Biochem. J. (1974) **140**, 193–203
Printed in Great Britain

Utilization of Gluconate by *Escherichia coli*

UPTAKE OF D-GLUCONATE BY A MUTANT IMPAIRED IN GLUCONATE KINASE ACTIVITY AND BY MEMBRANE VESICLES DERIVED THEREFROM*

By J. M. POUYSSÉGUR,† PELIN FAIK and H. L. KORNBERG
Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

(*Received* 15 *November* 1973)

1. From *Escherichia coli* strain K2.1.5°.8.9, which is devoid of 6-phosphogluconate dehydrogenase (*gnd*) and 6-phosphogluconate dehydratase (*edd*) activities, a mutant R6 was isolated that was tolerant to gluconate though still *edd*$^-$, *gnd*$^-$. 2. Measurements of the fate of labelled gluconate, of the conversion of gluconate into 6-phosphogluconate, and of the induction of gluconate kinase by the two organisms show that, although both inducibly form a gluconate-transport system, strain R6 is impaired in its ability to convert the gluconate thus taken up into 6-phosphogluconate; it was therefore used for study of the kinetics and energetics of gluconate uptake. 3. Suspensions of strain R6 induced for gluconate uptake took up this substrate via a 'high affinity' transport process, with K_m about 10μM and $V_{max.}$ about 25nmol/min per mg dry mass; a 'low affinity' system demonstrated to occur in certain *E. coli* mutants was not induced under the conditions used in this work. 4. The uptake of gluconate was inhibited by lack of oxygen and by inhibitors of electron transport; such inhibitors also promoted the efflux of gluconate taken up. 5. Membrane vesicles prepared from strain R6 also manifested these properties when incubated with suitable electron donors, at rates similar to those observed with whole cells. 6. The results indicate that the active transport of gluconate into the cells is the rate-limiting step in gluconate utilization by *E. coli*, and that the mechanism of this process can be validly studied with membrane vesicles.

The first step in the utilization of gluconate by *Escherichia coli* is its entry from the medium into the cells. Since mutants devoid of components of the phosphotransferase system (Kundig *et al.*, 1964) grow readily on gluconate, this step is not accompanied by the obligatory phosphoenolpyruvate-dependent phosphorylation of the substrate that accompanies the uptake of some carbohydrates (for review, see Roseman, 1972; Kaback, 1972; Kornberg, 1973). The entry of gluconate, taken up as such by the cells, into metabolic pathways requires the action of a kinase that catalyses its intracellular conversion into 6-phosphogluconate with concomitant formation of ADP from ATP. The uptake process, and the phosphorylation step that follows it, are catalysed by highly specific proteins that are inducibly formed after exposure of *E. coli* to gluconate. Although a mutation in a common regulatory gene may derepress both the enzymes that effect these steps, and also the enzyme catalysing the subsequent dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (3-deoxy-6-phospho-L-*glycero*-2-hexulosonate) (de Zwaig *et al.*, 1973; Zwaig *et al.*, 1973), this latter enzyme can be induced without inducing the uptake system or the kinase (Kornberg & Soutar, 1973).

Studies of the kinetic and other parameters that affect the uptake of carbohydrates by *E. coli* are usually performed by exposing suspensions of cells, suitably induced, to some isotopically labelled substrate and then measuring the appearance of label in the cells, which are removed from their medium by rapid filtration. However, data thus obtained may not measure only the uptake process: what is measured is the sum of the amounts of labelled materials that have been taken up and the progressively increasing amounts of labelled products that have been converted into cell components. Moreover, with increasing times of incubation, the loss of labelled material through its oxidation may also progressively distort the situation.

Two main means have been used to overcome these difficulties. Where a non-catabolizable substrate analogue is available, which can be taken up by induced cells but cannot be converted into cell components, a valid measure of the kinetics of the uptake process can be readily obtained. By using methyl [^{35}S]thiogalactoside as such an analogue of lactose, Rickenberg *et al.* (1956) were the first to use

* This paper is dedicated to Professor O. Hoffmann-Ostenhof on the occasion of his 60th birthday (18 October 1974).

† Present address: Unité de Virologie, Institut National de la Santé et de la Recherche Medicale, 1 Place du Professeur Joseph Renaut, 69-Lyon (8e), France.

this approach; their conspicuous success laid the foundation for much subsequent work. Where a non-catabolizable substrate analogue is not available, it may be possible to isolate a mutant that lacks the second step in substrate utilization. This approach was first used by Horecker *et al.* (1960) in their studies of galactose uptake by an *E. coli* mutant devoid of galactokinase activity. They found that the initial rate at which labelled galactose was taken up by the mutant was identical with that at which it was taken up by wild-type cells, although galactose accumulated only in the former organism. This led Horecker *et al.* (1960) to suggest that permeation is the rate-limiting step in galactose utilization.

The present paper reports the isolation of a mutant, R6, of *E. coli* K12, that is unimpaired in its ability inducibly to take up [^{14}C]gluconate but is greatly impaired in its ability to convert the gluconate thus taken up into 6-phosphogluconate. This mutant is therefore analogous to the galactokinase-less mutant described by Horecker *et al.* (1960). It was used for a study of the kinetic and energetic parameters that affect the uptake of gluconate by *E. coli*. The kinetic parameters measured with the mutant impaired in gluconate kinase activity are not significantly different from those determined with wild-type cells. Because gluconate does not accumulate in wild-type cells, it thus appears that the active transport of gluconate is also the rate-limiting step in gluconate utilization. The active transport of a variety of amino acids and carbohydrates can also be demonstrated with membrane vesicles prepared from appropriately induced cells (Kaback, 1960, 1970, 1972); the rate of gluconate uptake and the energetics of this process were therefore studied also with such membrane vesicles. It is evident that such preparations can take up gluconate, by a process dependent on a flux of electrons and protons, at rates and to the extent observed with whole cells.

Just before submission of this paper, a brief report appeared (Robin & Kepes, 1973) of measurements of gluconate uptake with an *E. coli* mutant deficient in gluconate kinase, derived from the DF1070 mutant of *E. coli* strain K10 (Fraenkel, 1968*a*) Although the *E. coli* K12 mutant R6 described in our present paper was derived in part from the same strain DF1070 as used by Robin & Kepes (1973), there appear to be some differences in the behaviour of the two mutants. Comparison of the two mutants will require further study, and particularly will require their genetic analysis.

Experimental

Organisms used

Strain K2.1.5^c.8.9. of *E. coli* K12 was prepared by crossing the Hfr C strain DF 1070, devoid of 6-phosphogluconate dehydratase (*edd*) and of 6-phosphogluconate dehydrogenase (*gnd*) (Fraenkel, 1968*a*) with the F$^-$ strain K2.1t.5^c (*his*, *arg*, *thr*, *leu*, *pps*, *iclR*, *str*; Kornberg, 1970*a*), selecting recombinants for His$^+$ and screening such recombinants for their inability to grow on gluconate and their lack of the two enzymes of 6-phosphogluconate metabolism. The isolation of the mutant R6, impaired in gluconate kinase activity, is described in the Results section. Cultures of strains K2.1.5^c.8.9 and R6 have been deposited with the *E. coli* Genetics Stock Center, Department of Microbiology, Yale University, New Haven, Conn. 06520, U.S.A.

The conditions used for the aerobic growth of the organisms, the preparation of ultrasonic extracts and the assay of enzymes of gluconate metabolism were as described previously (Pouysségur & Stoeber, 1972; Kornberg & Soutar, 1973). Cells were induced by the addition of 5mM-sodium gluconate to cultures growing on either nutrient broth or minimal media containing salts (Ashworth & Kornberg, 1966) and 20mM-glycerol as carbon source.

Osmotic shock

Cells harvested in the exponential growth phase (0.5–0.65mg dry mass/ml) were shocked by the method of Heppel (1969). After three washings with 30mM-Tris–HCl buffer, pH7.3, the cells were gently agitated for 15min with 80vol. of the same buffer containing 20% (w/v) sucrose and 0.1mM-EDTA, collected by centrifugation, and cold-shocked in 80vol. of ice-cold 0.1mM-$MgCl_2$. After centrifugation, the cells were resuspended in 50mM-potassium phosphate buffer, pH7.0, at a concentration of 2mg dry mass/ml and used within 2h of the cold-shock treatment.

Preparation of membrane vesicles

The strain R6 was grown on nutrient broth in the presence of 10mM-sodium gluconate. Spheroplasts and membrane vesicles were prepared by the lysozyme–EDTA method described by Kaback (1971). Membranes were stored in 0.1M-potassium phosphate buffer, pH6.6, at a concentration of 4–6mg/ml under liquid N_2. They were thawed at room temperature when required.

Gluconate uptake by whole cells and vesicles

Cells were washed with 50mM-potassium phosphate buffer, pH7.0, and were resuspended in this buffer at 0.2mg dry mass/ml; chloramphenicol (50μg/ml) was added to inhibit any synthesis of proteins *de novo*. The suspensions were shaken gently at 25°C. The uptake reaction was started by the addition of sodium [6-^{14}C]gluconate (3.4Ci/mol) to a final

concentration of 0.05mM. At appropriate times, samples (0.5ml) were withdrawn, diluted into 2.5ml of 50mM-potassium phosphate buffer, pH7.0, at room temperature, filtered immediately on HA Millipore filters (0.45μm pore size) and washed again with 2.5ml of the same buffer. The filters were then air-dried, dissolved in 5ml of Bray's (1960) fluid, and the radioactivity was assayed with a Packard model 4000 liquid-scintillation spectrometer.

Vesicles were incubated for 15min at 25°C with 100mM-potassium phosphate buffer, pH6.6, containing 10mM-$MgSO_4$. At the end of this period the appropriate electron donor was added to a final concentration of 20mM and the reaction was started immediately thereafter by the addition of sodium [6-^{14}C]gluconate, to 0.05mM final concentration. Samples (0.1ml) were withdrawn, washed twice with 2.5ml of 0.1M-LiCl on HA Millipore filters, and their radioactivity was assayed as described above.

Assay of gluconate efflux from whole cells

Cells were allowed to accumulate labelled gluconate in a final volume of 1ml, containing 0.5ml of cells (2mg/ml) and 0.5ml of 500μM-sodium [6-^{14}C]gluconate. When the radioactivity of the cells had reached a steady state, the cells were spun down for 1min at 16000*g* in an Eppendorf 3200 Microfuge centrifuge. The pellet was quickly resuspended in 80ml of 50mM-potassium phosphate buffer, pH7.0, which had been warmed to 25°C. Samples (10ml) were withdrawn at measured times and were filtered; the filters were washed with 2.5ml of the phosphate buffer, air-dried, and the radioactivity was assayed.

Chromatographic analysis of cell contents

Cells induced with gluconate were washed twice with 50mM-potassium phosphate buffer, pH7.0, and incubated at 1.4mg dry mass/ml with 0.3mM-sodium [6-^{14}C]gluconate. At various times, 1ml of the suspensions was spun down; the pellet was washed with the phosphate buffer, resuspended in 0.5ml of water and boiled for 5min. After centrifugation, the supernatant solution was concentrated to dryness under vacuum and the residue was resuspended in 0.1ml of water. Samples (10–30μl), together with authentic sodium [^{14}C]gluconate and 6-phosphogluconate, were applied to Whatman no. 1 paper and chromatographed overnight in the butanol–propionic acid–water solvents of Benson *et al.* (1950). The radioactive materials on the chromatogram were located by radioautography with Kodak Blue Brand X-ray film. Phosphate esters were detected by the procedure described by Steinitz (1961).

Induction of gluconate kinase and measurement of 6-phosphogluconate formation

To cultures (500ml) of organisms growing aerobically at 37°C on nutrient broth was added 5mM-sodium gluconate when the cell density had reached 0.3mg dry mass/ml. Samples, of a size sufficient to contain approx. 15mg dry mass of cells, were withdrawn at known times (see Fig. 2) and were centrifuged at 4°C and 20000*g* for 5min. The 6-phosphogluconate content of samples of these supernatant solutions was assayed by incubating them at 25°C with 50μmol of Tris–HCl buffer, pH8.0, 3μmol of $MgCl_2$, 0.25μmol of $NADP^+$ and 25μg of crystalline 6-phosphogluconate dehydrogenase, in a volume of 1ml, until no further increase in extinction at 340nm occurred. A ΔE_{340} of 0.622 was taken to be equivalent to 0.1μmol of 6-phosphogluconate. This measurement represented the 6-phosphogluconate liberated by the cells into the media.

The cells were taken up in 4ml of 10mM-potassium phosphate buffer, pH7.5. A sample (1ml) of this suspension was removed and was placed in boiling water for 5min: the 6-phosphogluconate content of the supernatant obtained from this treatment, which represented that of the cell contents, was assayed as described above. To the remaining 3ml of cell suspensions was added a further 15ml of the phosphate buffer, the cells were collected by centrifugation, and the resultant pellet of washed cells was taken up in 4ml of the phosphate buffer supplemented with $MgCl_2$ to 1mM. These suspensions were cooled in ice and exposed to the output of a MSE 100W sonicator for 45s; the virtually clear suspensions were centrifuged at 4°C and 20000*g* for 5min and the gluconate kinase activities of the supernatant solutions thus obtained were assayed as described (Pouysségur & Stoeber, 1972).

Measurement of 6-phosphogluconate phosphatase activity

The possible phosphatase activity of strain R6 was measured in two ways. In one procedure, crude sonic extracts of induced cells were incubated for 20min at 25°C with 10mM-sodium 6-phosphogluconate in buffers at pH5.5 (acetate), 7.0 (Tris–HCl) and 9.5 (ethanolamine–HCl), with and without 1mM-$MgCl_2$; any phosphate released was measured by the procedure of Fiske & SubbaRow (1925). In the second procedure, a thick suspension (5mg dry mass/ml) of induced strain R6 was rendered permeable by the method of Reeves & Sols (1973) and was incubated at 25°C for 30min with 2mM-sodium 6-phosphogluconate in 50mM-Tris–HCl, pH8.0. Samples were withdrawn at zero time and after 15 and 30min, boiled and their 6-phosphogluconate contents were

measured as described above. A control incubation mixture contained all ingredients but boiled cells.

Measurement of oxygen uptake

Rates of oxygen uptake by cell suspensions were measured with a Clark electrode at 25°C, as described by Estabrook (1967).

Chemicals

Sodium [6-^{14}C]- and [U-^{14}C]-gluconate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Lithium salts of D(–)- and L-lactic acid were obtained from Sigma (London) Chemical Co. Ltd., Kingston on Thames, Surrey, U.K. Crystalline 6-phosphogluconate dehydrogenase (yeast) was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. D-Xylonate and 3-deoxy-3-fluoro-D-gluconate (calcium salts) were generous gifts from Dr. M. McKillen (Dublin) and Professor N. F. Taylor (Windsor, Ont.) respectively. All other chemicals were analytical-grade reagents of commercial origin.

Results and Discussion

Isolation of E. coli mutant R6

The mutant K2.1.5^c.8.9 of *E. coli* K12 (Kornberg, 1970*b*) lacks the ability to form the enzymes that catalyse the oxidation and the dehydration of 6-phosphogluconate, 6-phosphogluconate dehydrogenase (*gnd*, EC 1.1.1.43) and 6-phosphogluconate dehydratase (*edd*, EC 4.2.1.12). A consequence of this double dysfunction (see Scheme 1 of Kornberg & Soutar, 1973) is that the addition of gluconate to cultures of this mutant leads to the intracellular accumulation of 6-phosphogluconate. As has been noted previously with other phosphorylated compounds (Kurahashi & Wahba, 1958; Englesberg & Baron, 1959; Nikaido, 1961; Englesberg *et al.*, 1962; Cozzarelli *et al.*, 1965; Böck & Neidhardt, 1966; Fraenkel, 1968*b*; Kelker *et al.*, 1970; Gay & Rapoport, 1970; Pouysségur & Stoeber, 1971; Kornberg, 1972; Solomon & Lin, 1972; Ferenci & Kornberg, 1973; Faik & Kornberg, 1973), this accumulation exerts a toxic (though not lethal) effect and growth of the mutant is speedily arrested. By the same token, cultures of the mutant strain K2.1.5^c.8.9 spread on agar plates containing minimal medium and a readily utilizable carbon source (such as succinate or glycerol) do not grow if the media also contain gluconate. However, after 2–3 days at 37°C, some 50–100 colonies appeared from about 10^8 cells thus plated. They were picked and tested for their ability to grow on plates containing glycerol plus gluconate, and gluconate alone. Of those that grew on the former but not on the latter medium, one colony (designated R6) was further purified by repeated isolation of single colonies and was used for subsequent work.

The mutant R6 which (apart from its tolerance of gluconate) carries the same genetic markers as does its parent K2.1.5^c.8.9 (F$^-$, *edd*, *gnd*, *pps*, *iclR*, *argH*, *str*), was only slightly retarded by the presence of 5mM-gluconate in its rate of growth on nutrient broth, on 20mM-glycerol, or on 10mM-glucose, fructose or succinate; however, it did not grow on gluconate alone, nor did extracts of cells that had grown in the presence of gluconate contain 6-phosphogluconate dehydrogenase or 6-phosphogluconate dehydratase activities. The acquisition of the ability of strain R6 to tolerate the presence of gluconate in growth media was therefore not due to a reversion that enabled 6-phosphogluconate to be catabolized; it must have involved a change either in the process whereby gluconate enters the cells, or in the intracellular conversion of gluconate into 6-phosphogluconate.

That the latter is the case is indicated by the data of Fig. 1. A washed suspension of a culture of strain R6, grown on nutrient broth in the absence of added gluconate, took up little ^{14}C when incubated with [^{14}C]gluconate (open triangles); in contrast, a suspension of cells that had grown for approximately one generation on nutrient broth supplemented with 5mM-unlabelled gluconate took up [^{14}C]gluconate rapidly (closed circles), at about the same rate and to about the same extent as did a suspension of the parent organism K2.1.5^c.8.9, similarly grown (open circles).

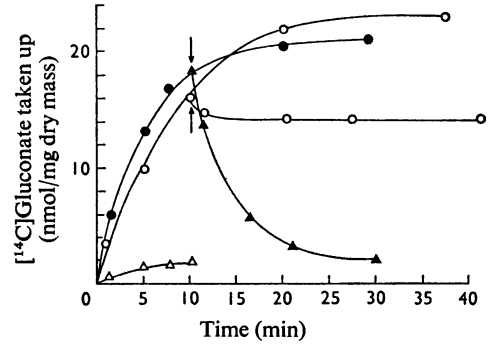

Fig. 1. *Uptake of* [^{14}C]*gluconate by washed suspensions of strains K*2.1.5^c.8.9 *and its mutant R6*

The time-course of uptake of 0.05mM-sodium [^{14}C]gluconate by washed suspensions of nutrient-grown strain R6 (△), and by strain R6 grown on nutrient in the presence of 5mM-gluconate (●), was measured as described in the Experimental section. To a duplicate flask of strain R6 induced for gluconate uptake was added 10mM-sodium [^{12}C]gluconate after 10min (▲). The experiments with induced cells were repeated with strain K2.1.5^c.8.9 (○). Arrows show where [^{12}C]gluconate was added.

This shows that the uptake system(s) for gluconate are inducible, and are present in both types of mutant. However, the fate of the gluconate taken up is abnormal in strain R6: whereas the addition of 5mM-unlabelled sodium gluconate to the suspension of K2.1.5^c.8.9, incubated with 0.05mM-[^{14}C]gluconate for 10min, removed from the cells less than 20% of the labelled material taken up, over 80% of the accumulated ^{14}C was washed out of the R6 cells. The simplest explanation of these findings is that neither type of mutant is impaired in the uptake of [^{14}C]gluconate, but that only strain K2.1.5^c.8.9 converts the gluconate taken up into 6-phosphogluconate. This view is supported by three types of experiment. In the first, cultures of the two mutants were incubated with sodium [^{14}C]gluconate and the material accumulated by the cells was analysed by chromatography. Over 80% of the labelled contents of strain K2.1.5^c.8.9 were found to co-chromatograph with authentic 6-phosphogluconate, whereas over 90% of the radioactivity present in R6 cells remained as gluconate. Clearly, strain R6 did not effect the net conversion into 6-phosphogluconate of gluconate that had entered the cells, which was evident in its parent organism.

This is shown also by measurements of the differential rates of synthesis (Monod, 1956) of gluconate kinase by cultures of the two strains, growing on either nutrient broth or minimal medium containing glycerol as carbon source, when 5mM-gluconate was added to them (Fig. 2). It is apparent that the nature of the growth substrate exerts a considerable influence on the induction of the kinase: the rates at which the enzyme activities rise after addition of gluconate are lower in the presence of glycerol than in that of nutrient broth. Since, in general, glycerol exerts little catabolite repression on enzymes of carbohydrate metabolism (Magasanik, 1961), it is possible that this repressive effect is a physiological 'feedback' associated with the metabolic role of gluconate kinases. In the Entner–Doudoroff pathway, which forms the predominant route for gluconate catabolism in *E. coli* (Zablotny & Fraenkel, 1967), gluconate kinase initiates a sequence of reactions that results in the cleavage of the C_6 compound 2-keto-3-deoxy-6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate. Since this triose phosphate is interconvertible with the C_3 product of glycerol catabolism dihydroxyacetone phosphate, the repression of gluconate kinase synthesis observed during growth on glycerol may be a further example of a well-known phenomenon. It is also apparent from Fig. 2 that, under both conditions tested, strain R6 formed much less gluconate kinase than did its parent organism. The kinetic parameters of the gluconate kinase formed by strain R6 were, however, identical with those of the enzyme formed by the parent mutant: in both cases, the K_m for gluconate was determined as 0.11mM, and the K_m for Mg-ATP was 0.9mM.

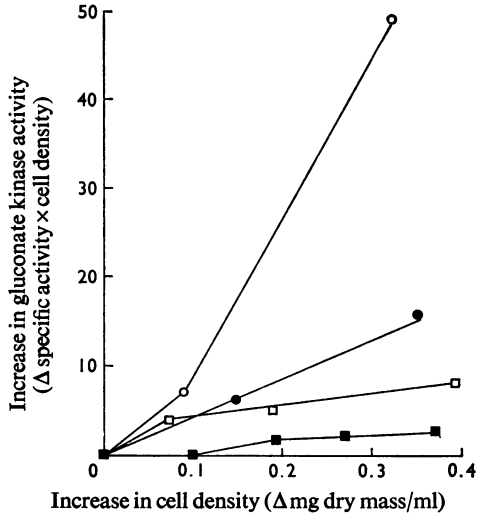

Fig. 2. *Induction of gluconate kinase in cultures of strain K2.1.5^c.8.9 and its mutant R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5^c.8.9 growing on nutrient broth (○) or glycerol (●), and to cultures of strain R6 growing on nutrient broth (□) or glycerol (■). The cell densities and gluconate kinase activities of samples, taken thereafter, were measured as described in the Experimental section. The results are plotted to illustrate the differential rates of synthesis of the enzyme (Monod, 1956).

In a third experiment, the net rates of 6-phosphogluconate formation in cultures of the two strains of mutants were measured. When 5mM-gluconate was added to cultures of strain K2.1.5^c.8.9 that were growing either on nutrient broth or on glycerol, 6-phosphogluconate rapidly appeared in the cells and, in 100min, increased to nearly 18mM in the presence of nutrient broth, and 8mM in the presence of glycerol (calculated on the assumption that 1mg dry mass of *E. coli* K12 contains 3μl of cell water; see Roberts *et al.*, 1955; Winkler & Wilson, 1966) (Fig. 3). Surprisingly, significant amounts of 6-phosphogluconate appeared as such also in the suspending medium; the amounts that thus appeared reflected the intracellular concentrations of this compound (Fig. 4). In contrast, the intracellular 6-phosphogluconate content of strain R6 increased to less than 7mM and 5mM respectively under these conditions (from an initial value of about 4mM) and only traces of 6-phosphogluconate appeared in the media. This latter observation also shows that the low intracellular concentrations of 6-phosphogluconate found with strain R6 are not due to an accelerated efflux of 6-phosphogluconate that might have been formed at a normal rate.

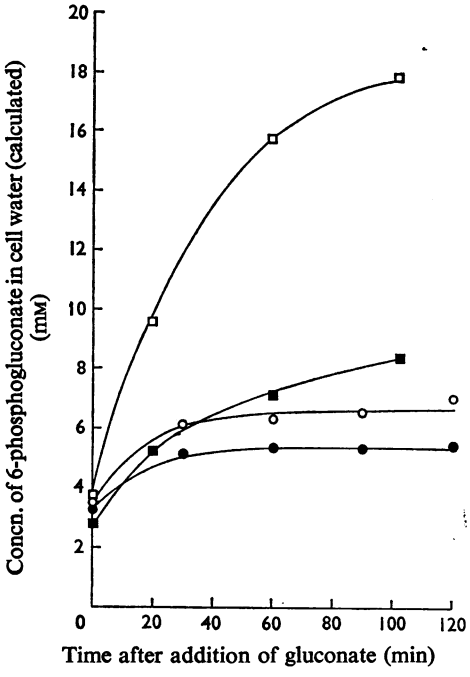

Fig. 3. *Accumulation of 6-phosphogluconate by cultures of strain K2.1.5^c.8.9 and its mutant R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5^c.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). The 6-phosphogluconate content of samples of cells, taken thereafter, was measured as described in the Experimental section. For the purposes of this calculation, it was assumed that 1 mg dry mass of cells contains 3 μl of water.

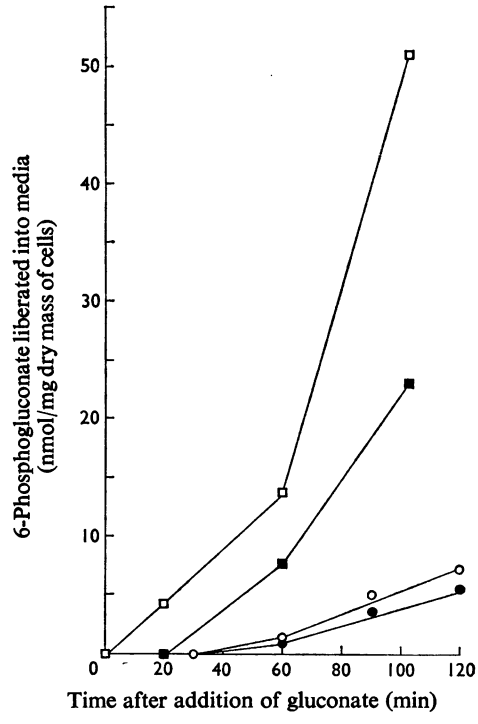

Fig. 4. *Appearance of 6-phosphogluconate in media after addition of gluconate to cultures of strains K2.1.5^c.8.9 and R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5^c.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). Samples were taken thereafter and centrifuged; the 6-phosphogluconate content of the supernatant solutions was assayed as described in the Experimental section.

It is conceivable that the 6-phosphogluconate formed from the gluconate taken up by strain R6 might be rapidly hydrolysed again to gluconate, which might be retained. Although there is no direct evidence against this, we have failed to find any increased rate of hydrolysis of 6-phosphogluconate by extracts of strain R6 or by suspensions of this organism made permeable to external 6-phosphogluconate by the procedure of Reeves & Sols (1973). It is thus likely that strain R6 indeed differs from its parent organism in its ability enzymically to convert into 6-phosphogluconate the gluconate that has been taken up by the cells. Since, in consequence, it takes up labelled gluconate at a normal rate and retains it predominantly as such (Fig. 1), this organism is admirably suited to a study of the kinetics of the uptake process. The experiments with whole cells reported in the next section were therefore done with this mutant.

Uptake of gluconate by strain R6

The initial rates at which 0.05mM-sodium [^{14}C]-gluconate was taken up by washed suspensions of strain R6 (that had been induced to form the uptake system) were measured over the range 1–1000μM. As shown in the inset of Fig. 5, the rates increased with increasing gluconate concentration up to about 60μM, after which the uptake system appeared to be virtually saturated. The K_m of this process is about 10μM and the $V_{max.}$ about 25nmol/min per mg dry mass, values which are identical with the parameters previously determined in wild-type *E. coli* (Faik & Kornberg, 1973). This is particularly significant, since a mutant of *E. coli* has been described (Faik

& Kornberg, 1973) that is capable of forming a gluconate-uptake system with low affinity (K_m above 100μM) for gluconate and that lacks the high-affinity system. Since no increases in the initial rates of gluconate uptake by strain R6 were observed at gluconate concentrations between 60 and 1000μM, it is safe to conclude that the low-affinity uptake system was not induced under the conditions used with strain R6 and that the properties measured here are indeed those of the high-affinity system.

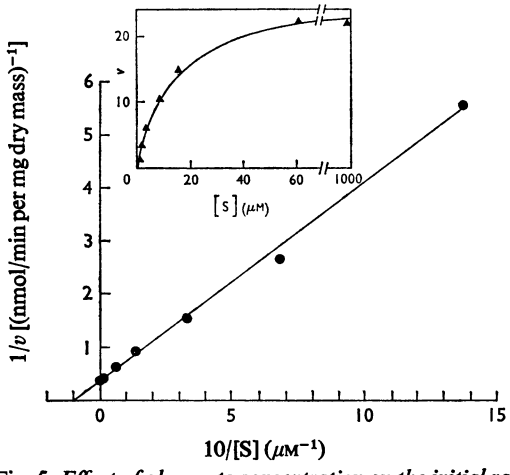

Fig. 5. *Effect of gluconate concentration on the initial rates of gluconate uptake by strain R6*

Initial rates of uptake (v = nmol/min per mg dry mass) were calculated from the amounts of [^{14}C]gluconate taken up by washed suspensions of induced strain R6 10, 25 and 40s after addition of labelled gluconate, and are plotted in the inset figure against the gluconate concentration added ([S] = μM). The reciprocals of these rates (×10) are plotted on the larger figure.

The uptake system is highly specific for gluconate: the inclusion of the sodium salts of all available hexonic, pentonic and hexuronic acids, and of 2-oxogluconic acid, 5-oxogluconic acid, 2-oxo-3-deoxygluconic (3-deoxy-L-*glycero*-2-hexolusonic) acid and glucaric acid at 1mM did not decrease significantly the rate at which strain R6 took up 50μM-sodium [^{14}C]gluconate; only 1mM-3-deoxy-3-fluorogluconate inhibited by 50%. A similar specificity appears to be exhibited by the induction process: only the addition of gluconate itself to cultures of R6 growing on glycerol or nutrient broth induced the uptake system.

It is apparent from Fig. 1 that, in the steady state, strain R6 takes up over 20nmol of gluconate/mg dry mass when incubated with that substance at 50μM: this corresponds to a concentration of over 6mM-gluconate in the cell water. Clearly the uptake and retention of gluconate to this extent (over 100-fold) must require the expenditure of metabolic energy. The decreased rate of uptake found under anaerobic conditions, and the strong inhibition exerted by amytal, cyanide, and a variety of agents known to interfere with energy conservation (Table 1), indicate that this energy for gluconate uptake is derived from the activity of the respiratory chain. The inhibition observed with *p*-chloromercuribenzoate and its reversal by 2-mercaptoethanol further indicate that a

Table 1. *Comparison of the effect of inhibitors on gluconate uptake by intact cells and membrane vesicles prepared from strain R6*

The inhibitors, dissolved in water (or, where marked *, in ethanol) were incubated for 15min at 25°C with the cells and membrane vesicles before addition of 0.05mM-sodium [^{14}C]gluconate and 0.05mM-sodium [^{14}C]gluconate and 20mM-L-lactate respectively. The amounts of labelled gluconate taken up in a further 15min were measured as described in the Experimental section. —, not tested.

Inhibitor used	Concn. added (mM)	Inhibition (%)	
		Cells	Vesicles
NaN_3	1.0	50	—
	10	93	0
KCN	1.0	60	0
	10	76	79
Sodium arsenate	1.0	12	—
	10	30	0
2,4-Dinitrophenol	0.05	30	—
	0.25	90	97
Carbonyl cyanide *m*-chlorophenylhydrazone*	0.001	79	—
	0.01	98	97
Amytal*	0.1	21	80
Anaerobic conditions	—	55	—
p-Chloromercuribenzoate	0.1	80	94
β-Mercaptoethanol	1.0	0	—
p-Chloromercuribenzoate + β-mercaptoethanol	0.1 + 1.0	12	—
Ethanol	—	0	0

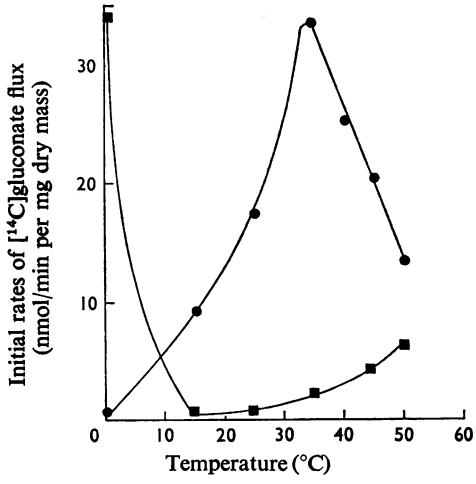

Fig. 6. *Temperature dependence of uptake* (●) *and efflux* (■) *of gluconate by suspensions of strain R6*

For conditions see the text.

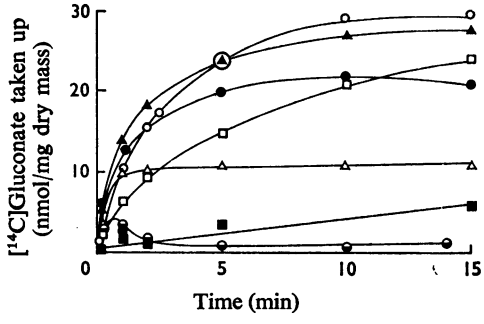

Fig. 7. *Effect of temperature on the time-course of gluconate uptake by strain R6*

Flasks containing 50mM-potassium phosphate buffer, pH7.0, were incubated for 10min at 0°C (■), 15°C (□), 25°C (○), 35°C (▲), 40°C (●), 45°C (△) and 50°C (◒) before addition of a suspension of induced strain R6. Sodium [14C]gluconate (0.05mM) was added 2min thereafter. Samples were taken at times indicated and washed at room temperature; for details see the Experimental section.

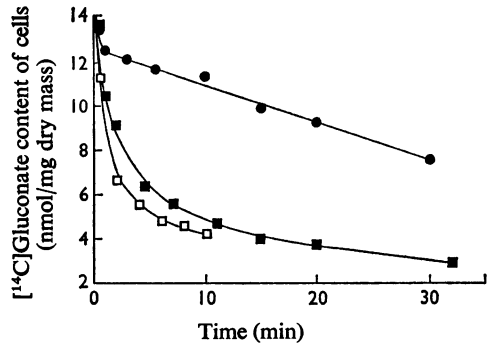

Fig. 8. *Time-course of gluconate efflux from strain R6*

Cells were allowed to accumulate [14C]gluconate as described in the text and diluted in a large volume of 50mM-potassium phosphate buffer, pH7.0, alone (●) or in buffer that also contained 5mM-sodium [12C]gluconate (■) or 0.25mM-dinitrophenol (□).

thiol group may be associated with the site that recognizes gluconate, or with the energetic coupling. The initial rates of uptake of 0.05mM-gluconate by washed suspensions of strain R6 that had been grown on nutrient broth plus gluconate at 37°C were not greatly affected by changes in pH over the range 6–8; this behaviour thus differs from that of the apparently similar mutant briefly reported by Robin & Kepes (1973). At the pH of 7.0 usually used in our work, the rate of gluconate uptake by strain R6 was very low when measured at 0°C and increased rapidly with increase in temperature to a peak at about 35°C, above which it declined (Fig. 6). There was a concomitant rise in the steady-state concentrations of gluconate maintained by the cells between 0°C and 35°C, and a sharp decline thereafter (Fig. 7). The behaviour shown in Fig. 6 is characteristic of an active-transport process, which exhibits a temperature coefficient much greater ($Q_{10}>2$) than would passive diffusion, the rate of which is proportional to the absolute temperature.

The sharp decrease above 35°C in the quantities of gluconate retained by strain R6 can be only partially accounted for by an increased rate of gluconate efflux from the cells. That an alteration in some energy-dependent process in gluconate retention might be involved is supported by measurement of the effect of 2,4-dinitrophenol on gluconate efflux (Fig. 8). In the absence of this proton-conducting agent (Harold, 1972) a culture of strain R6 that had accumulated [14C]gluconate lost gluconate at only 0.04 nmol/min per mg dry mass when diluted into a large volume of 50mM-potassium phosphate buffer without change in pH or temperature. As shown also in Fig. 1, the addition of [12C]gluconate greatly accelerated this exit reaction: clearly the dilution of cellular radioactivity observed reflected the rapid uptake of the unlabelled gluconate and the consequent displacement of the labelled material already present. The efflux reaction involved in this exchange could be a passive diffusion, since it exhibits the small temperature coefficient expected of such a process (Fig. 6), and could involve the activity of a mobile carrier protein (Kepes, 1973). However, no such exchange can account for the

stimulation of gluconate efflux observed after addition of 2,4-dinitrophenol: presumably some energy-requiring process must operate to retain the gluconate taken up, possibly by decreasing the affinity of the gluconate 'carrier' for the substrate present internally (Koch, 1964) or by providing the power for a re-capture process at the cell surface (Halpern, 1967).

The uptake of a number of carbohydrates by *E. coli* appears to involve 'binding proteins' (for review, see Lin, 1971), which can be liberated from the periplasmic space by EDTA treatment followed by osmotic shock (Heppel, 1969). In order to investigate whether such 'binding proteins' played any part in the uptake of gluconate, cultures of wild-type *E. coli* K12 were osmotically shocked. In conditions where at least 95% of the shocked cells remained viable (Anraku & Heppel, 1967) there was a decrease, by 75–80%, in the initial rates of gluconate uptake, which could be partially restored by addition of the concentrated shock fluid (Table 2). However, this partial restoration of uptake could also be achieved by the addition of shock fluid derived from cells that had not been induced for gluconate uptake. It appeared to reside in some components of the fluids that were stable to boiling for 10min, and was lost when shock fluid from induced cells was dialysed. It is therefore unlikely that a gluconate-specific 'binding protein' was released in the shock fluid; indeed, our attempts by equilibrium dialysis to detect a protein in concentrated shock fluids that would bind labelled gluconate were unsuccessful. Rather it is likely that the osmotic cold-shock procedure impairs the energy metabolism of the cells, perhaps by the loss of small molecules: this is also indicated by the major decrease in cellular respiration upon cold-shock treatment (Table 2).

Table 2. *Effect of cold osmotic-shock treatment on the uptake of gluconate and of oxygen by wild-type E. coli K12*

Gluconate uptake and respiration were measured within the 2h after the EDTA–osmotic-shock treatment. Cells were grown on nutrient broth supplemented with 5mM-sodium gluconate as inducer. For measurements of oxygen uptake, nutrient broth was used as substrate. Shock fluid (0.6mg of protein/ml) was concentrated under vacuum about 40-fold and added at a final concentration of 2mg of protein/ml.

	Relative rates (%) of	
	Gluconate uptake	Oxygen uptake
Experiment 1.		
Cells	100	100
σhocked cells	20	16
Shocked cells+fluid	54	16
Experiment 2.		
Cells	100	100
Shocked cells	26	20
Plus fluid	62	20
Plus boiled fluid	78	—
Plus fluid from non-induced cells	99	—

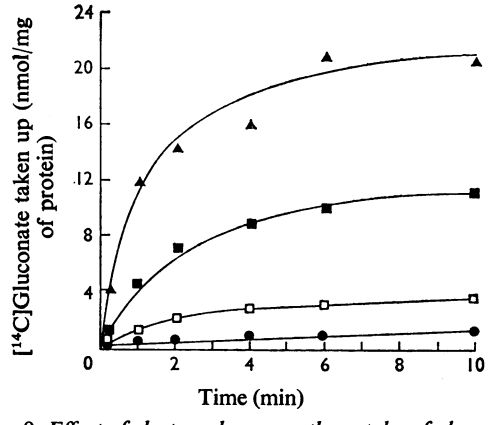

Fig. 9. *Effect of electron donors on the uptake of gluconate by membrane vesicles from strain R6*

The uptake of 0.05mM-sodium [^{14}C]gluconate by vesicles (0.4mg of protein/ml of buffer) was measured, as described in the Experimental section, in the absence of added electron donors (●) and in the presence of D-lactate (□), of L-lactate (■) and of ascorbate+phenazine methosulphate (▲).

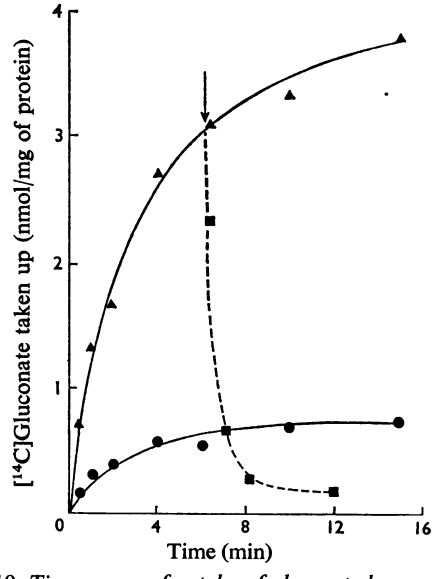

Fig. 10. *Time-course of uptake of gluconate by membrane vesicles from strain R6*

The uptake of 0.05mM-sodium [^{14}C]gluconate by membrane vesicles was measured in the absence of added donors (●) and in the presence of D-lactate (▲). At the time indicated by an arrow, 5mM-sodium [^{12}C]gluconate was added to a portion of the latter (■).

Gluconate uptake by membrane vesicles

In common with other carbohydrates actively taken up by *E. coli* (for review, see Kaback, 1970; Kornberg, 1973), gluconate can be taken up and retained against a concentration gradient by membrane vesicles prepared from induced cells, provided that a suitable source of metabolic energy is also supplied (Fig. 9). The optimum source of this metabolic energy appears to differ with different carbohydrates. Thus the uptake of gluconate by membrane vesicles prepared from strain R6 was stimulated nearly 30-fold by the presence of ascorbate and phenazine methosulphate, tenfold by L-lactate, and only 3–4-fold by D-lactate; this preference for L- over D-lactate differs from the preference for D-lactate exhibited by *E. coli* vesicles taking up, e.g., galactosides (Kaback, 1970, 1972). With ascorbate+phenazine methosulphate as electron donor, vesicles concentrated external 0.05mM-gluconate over 100-fold, to the approx. 6mM concentration that had also been observed with whole cells (Fig. 1). Further, and also as observed with whole cells (Figs. 1 and 8), the addition of [^{12}C]gluconate to vesicles that had taken up [^{14}C]gluconate rapidly displaced from them over 90% of the radioactive material, in 2min (Fig. 10).

Stimulation of gluconate uptake was observed also with succinate or α-glycerophosphate as electron donors, but these materials were less effective than ascorbate+phenazine methosulphate or L-lactate; NADH, which is the preferred electron donor for the uptake of some carbohydrates by *Bacillus subtilis* membrane vesicles (Matin & Konings, 1973), did not stimulate gluconate uptake by vesicles prepared from *E. coli* strain R6 (Table 3). With L-lactate as electron donor, the K_m for gluconate uptake was 5×10^{-5}M, which is of the same order of magnitude as that found for uptake by whole cells.

As noted with whole cells, the uptake of gluconate by membrane vesicles is strongly inhibited by inhibitors of electron transfer, and the pattern of inhibition observed is similar to that observed with whole cells (Table 1). In both cases, the uptake process is powerfully inhibited by proton-conducting agents, such as carbonyl cyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol; the lack of effect by arsenate is consistent with the view (Klein & Boyer, 1972) that ATP or other high-energy phosphate compounds are not directly involved in the transport process.

The close parallel between the kinetic and energetic parameters that characterize the uptake of gluconate by whole cells and that by membrane vesicles support the use of the latter type of experimental material for studies of active transport, and also show that no indispensible components of the uptake process have been lost in the preparation of membrane vesicles. Comparison of the rates of gluconate uptake by suspensions of induced strain R6, its parent, and its grandparent organisms also confirm that, provided measurements are made over brief periods after addition of a labelled substrate, the measured rates of appearance of label in the cells are very close to the 'true' rates at which the substrate is taken up, and that this uptake process is thus the rate-limiting step in gluconate utilization. This leads to the comforting conclusion that, although mutants devoid of the first catabolic enzyme subsequent to the uptake step provide a powerful and unambiguous means for investigating that uptake process, they are not essential for determining its kinetic parameters.

Table 3. *Effect of various energy sources on the gluconate uptake by membrane vesicles from strain R6*

Samples (0.1 ml) containing membranes (0.2 mg of protein) from strain R6 were incubated, and the amounts of label taken up in 15min from 0.05mM-sodium [^{14}C]gluconate were measured, as described in the Experimental section.

Energy source added	Concn. added (mM)	[^{14}C]Gluconate taken up (nmol/mg of protein)
None	—	1
D-Lactate	20	3
L-Lactate	20	10
Ascorbate	20	1.5
Phenazine methosulphate	0.2	1
Ascorbate+phenazine methosulphate	20+0.2	27
Succinate	30	3
DL-α-Glycerophosphate	20	3
NADH	20	1.5
NADH+phenazine methosulphate	20+0.2	1.5
NADPH	20	1.5
Phosphoenolpyruvate	20	1
ATP	10	1.5

We thank Miss Susan Dilks for preparing the diagrams, the British Council for the award of a scholarship to J.P., the Medical Research Council for a studentship to P. F., and the Science Research Council for financial support under grant B/SR/7246.

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