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D-GLUCONATE TRANSPORT IN *ARTHROBACTER PYRIDINOLIS*

METABOLIC TRAPPING OF A PROTONATED SOLUTE

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Summary

D-Gluconate uptake was studied in whole cells of *Arthrobacter pyridinolis*; the uptake activity was inducible, mutable and showed saturation kinetics (K_m = 5 μM). Uptake of D-gluconate was not mediated by a phospho*enol*-pyruvate : hexose phosphotransferase system, nor was it directly energized by ATP. A transmembrane pH gradient, ΔpH, of —63 mV was generated by *A. pyridinolis* cells at pH 6.5, while at pH 7.5, ΔpH = 0. Addition of 8 μM D-gluconate significantly reduced the ΔpH. The transmembrane electrical potential, $\Delta\psi$, which was —87 mV over a range of pH from 5.5 to 7.5, was unaffected by the presence of substrate. D-Gluconate accumulated at the same rate and as the protonated solute, at both pH 6.5 and 7.5. Experiments in which a diffusion potential was generated in cyanide-treated cells, indicated that the $\Delta\psi$ did not energize transport. Rather, the rate of D-gluconate uptake correlated with and appeared to be determined by the rate of D-gluconate metabolism: (a) treatment of cells with valinomycin or nigericin, under conditions in which there was a loss of intracellular potassium, inhibited both D-gluconate uptake and the metabolism of pre-accumulated D-gluconate; (b) the effects of cyanide and azide on D-gluconate uptake were much more severe at pH 6.5 than pH 7.5, a pattern which paralleled the effects of these inhibitors on D-gluconate metabolism; (c) extraction and chromatography of intracellular label from D-gluconate uptake revealed that accumulation of unaltered D-gluconate was negligible; (d) a series of mutant strains with lower D-gluconate kinase activities also exhibited low rates of D-gluconate uptake; (e) spontaneous

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; $TPMP^+$, triphenylmethylphosphonium; Tris, tris(hydroxymethyl)aminomethane.

revertants of these mutant strains consistently regained both D-gluconate kinase activity and wild type levels of uptake.

Introduction

In preliminary experiments, *Arthrobacter pyridinolis* was found to utilize D-gluconate as sole carbon source for growth. Utilization of D-gluconate by whole cells occurred in mutants lacking the phospho*enol*pyruvate : hexose phosphotransferase system, and did not depend upon exogenous L-malate. These observations were surprising in view of previous work on *A. pyridinolis*. The only other hexoses used as sole carbon source, D-fructose and L-rhamnose, were phosphotransferase system substrates [1—3]. A respiration-coupled transport system was also operative for these two substrates [3,4], and was the sole transport mechanism for a variety of other solutes such as D-glucose [5] and several amino acids [6]. Growth on non-phosphotransferase substrates was always dependent upon the presence of L-malate or a direct precursor thereof in the medium [3,5,6]. The L-malate was oxidized concomitantly with solute transport. The requirement for exogenous L-malate reflected an inability of *A. pyridinolis* to generate sufficient dicarboxylic acids from metabolism of hexoses or certain amino acids; this inability was in turn due to a natural paucity in anaplerotic enzymes [7]. Thus the finding of a non-phosphotransferase substrate, D-gluconate, which could serve as sole carbon source in the absence of L-malate was surprising. The studies reported here were undertaken to elucidate how D-gluconate transport in *A. pyridinolis* might differ from the respiration-coupled active transport systems studied previously. The results suggest that transport of D-gluconate in this organism depends upon its subsequent metabolism, and hence is not a true active transport system.

Methods

Bacterial growth conditions and mutagenesis procedure. A. pyridinolis was grown with shaking at 30° C in the mineral salts medium, previously described [7], with carbon sources added from separate sterile solutions to a final concentration of 0.05 M. *A. pyridinolis* was mutagenized as described elsewhere [8]. After penicillin enrichment and replica plating, mutant strains were isolated which could utilize D-fructose but not D-gluconate for growth.

Chemicals. D-[U-^{14}C]Gluconate (sodium salt, 3.9 Ci/mol), was obtained from Amersham Searle Corp. [1-^{14}C]Butyric acid (20 Ci/mol) and [^{3}H]triphenylmethylphosphonium ($TPMP^+$) (18.4 Ci/mol) were purchased from New England Nuclear Corp. ATP, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), D-gluconic acid lactone, *N,N'*-dicyclohexylcarbodiimide (DCCD), luciferin-firefly tails, NADP, 6-phosphogluconate, 6-phosphogluconate dehydrogenase, sodium azide, and valinomycin were purchased from Sigma Chemical Co. $TPMP^+$ was obtained from ICN-K and K Laboratories, Inc. Nigericin was the generous gift of Dr. H.R. Kaback. All other reagents were purchased from commercial suppliers at the highest purity available.

Chromatography. Cells, suspended to 0.05 mg protein/ml in 50 mM Tris,

pH 6.5 or 7.5, with 40 μg chloramphenicol/ml, were incubated for 0.5–3.0 min with 4 mM D-[U-^{14}C]gluconate (25 Ci/l) added to a final concentration of 8 μM, filtered through Millipore HA filters (0.45 μm), and washed free of the radioactive medium with the appropriate buffer. Intracellular label was extracted with 80° C water as described by Guymon and Eagon [9]. Descending chromatography was performed using Whatman No. 1 paper with ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3) [9]. Two-dimensional descending paper chromatography was performed as described by de Torrontegui et al. [10] and ascending chromatography was done as described by Robin and Kepes [11]. To account for D-gluconate nonspecifically bound to the filters and extracted in the above procedure, controls were included for all experiments using heat-killed cells of *A. pyridinolis*. These cells neither accumulated nor metabolized D-gluconate.

Determination of CO_2 evolution. CO_2 evolution from metabolism of D-[U-^{14}C]gluconate by whole cells of *A. pyridinolis* was measured as described by Cederbaum et al. [12]. Cells were harvested by centrifugation (13 000 × *g* for 10 min), washed twice in 0.05 M Tris, pH 6.5 or 7.5, and resuspended in the same buffer to 0.05 mg protein/ml. 4 mM D-[U-^{14}C]gluconate (25 Ci/l) was added to a final concentration of 8 μM. Solute uptake was allowed to proceed for 5 min; accumulation of label at either pH corresponded to 103 nmol/mg protein. Cells were then separated from the radioactive medium by centrifugation, resuspended to the initial volume, and CO_2 trapping was initiated.

Determination of ΔpH and $\Delta\psi$. $\Delta\psi$ was measured as a function of the accumulation of 25 μM [^{3}H]TPMP$^+$ using a filtration assay [13]. Conditions for individual experiments are given in the figure legends. Determination of ΔpH across the membrane of whole cells was determined from the distribution of 62.5 μM [1-^{14}C]butyric acid assayed by a flow dialysis procedure devised by Ramos et al. [14], employed for whole cells as previously described [15]. A control, to obtain values for nonspecific binding of butyric acid, conducted for each experiment using either heat-killed cells or cells treated with 40 μM CCCP, gave identical results. The internal pH was calculated by the method of Waddell and Butler [16]; ΔpH was the difference between internal and external pH. Data from a typical flow dialysis experiment are shown in Fig. 1. Butyrate uptake is not carrier mediated nor is it metabolized by *A. pyridinolis*, so that the slopes of the two curves are the same. Its uptake is stoichiometric with protons and responds to the ΔpH (data not shown). Use of metabolizable acids, e.g. acetic acid, yielded patterns in which the curve for the live cells has a much steeper slope than that for the control cells, after the initial equilibration.

Enzyme assays. Enzyme assays were carried out using dialyzed extracts prepared from *A. pyridinolis* cells as described previously [8]. Both 6-phosphogluconate dehydrogenase (EC 1.1.1.44), and D-gluconate kinase (EC 2.7.1.12), were assayed as described by Eisenberg and Dobrogosz [17].

Other assays. Cellular potassium concentrations were determined as a function of the extracellular concentration by the procedure of Harold et al. [18], except that cells were harvested by centrifugation rather than by filtration. Potassium was measured with a Beckman KLiNa Flame spectrophotometer. The intracellular water volume for cells of *A. pyridinolis* was determined to be

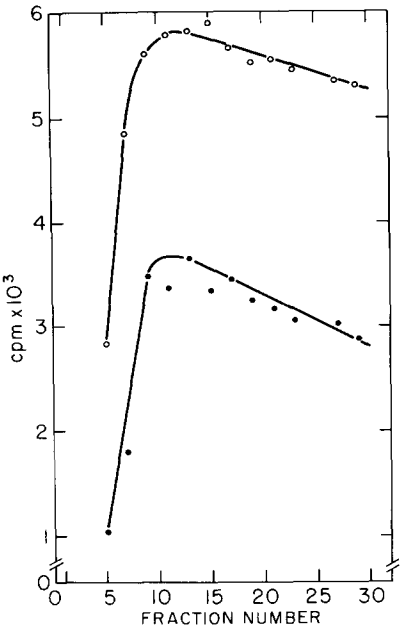


Fig. 1. Butyrate accumulation by whole cells of *A. pyridinolis* as assayed by the flow dialysis procedure. Cells grown on 50 mM D-gluconate were washed twice and resuspended in 25 mM potassium phosphate (pH 6.5) to approximately 5 mg protein/ml. The assay was performed as described in Methods. ○, radioacitivity recovered in the dialysate fractions from heat-killed cells, and ●, from live cells. 5 mM [1-^{14}C]-butyric acid (100 μCi/ml) was added to 0.8 ml of cell suspension, giving a final concentration of 62.5 μM.

7.5 μl/mg protein using the method described by Stock et al. [19]. For this and all other assays protein concentrations were determined by the method of Lowry et al. [20], using egg white lysozyme as a standard. ATP was extracted from cells as described by Cole et al. [21], and intracellular ATP concentrations were then determined using a firefly assay in a Beckman LS-230 spectrometer with the coincidence turned off [22]. The assay was linear over a range of $1 \cdot 10^{-9}$—$1 \cdot 10^{-6}$ M ATP. Uptake of D-[U-^{14}C]gluconate by whole cells was assayed by a membrane filtration method as described elsewhere [1], using cell suspensions of 0.05 mg protein/ml. Electrogenic potassium-diffusion potentials were generated as described by Kashket and Wilson [23—25], using cells suspended in potassium-free buffers. Proton movements associated with D-gluconate uptake by aerobic suspensions of whole cells were recorded using a Beckman (model 39012) combination electrode connected to a Beckman Expandomatic pH meter and strip chart recorder. Conditions for individual traces are described in the figure legends. All assays were performed at 25° C.

Results

A. pyridinolis grew on D-gluconate with a doubling time of 135 min, over a pH range from 5.5 to 7.5. There was no growth above pH 7.5. D-Gluconate uptake was inducible and exhibited saturation kinetics, with an apparent K_m of 5 μM (Fig. 2). Uptake of D-gluconate by whole cells was linear with time over a

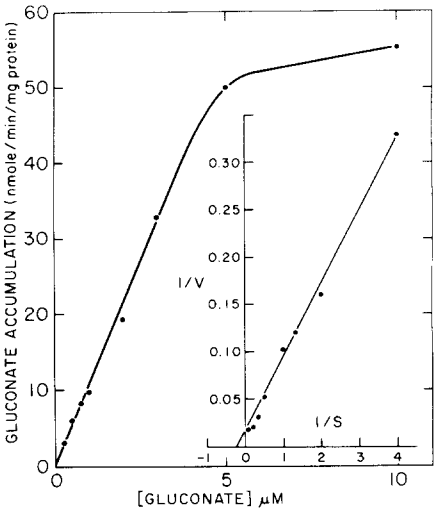
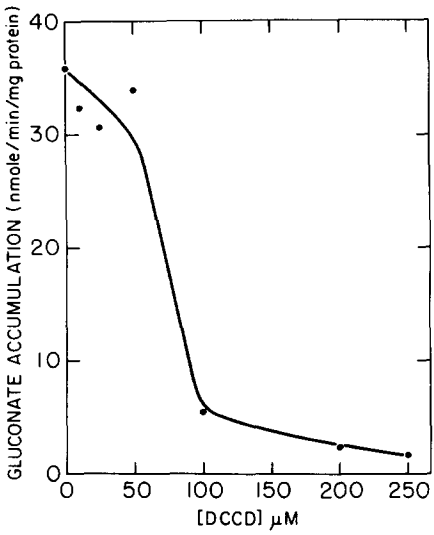


Fig. 2. Accumulation of D-gluconate by whole cells of *A. pyridinolis* as a function of D-gluconate concentration. D-Gluconate-grown cells of *A. pyridinolis* were washed and resuspended in mineral salts medium, and incubated with 40 μg chloramphenicol/ml for 10 min. D-[U-^{14}C]Gluconate was added to the concentrations shown from either 0.25 mM (4 μCi/ml) or 4.0 mM (25 μCi/ml) solutions, and accumulation was stopped by filtration and washing after 1 min of incubation.

period of 2—3 min. Since D-gluconate was a metabolizable substrate, 30-s or 1 min time points were used whenever possible. The initial rate of D-gluconate uptake was invariant over a pH range of 5.5—7.5. The apparent K_m remained 5 μM. Addition of 20 mM L-malate did not affect the initial rate of D-gluconate uptake by *A. pyridinolis* cells suspended in mineral salts medium over this pH range. D-Gluconate, at a concentration of 8 μM, was taken up at an initial rate of 58.1 nmol/min per mg protein in the absence of exogenous L-malate, and at a rate of 55.9 nmol/min per mg protein in its presence. A strain of *A. pyridinolis*, AP 243, which lacks enzyme I of the phospho*enol*pyruvate : hexose phosphotransferase system [26], and hence unable to utilize any phosphotransferase substrate, grew on D-gluconate at the same rate as did the wild type organism. Thus, as expected from preliminary experiments, D-gluconate was neither a phosphotransferase substrate, nor was its uptake stimulated by L-malate; it was accumulated by an inducible, saturable transport process.

D-Gluconate uptake was inhibited by DCCD, an adenosine triphosphatase inhibitor [27], only at concentrations of DCCD above 50 μM (Fig. 3). As illustrated in Fig. 4, 50 μM DCCD, a concentration which did not affect D-gluconate uptake, prevented D-gluconate uptake (data not shown). CCCP, which inhibited D-gluconate-dependent ATP synthesis. Similarly, arsenate inhibited ATP synthesis from D-gluconate metabolism (Fig. 4) without inhibiting the initial rate of D-gluconate-dependent ATP synthesis to about the same extent as the other inhibitors, did inhibit D-gluconate uptake by more than 90%. Thus there was no correlation between generation of cellular ATP and the rate of D-gluconate uptake, and the transport process did not appear to be energized directly by ATP.

A variety of bacterial transport systems which are not energized directly by the phosphate bond energy of phospho*enol*pyruvate or ATP are believed to be



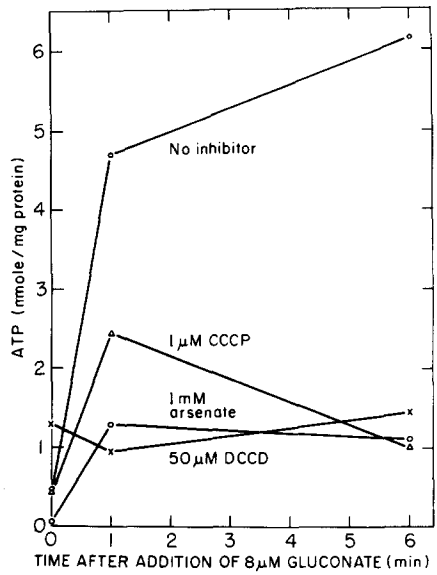


Fig. 3. Effect of DCCD on D-gluconate uptake by whole cells of *A. pyridinolis*. Cells were prepared as in the legend to Fig. 2, and DCCD was added to the indicated concentrations. After 5 min, D-[U-^{14}C]-gluconate was added to 8 μM final concentration from a 4 mM (25 μCi/ml) solution. DCCD was in an ethanol solution giving a final concentration of 0.1% (v : v) of ethanol in the reaction flask. This concentration of ethanol had no effect on D-gluconate accumulation.

Fig. 4. The effect of DCCD, arsenate and CCCP on ATP synthesis from D-gluconate metabolism. D-Gluconate-grown cells of *A. pyridinolis* were washed twice with and resuspended in 25 mM potassium phosphate, pH 6.5. Aliquots were preincubated for 10 min with chloramphenicol, and for 5 min with the indicated inhibitor. D-Gluconate was added to a final concentration of 8 μM. Intracellular ATP concentrations were determined, as described in Methods, at 0, 1 and 6 min after addition of D-gluconate.

energized by the protonmotive force. According to Mitchell's chemiosmotic hypothesis [28—30], the oxidation of an electron donor by the respiratory chain, or ATP hydrolysis, results in the extrusion of protons from the cell. This generates a protonmotive force ($\Delta\mu_{H^+}$), consisting of a transmembrane electrical potential ($\Delta\psi$), and a transmembrane proton gradient (ΔpH), interior both negative and alkaline. We examined the protonmotive force generated by D-gluconate-grown cells of *A. pyridinolis* to facilitate a subsequent evaluation of the role of this force in the transport process. The ΔpH and $\Delta\psi$ were measured for cells suspended in 25 mM potassium phosphate buffer over an external pH range of 5.5—7.5 (Fig. 5). ΔpH, as determined by butyric acid distribution, decreased from —77 mV at pH 5.5 to 0 mV at pH 7.5; addition of 8 μM D-gluconate caused an appreciable decrease in the magnitude of the ΔpH measured over the same range of pH. At pH 5.5 addition of D-gluconate decreased the magnitude of ΔpH from —77 mV to —49 mV, and at pH 6.5 it was diminished from —63 mV to —15 mV when D-gluconate was present. With D-gluconate present there was no measurable ΔpH at pH values above pH 7.0. $\Delta\psi$, determined by $TPMP^+$ accumulation, remained essentially constant from pH 5.5 to pH 7.5 at —87 mV and was unaffected by addition of substrate. The total protonmotive force, $\Delta\mu_{H^+}$, therefore paralleled the ΔpH curve.

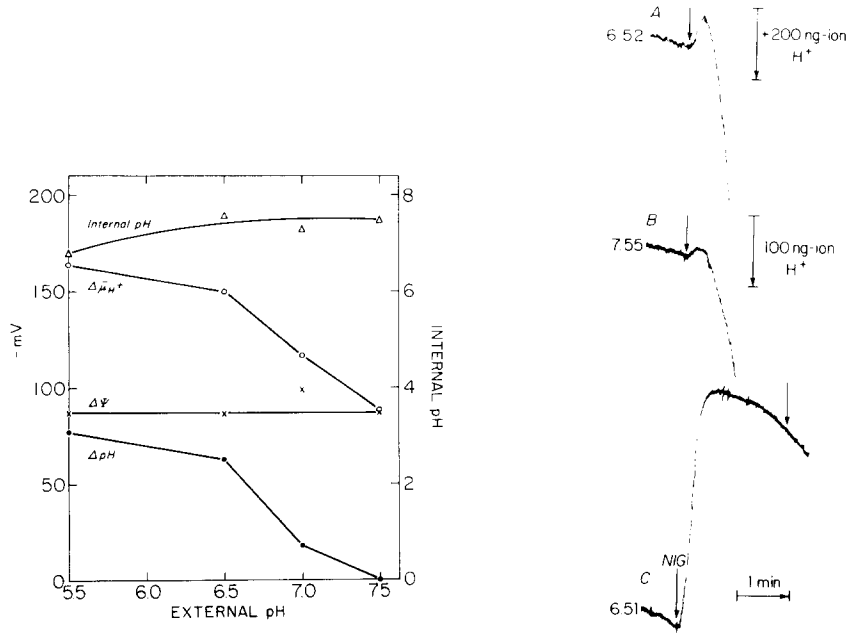


Fig. 5. Magnitude of ΔpH, $\Delta\psi$, and $\Delta\mu_{H^+}$ of whole cells of *A. pyridinolis*. Washed, D-gluconate-grown cells were resuspended in 25 mM potassium phosphate at the indicated pH. ΔpH, $\Delta\psi$, $\Delta\mu_{H^+}$ and internal pH were determined as described in Methods and the legend to Fig. 1. For $\Delta\psi$ determinations 25 μM [^{3}H]TPMP$^+$ was added from a 5.0 mM (100 μCi/ml) solution.

Fig. 6. D-Gluconate-dependent proton uptake by *A. pyridinolis*. D-Gluconate-grown cells were washed twice with 50 mM Tris (pH 6.5) or 50 mM 2-amino-3-methyl-1,3-propandiol, pH 7.5, and once with 3 mM Tris (pH 6.5) or 2-amino-3-methyl-1,3-propandiol, pH 7.5. Cells were resuspended to 0.25 mg protein/ml in the 3-mM buffers containing 100 mM KCl to a final volume of 20 ml, and incubated for 30 min at 30°C. The initial pH was adjusted to that shown on the left side of the trace. D-Gluconate was added to a final concentration of 10 μM at the unmarked arrows. The recorder was set for a full scale deflection of 0.2 pH units. Plot A, pH 6.5; plot B, pH 7.5; plot C, pH 6.5 with 0.1 μM nigericin added as shown (NIG); the scale of plot C is the same as plot A.

At both pH 6.5 where a ΔpH existed, and at pH 7.5 where there was no proton gradient, addition of D-gluconate to cells in lightly buffered suspensions caused proton uptake, alkalinizing the medium (Fig. 6, plots A and B). The secondary acidification of the medium which immediately followed the alkalinization was presumably the result of catabolism of internalized D-gluconate. From the data in Fig. 6, an initial rate of proton uptake of 77 natoms/min per mg protein was calculated; since under these conditions the initial rate of D-gluconate uptake was 54 nmol/min per mg protein, an approximate H^+/D-gluconate ratio of 1.4 was obtained. This ratio may be somewhat overestimated due to aerobic conditions during the pH tracing. These results, along with the diminution of the ΔpH by addition of D-gluconate, indicated that proton uptake occurred concomitantly with D-gluconate uptake, suggestive that D-gluconate was taken up as the neutral (protonated) solute.

Experiments were then undertaken to further document the role of protons in D-gluconate uptake. Nigericin, which allows an electroneutral exchange of

K^+ for H^+ across the membrane [30—32] was utilized. To interpret any effects of nigericin upon transport it was first necessary to know the magnitude and directionality of the potassium gradients maintained across the cell membrane under various conditions. Under normal growth conditions, *A. pyridinolis* accumulated potassium against a concentration gradient (data not shown). However, at an external K^+ concentration of 0.2 M internal and external potassium concentrations were equal; therefore, under these conditions, addition of nigericin should abolish an existing proton gradient. Indeed, when cells were suspended in 0.2 M Tris (pH 6.5) containing 0.2 M KCl, addition of nigericin inhibited D-gluconate uptake by more than 75% (Fig. 7A). Also, addition of nigericin to lightly buffered cell suspensions at pH 6.5 caused immediate and dramatic alkalinization of the medium (Fig. 6C), and subsequent addition of D-gluconate did not cause further proton uptake. By constrast, nigericin failed to inhibit uptake of D-gluconate by cells suspended in 0.2 M Tris (pH 7.5) with 0.2 M KCl present. Since neither ΔpH nor a potassium ion gradient exists at pH 7.5 under these conditions, nigericin should indeed cause no inhibition. Cells were then resuspended in a potassium-free buffer, thus imposing a potassium gradient, $K^+_{in} > K^+_{out}$ so that nigericin would cause proton influx, coupled to potassium efflux along this gradient, even at alkaline pH. Inhibition of D-gluconate transport by nigericin was observed at both pH 6.5 and 7.5 in the absence of external potassium (Fig. 7B). Thus, even at pH 7.5, extracellular protons (and possibly also intracellular potassium) were apparently required for D-gluconate uptake.

However, even if proton movements necessarily occurred with D-gluconate uptake, it seemed unlikely that a ΔpH of only —15 mV to —63 mV at pH 6.5 could be sufficient to energize the observed levels of accumulation if the solute were transported unchanged. Moreover, the rate of D-gluconate transport at pH 7.5, where ΔpH = 0, was as great as that at pH 6.5. Therefore, the possible role of the $\Delta\psi$, the slightly larger component of energization at pH 6.5, and the

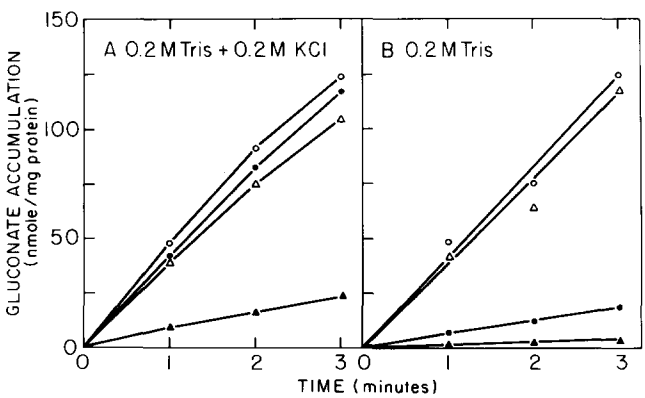


Fig. 7. Effects of nigericin on D-gluconate uptake in D-gluconate-grown cells of *A. pyridinolis*. Cells were washed twice and resuspended in: (A) 0.2 M Tris + 0.2 M KCl, or (B) 0.2 M Tris buffer, at pH 6.5 (△,▲) or pH 7.5 (○,●). Aliquots were preincubated for 10 min with chloramphenicol. Nigericin was added to 0.1 μM (●,▲), or an equivalent amount of ethanol was added (○,△). After 5 min, 4 mM D-[U-^{14}C]-gluconate was added to a final concentration of 8 μM, and uptake was determined as in the legend to Fig. 2.

sole component at pH 7.5, was investigated. Addition of valinomycin to cells of *A. pyridinolis*, in potassium-free buffer would cause an electrogenic efflux of K^+ from the cells generated a $\Delta\psi$. *A. pyridinolis* cells, suspended in 50 mM Tris, pH 6.5, were preincubated with 10 mM NaCN for 30 min to inhibit respiration, deplete endogenous energy reserves and thus lower the initial $\Delta\psi$. As shown in Fig. 8, addition of 2 μM valinomycin caused a marked stimulation of $TPMP^+$ uptake, presumably reflecting the diffusion potential resulting from potassium efflux. The increase in $\Delta\psi$, however, did not cause stimulation of D-gluconate uptake, but rather, caused a small inhibition of uptake beyond the level of inhibition already caused by the cyanide treatment. Similar results were observed at pH 7.5; generation of a diffusion potential stimulated $TPMP^+$ uptake, while inhibiting uptake of D-gluconate beyond inhibition already caused by the cyanide incubation (data not shown). Thus D-gluconate uptake did not appear to be directly energized by the membrane potential at either pH 6.5 or 7.5.

We had noted in the foregoing experiments that D-gluconate transport at pH 6.5 was much more sensitive to cyanide than at pH 7.5. This observation was reproducible and extended to inhibition by azide (Fig. 9). The latter respiratory inhibitor had very little effect on D-gluconate uptake at pH 7.5, but inhibited markedly at pH 6.5. In the absence of energization by ATP, phospho*enol*pyruvate, the $\Delta\psi$, or an appreciable ΔpH, it seemed possible that D-gluconate metabolism could be a critical determinant in the rate of D-gluconate uptake. It was further possible that metabolism of D-gluconate at pH 7.5 was more fermentative than at pH 6.5. Extraction and chromatographic analysis of intracellular label accumulated from radioactive D-gluconate, as described in Methods was consistent with a rapid and rate-determining metabolism of internalized D-gluconate. Upon correction for nonspecific binding of D-[U-^{14}C]-

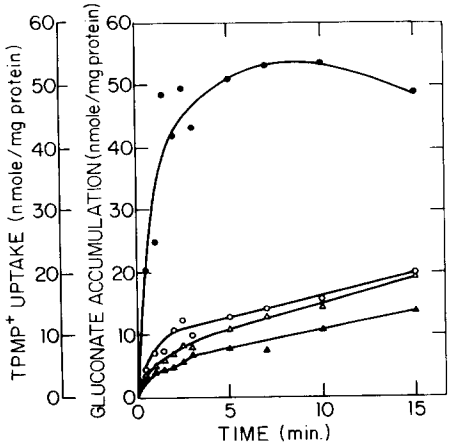


Fig. 8. Effect of a valinomycin-induced diffusion potential on D-gluconate accumulation by *A. pyridinolis* (pH 6.5). Cells were washed twice with and resuspended in 50 mM Tris, pH 6.5. They were then preincubated with 40 μg chloramphenicol/ml and 10 mM NaCN for 10 min. Aliquots were incubated with 25 μM [^{3}H]$TPMP^+$ (○,●) or 8 μM D-[^{14}C]gluconate (△,▲), in the presence (closed symbols) or absence (open symbols) of 2 μM valinomycin. $TPMP^+$ and D-gluconate uptake were stopped by filtration and washing. [^{3}H]$TPMP^+$ and D-[^{14}C]gluconate were added from 5 mM (100 μCi/ml) and 4 mM (25 μCi/ml) solutions, respectively.

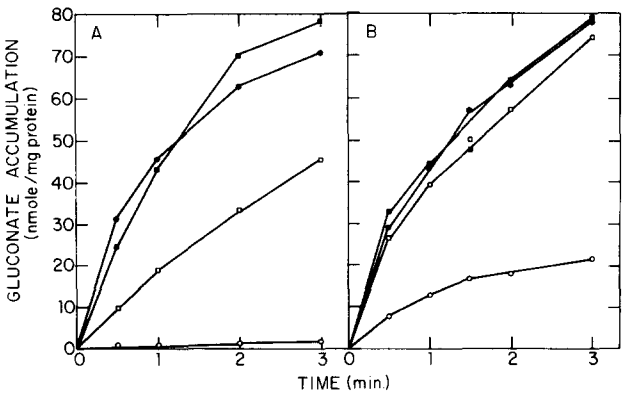


Fig. 9. Effect of respiratory inhibitors on D-gluconate accumulation. Cells were washed with and resuspended in 50 mM Tris buffer at pH 6.5 (○,●), or pH 7.5 (□,■). Aliquots of the cell suspension were incubated in the presence (open symbols) or absence (closed symbols) of 10 mM NaCN (A), or 10 mM NaN_3 (B) and 8 μM D-[U-^{14}C]gluconate; accumulation was assayed as described in the legend to Fig. 2.

gluconate, less than 1% of the intracellular label was found to comigrate with unmodified D-gluconate. Thus, the uptake of D-gluconate did not appear to represent true active transport with concentrative uptake of unmodified solute. It was therefore not surprising that we were repeatedly unable to prepare isolated membrane vesicles that exhibited D-gluconate uptake in the presence of artificial electron donors but in the absence of cytoplasmic enzymes. For a further documentation of the role of metabolism in determining the rate of D-gluconate uptake, the metabolism of accumulated D-gluconate was measured by monitoring evolution of $^{14}CO_2$ from cells which had been preloaded with D-[U-^{14}C]gluconate. Cells accumulated the same amount of D-gluconate during a 5 min incubation at either pH 6.5 or 7.5. As shown in Table I, CO_2 evolution was higher at pH 7.5, and less sensitive to the respiratory inhibitors azide and cyanide at the more alkaline pH, thus paralleling the effects observed upon uptake. Valinomycin inhibited CO_2 evolution at both pH 6.5 and 7.5.

TABLE I

THE EFFECTS OF VARIOUS INHIBITORS ON $^{14}CO_2$ EVOLUTION FROM CELLS PRE-LOADED WITH D-[^{14}C]GLUCONATE

The assay was performed as described in Methods. Accumulation at both pH values was 103 nmol/mg protein, which correspond to approximately 54 000 cpm. n.d., not determined.

Concentration of inhibitor added	$^{14}CO_2$ evolution from D-gluconate (cpm)			
	5 min		10 min	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5
None	8 166	12 206	10 212	15 755
Valinomycin, 2 μM	3 287	4 215	5 059	n.d.
Nigericin, 0.2 μM	3 898	7 391	4 202	7 361
Sodium azide, 10 mM	n.d.	n.d.	7 351	20 052
Sodium cyanide, 10 mM	n.d.	n.d.	3 469	16 217

The effects were similar to those of valinomycin on D-gluconate uptake, and suggested that those effects could be related to influences of the loss of intracellular potassium on D-gluconate metabolism. Under these same conditions nigericin also severely inhibited both uptake and metabolism of D-gluconate. Thus it appeared that a loss of intracellular potassium inhibited both D-gluconate uptake and the metabolism of internalized D-gluconate in concert.

The relationship between D-gluconate metabolism and the rate of D-gluconate uptake was also illustrated in studies of mutant strains of *A. pyridinolis* which could not grow on D-gluconate. The wild type and six such mutant strains were grown on D-fructose to the midlogarithmic phase, and were then induced for D-gluconate catabolism by the addition of 5 mM D-gluconate and incubation for an additional 2 h. Cells were assayed for their ability to take up D-gluconate, and extracts were prepared for assays of D-gluconate kinase and 6-phosphogluconate dehydrogenase activities (Table II). One strain, KM3, had wild type levels of both enzymes and significantly lower activity of D-gluconate uptake, this strain could be a mutant with a defective carrier protein. All the other strains had lower D-gluconate kinase activity and lower ability to transport D-gluconate. As with wild type cells addition of 20 mM L-malate did not stimulate the initial rate of uptake in these mutant strains (data not shown). It appeared that a functional D-gluconate kinase, the first enzyme of the metabolic pathway, was essential for D-gluconate uptake. Spontaneous revertants of strains KM1 and KM6, were selected for their ability to utilize D-gluconate as a carbon source (reversion to wild type phenotype). Of nine revertant strains isolated from KM1, and six from KM6, all but one (KM104) regained wild type levels of both D-gluconate kinase and transport activity (Table III). KM104, a partial revertant for both activities, grew much more slowly than the other revertant strains on D-gluconate. Thus, in studies of strains which could not utilize D-gluconate and of revertants of these strains, there was a correlation between the level of D-gluconate kinase activity and the rate of D-gluconate uptake.

TABLE II

D-GLUCONATE ACCUMULATION, D-GLUCONATE KINASE ACTIVITY, AND 6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY IN WILD TYPE AND MUTANT STRAINS OF *A. PYRIDINOLIS*

Cells were grown to midlogarithmic phase in mineral salts medium with 50 mM D-fructose as carbon source and then induced with 5 mM D-gluconate for 2 h. All assays are described in Methods. The initial rate of D-gluconate uptake by uninduced cells was 1.1 nmol/min per mg protein for all strains, wild type or mutant. The initial rate of D-gluconate accumulation was assayed as described in the legend to Fig. 1 except that cells were suspended in 50 mM Tris (pH 6.5) and were allowed to accumulate D-[^{14}C]-gluconate for 30 s. WT, wild type strain.

Strain	Initial rate of D-gluconate accumulation (nmol/min per mg protein)	D-Gluconate kinase activity (μmol/min per mg protein)	6-Phosphogluconate dehydrogenase activity (μmol/min per mg protein)
WT	24.38	23.0	4.64
KM1	8.16	3.8	3.03
KM2	8.08	5.2	3.65
KM3	5.60	23.9	4.37
KM4	6.49	5.4	2.89
KM5	6.25	8.8	7.35
KM6	6.55	5.8	5.34

TABLE III

D-GLUCONATE ACCUMULATION AND D-GLUCONATE KINASE ACTIVITY OF SPONTANEOUS REVERTANTS OF *A. pyridinolis* STRAINS KM1 AND KM6

Revertants were isolated and all assays performed as described in Methods. Cells were grown on D-gluconate as carbon source. Initial rate of D-gluconate accumulation was determined as described in Table II. WT, wild type strain.

Strain	Initial rate of D-gluconate accumulation (nmol/min per mg protein)	D-Gluconate kinase activity (μmol/min per mg protein)
WT	47.3	33.0
KM101	37.8	35.4
KM102	38.3	35.3
KM103	50.0	30.3
KM104	12.6	16.1
KM105	46.3	29.8
KM106	60.5	29.4
KM107	51.1	36.4
KM108	51.2	30.0
KM109	43.4	28.8
KM601	64.2	28.0
KM602	50.9	27.9
KM603	53.1	30.5
KM604	56.8	30.2
KM605	49.1	32.3
KM606	47.3	54.4

Discussion

D-Gluconate uptake in *A. pyridinolis* was a saturable and inducible process as it is in *Escherichia coli* [11] and *Bacillus subtilis* [33]. The uptake activity was mutable, with strain KM3 having a low activity of D-gluconate uptake while possessing wild type levels of D-gluconate kinase and 6-phosphogluconate dehydrogenase. Therefore, the D-gluconate transport system in *A. pyridinolis* was clearly carrier mediated. Neither a phosphotransferase system nor a direct involvement of ATP was found. Experiments in which a diffusion potential was generated in cyanide-treated cells indicated that D-gluconate uptake was not energized by a $\Delta\psi$ or a ΔpH at pH 7.5. Transport could be only modestly energized by the small ΔpH of -15 mV, in the presence of 8 μM D-gluconate, existing at pH 6.5, if D-gluconate was transported as unaltered substrate.

Several lines of evidence indicated that in *A. pyridinolis*, at both pH 6.5 and 7.5, the rate of D-gluconate uptake was largely determined by the rate of substrate metabolism. First, the patterns of inhibition of D-gluconate uptake by valinomycin and nigericin, under conditions in which potassium was caused to efflux from the cells, paralleled their effects on the metabolism of internalized D-gluconate. Loss of intracellular potassium has been shown, in other cell types, to inhibit pyruvate kinase activity (EC 2.7.1.40) [34]. Second, respiratory inhibitors prevented both the uptake and metabolism of D-gluconate much more severely at pH 6.5 than at pH 7.5. A much more rapid and perhaps fermentative metabolism of D-gluconate appeared to occur at the more alkaline

pH. Third, chromatographic analysis revealed that virtually none of the intracellular label accumulated from the initial uptake of D-[U-^{14}C]gluconate was unaltered substrate. Fourth, mutant strains selected for their inability to grow on D-gluconate had both lower D-gluconate kinase activity and decreased ability to transport D-gluconate, which as in the wild type, was not stimulated by addition of L-malate. Spontaneous revertants to a gluconate$^+$ phenotype coordinately regained both the ability to transport D-gluconate and D-gluconate kinase activity. This indicated that the original mutations occurred at one locus and were not separate mutations in the genes for the carrier and D-gluconate kinase.

Apparently, the D-gluconate transport system of *A. pyridinolis* is independent of L-malate because it is only slightly, if at all, respiration coupled (at acid pH only), and is more importantly coupled to D-gluconate metabolism. By contrast, D-gluconate uptake by *E. coli*, which is a proton symport system [35], was shown, in membrane vesicles, to be energized totally by a ΔpH at pH 5.5, and by a $\Delta\psi$ at pH 7.5 [36,37]. A relationship between transport and catabolism, similar to that reported here, has been described as a facilitated diffusion system for glycerol accumulation in *E. coli* by Lin and his colleagues [38—41]. Both the 'facilitator' and glycerol kinase activities were inducible in *E. coli*, and their apparent K_m values were approximately the same [38,41]. A mutation in the kinase prevented both the utilization and permeation of glycerol [41].

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