (mM)	
1	Glucose	2	None	—	13.2
	Glucose	2	None	—	8.7
	+ citrate	5	Citrate	10	5.8
2	Glucose	2	None	—	49.0 (42)
	Glucose	2	None	—	25.3 (32.3)
	+ citrate	5	Citrate	10	20.8 (23.2)

Fig. 8. Effect of external glucose concentration on the uptake of material from glucose by glucose-grown *P. aeruginosa* 2F32

Initial rates of transport were measured in duplicate from samples taken at 15, 30, 45 and 60 s after glucose addition, as described in the Experimental section. For concentrations up to and including $35 \mu\text{M}$ the bacterial density was $4 \mu\text{g dry wt./ml}$. Above this concentration $380 \mu\text{g dry wt./ml}$ was employed.

Fig. 9. Lineweaver-Burk plot of the initial rate of uptake of material from glucose by gluconate- and glycerol-grown *P. aeruginosa* 2F32

Initial rates of transport were measured in duplicate from samples taken at 15, 30, 45 and 60 s after glucose addition, by the technique described in the Experimental section. The bacterial densities used were 380 and $410 \mu\text{g/dry wt./ml}$ for glycerol- ($\circ$) and gluconate-grown ($\bullet$) cells respectively.

The variation in the initial rate of ^{14}C uptake by glucose-grown cells in response to increasing external glucose concentration is shown in Fig. 8. Clearly the involvement of two components is suggested. A Lineweaver & Burk (1934) plot of the data for the system saturated at low substrate concentration gave a K_m value of $8 \mu\text{M}$. This is henceforth referred to as the 'low- K_m ' transport system. The K_m for the second process capable of being saturated with glucose is estimated at approx. 2 mM . An accurate measurement was not possible because of the relative errors involved when the activity due to the low- K_m transport system was subtracted from the total activity measured.

Similar experiments were performed with glycerol- and gluconate-grown cells (Fig. 9). No uptake of ^{14}C by the low- K_m component could be detected but, as indicated, uptake was mediated by a process with K_m of approx. 1 mM in both types of cell.

At this stage of the investigation it was recognized that the organism could accumulate glucose via two independent systems. One of these was an active transport system of broad specificity, characterized as the methyl α -glucoside system and present only in the glucose-grown organism, and probably identical with the low- K_m glucose-transport system. The nature

Table 9. Oxidation of carbohydrates by a membrane preparation from glucose-grown *P. aeruginosa* 2F32

Membrane preparations were produced as described in the Experimental section. Activity was measured as described by Ng & Dawes (1973) except that the reaction was initiated with substrate after measurement of the endogenous rate.

Substrate	K_m (mM)
Glucose	1
2-Deoxyglucose	3
6-Deoxyglucose	1.6
Glucosamine	6
Xylose	25
Galactose	44

of the second system was next investigated and since, as will be seen subsequently, it involves the extra-cellular activity of glucose dehydrogenase, characterization of uptake via this system necessitated a study of this enzyme.

Specificity of glucose dehydrogenase

Glucose dehydrogenase, which is present in glucose-, gluconate- and glycerol-grown cells, is a membrane-bound enzyme (Campbell *et al.*, 1962) and membrane preparations derived from glucose-grown cells as described in the Experimental section have a K_m for glucose oxidation of 1 mM (Table 9). Also recorded are the K_m values for the oxidation of various glucose analogues by such preparations. Williams & Eagon (1959) and Eagon (1971) have presented evidence that 2-deoxyglucose is a substrate for glucose dehydrogenase. Our work (see below) suggests that 6-deoxyglucose, galactose, glucosamine and xylose are also substrates for this enzyme. The correlation between the K_m for glucose oxidation and high- K_m component in glucose-, glycerol- and gluconate-grown cells, and the presence of glucose dehydrogenase in such cells, suggested that the glucose entry by the high- K_m component involved in some way the activity of this enzyme.

Transport studies with mutants deficient in glucose dehydrogenase

The relationship between transport and dehydrogenation of glucose was established with two mutants of *P. aeruginosa* 2F32 isolated after nitrosoguanidine treatment as described in the Experimental section. Two major classes of mutant appeared on the indicator media used, namely (1) non-acid-producing but of wild-type colony size and (2) acid-producing but with small colony size compared with the wild type. Our experiments made use of two independently

Table 10. Specific activities of glucose-metabolizing enzymes of *P. aeruginosa* 2F32 59A and 106B on various media

Growth medium	Organism	Specific activity ($\mu\text{mol/h per mg of protein}$)					
		Glucose dehydrogenase	Hexokinase	Glucose 6-phosphate dehydrogenase	6-Phospho-gluconate dehydrogenase	Gluconate kinase	Gluconate dehydrogenase
Glucose	59A	n.d.	27.0	26	1.5	4.0	10.0
	106B	n.d.	15.0	19	8.5	0.3	27.0
Glycerol	59A	n.d.	9.1	26	1.6	1.8	9.4
	106B	n.d.	—	87	—	—	19.5
Gluconate	59A	0.50	11.5	30	—	1.3	365
	106B	0.12	—	70	—	—	365
							Entner-Doudoroff system
							24
							28
							—
							—
							—

Table 11. Rate of utilization of glucose analogues by glucose-grown *P. aeruginosa* 2F32 (WT) and *P. aeruginosa* 2F32 59A and 106B

2-Deoxyglucose, 6-deoxyglucose and galactose were used at a final concentration of 6.25 mM. Glucosamine and xylose were at 30 mM final concentration. The procedure was as described in the Experimental section. n.d., Not detected.

Organism	Rate of utilization ($\mu\text{mol/h per g dry wt.}$)				
	2-Deoxyglucose	6-Deoxyglucose	Galactose	Glucosamine	Xylose
Wild type	5950	9160	515	11 000	4950
106B	180	92	33	n.d.	n.d.
59A	25	83	75	n.d.	n.d.

Fig. 10. Effect of glucose concentration on the uptake of material from glucose by glucose-grown *P. aeruginosa* 2F32 59A (○) and 2F32 106B (Δ)

Initial rates of transport were measured in duplicate from samples taken at 15, 30, 45 and 60 s after glucose addition, by using the procedure described in the Experimental section. For concentrations up to and including 64 μM the bacterial density was 4 $\mu\text{g dry wt./ml}$. Above this concentration 400 $\mu\text{g dry wt./ml}$ was used.

isolated mutants, *P. aeruginosa* 2F32 106B and *P. aeruginosa* 2F32 59A, that are typical non-acid-producers. These mutants are deficient in glucose dehydrogenase although the other major glucose-catabolizing enzymes are present (Table 10).

A comparison of the rates of utilization of presumed substrates for glucose dehydrogenase by whole cells of glucose-grown wild-type and mutant (Table 11) revealed the mutants to have depressed rates of utilization of these compounds. Similarly, cell extracts prepared from the mutants did not oxidize the analogues. These observations with whole cells and extracts clearly show that glucose dehydrogenase is responsible for the oxidation of the analogues in the wild-type organism, and that this activity is absent in the mutants.

The variation of glucose transport, in response to

increasing glucose concentrations, by glucose-grown mutants is shown in Fig. 10. Clearly the low- K_m system is present but the high- K_m system is absent. Further transport studies, summarized in Table 12, demonstrated that the active transport system capable of accumulating methyl α -glucoside and related compounds was present in the glucose-grown mutants but repressed when they were grown on glycerol or gluconate. The high- K_m system was absent from glycerol- or gluconate-grown cells, indicating a requirement for glucose dehydrogenase activity for the high- K_m system to be operative.

Further experiments were concerned with the role of glucose dehydrogenase in glucose uptake, namely its site of substrate access. Comparison of the rates of glucose utilization and of glucose transport by gluconate-grown wild-type cells (glucose uptake via

Table 12. Properties of *P. aeruginosa* mutants defective in glucose dehydrogenase

The organisms were trained to each growth substrate by at least six transfers on the appropriate substrate. The results are compiled from a series of experiments. Glucose was used at concentrations of 5 mM and 10 μ M to measure transport by the low K_m and high K_m transport systems respectively. Transport of methyl α -glucoside and galactose was measured at 2.5 mM and of 2-deoxyglucose at 0.1 mM. n.d., Not detected. n.p., Not performed.

Organism	Growth substrate	Specific activity of glucose dehydrogenase (μ mol/h per mg of protein)	Rate of transport (μ mol/min per g dry wt.)					
			Glucose		Methyl α -glucoside	Galactose	2-Deoxyglucose	
			Low K_m	High K_m				
2F32	Glucose	15	18	15	10.5	15.6	8.9	
	Gluconate	7	n.d.	22	n.d.	n.d.	n.p.	
	Glycerol	11	n.d.	22	n.d.	n.d.	n.p.	
2F32 106B	Glucose	n.d.	41	n.d.	13.5	16.8	15.3	
	Gluconate	0.1	n.d.	n.d.	n.d.	n.p.	n.p.	
	Glycerol	n.d.	n.d.	n.d.	n.d.	n.p.	n.p.	
2F32 59A	Glucose	n.d.	39	n.d.	12.5	14.6	14.1	
	Gluconate	0.5	n.d.	n.d.	n.p.	n.p.	n.p.	
	Glycerol	n.d.	n.d.	n.d.	n.p.	n.p.	n.p.	

the high- K_m system) revealed that the former exceeded the latter. The initial rate of transport was measured in duplicate over the first minute of incubation, and the rate of glucose consumption was determined with an identical incubation mixture from which samples (0.5 ml) were removed over a 40 min period; the initial glucose concentration was 5 mM and the cell density in each case 400 μ g/ml. The glucose concentration was measured by the anthrone method. The rates of 14 C uptake and of glucose utilization measured were respectively 23 and 285 μ mol/min per g dry wt. Similar observations were made with glucosamine, the conversion into glucosamic acid exceeding the rate of uptake of 14 C into gluconate-grown cells (1.2 and 180 μ mol/min per g dry wt. respectively) when measured at 20 mM external concentration.

The observations suggest that glucose dehydrogenase is acting extracellularly, converting glucose into gluconate, and that gluconate is then taken up. This was substantiated in experiments with glycerol- and gluconate-grown cells where apparent glucose transport, measured at 2.5 mM-glucose, was completely inhibited by the addition of gluconate (10 mM) 5 s before glucose. Addition of 2-oxogluconate (10 mM) to such cells produced only partial inhibition in gluconate-grown cells and no inhibition in glycerol-grown cells.

Discussion

Our results suggest that glucose entry into *P. aeruginosa* is mediated by an active transport system of rather broad specificity, since it is also capable of transporting methyl α -glucoside, 2-deoxyglucose, 6-deoxyglucose, xylose, glucosamine and galactose. These findings support the conclusion of previous workers (Romano *et al.*, 1970; Phibbs & Eagon, 1970) that the transport of glucose by this organism does not involve a phosphoenolpyruvate-dependent phosphotransferase system.

Glucose transport is subject to metabolic regulation since we have shown that both the activity and the level of the transport system are controlled. The exact contribution of these phenomena to the inhibition of glucose utilization and inhibition of induction of the glucose-catabolizing enzymes (Ng & Dawes, 1973) is as yet unclear, since mutants having altered transport properties have not yet been isolated. The observation that the methyl α -glucoside-transport system is absent in glycerol- and gluconate-grown cells, whereas the major glucose-catabolizing enzymes are present at activities characteristic of glucose-grown cells, demonstrates that the transport system can be regulated independently of these enzymes and it should thus be possible to isolate mutants constitutive for this transport system. Attempts have been made to induce this transport system under the conditions described in Table 8, except that glucosamine,

xylose, 2-deoxyglucose, 6-deoxyglucose, methyl α -glucoside or galactose were substituted for glucose (E. A. Dawes & M. Midgley, unpublished observations). However, no induction of the transport system or glucose 6-phosphate dehydrogenase by these analogues could be detected, although significant induction was produced by glucose.

The apparent lack of correlation between the presence of glucose- and methyl α -glucoside transport in cells grown on glycerol or gluconate is explicable on the basis that glucose dehydrogenase, present in such cells, acts extracellularly to produce gluconate, which is then transported to the cells. This is confirmed by the complete inhibition of 'glucose transport' by gluconate in such cells, although a more detailed examination of gluconate transport by this organism is warranted.

The large excess of glucose dehydrogenase activity over the rate of accumulation of ^{14}C from glucose is the probable reason for the linearity of the uptake process despite the postulated involvement of at least two steps. Whatever the subsequent components involved in entrance, the observation that glucose disappears from the medium far more rapidly than radioactive material is transported into the cell is incompatible with glucose dehydrogenase being orientated in the membrane so that it catalyses a group translocation. Eagon (1971) has proposed that 2-deoxyglucose entry into *P. aeruginosa* is by passive diffusion. He suggests that it is trapped intracellularly by the action of glucose dehydrogenase. Our results, however, are compatible with 2-deoxyglucose entering glucose-grown cells via the glucose-methyl α -glucoside active transport system, while glucose dehydrogenase acts extracellularly. This discrepancy probably arises from the different wash technique we have employed, since previous investigators have not taken into account the cold-lability of the intracellular pool of their organisms.

Although the concentration of glucose required for glucose dehydrogenase activity is physiologically significant when compared with that employed in growth media, it is difficult to judge the quantitative significance of this enzyme in glucose metabolism since the mutants defective in glucose dehydrogenase activity usually show wild-type growth rates on glucose. However, as we have demonstrated, such isolated organisms also display higher rates of glucose transport via the methyl α -glucoside-glucose-transport system (cf. Figs. 8 and 10) such that similar fluxes of glucose carbon atoms are recorded for both wild type and mutant. Thus the regulatory mechanisms operating have compensated for the deficiency of glucose dehydrogenase.

It seems unlikely that glucose transport is the only control site by which metabolic derivatives produced from organic acids exert their inhibitory effect on the induction of the glucose-metabolizing enzymes, and

the induction of the glucose-transport system, by glucose. For example, when succinate is added to bacteria growing on glycerol, the synthesis of glucose 6-phosphate dehydrogenase is rapidly turned off (E. A. Dawes & M. Midgley, unpublished work). Synthesis of this enzyme in such cells probably does not depend on glucose transport; thus further control sites are indicated, although no information about the level at which this control is exerted is at present available.

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