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# Proton Uptake Linked to the 3-Deoxy-2-oxo-D-gluconate-Transport System of *Escherichia coli*

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Genetic and kinetic evidence is presented to show that the carrier-mediated uptake of the anionic sugars 3-deoxy-2-oxo-D-gluconate and D-glucuronate by *Escherichia coli* involves the concomitant transport of protons.

Three functionally distinct mechanisms have been described to account for the energy-dependent accumulation of solutes into bacterial cells (Simoni & Postma, 1975: Hamilton, 1975): (*a*) the phosphoenolpyruvate-dependent group translocation of certain sugars (Kundig *et al.*, 1964); (*b*) the ATP-dependent periplasmic-binding-protein-mediated transport systems (Boos, 1974); and (*c*) the transport of a variety of solutes driven by the protonmotive force generated across the cytoplasmic membrane by either respiration or ATP hydrolysis (Harold, 1976). With reference to this last-mentioned mechanism, the chemiosmotic theory proposes that anions are accumulated by specific $H^+$ symporters in response to the pH gradient across the membrane, that the cation fluxes are mediated by a uniporter and depend only on the electrical potential gradient across the membrane, and that the flux of neutral substrates is mediated by $H^+$ symporters and is driven by the total protonmotive force (Mitchell, 1976). Indeed there is now ample evidence for proton movement associated with the transport of β-galactosides (West, 1970; West & Mitchell, 1972, 1973), arabinose and galactose (Henderson & Skinner, 1974), gluconate (Robin & Kepes, 1973), succinate (Gutowski & Rosenberg, 1975), lactate and alanine (Collins *et al.*, 1976) in *Escherichia coli.* In such studies comparatively little attention has been given to the possibility that the free carrier itself may be charged (Schuldiner *et al.*, 1975). Lack of such consideration probably leads to an underestimate of the complexity of the mechanisms coupling proton circulation to solute transport (Rottenberg, 1976). This point is particularly critical in the case of the transport of anionic sugars in *E. coli*, where the experimentally determined pH gradient across the membrane cannot account, by itself, for the observed sugar concentration gradient (Padan *et al.*, 1976).

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Previous work has provided an extensive genetic and kinetic appreciation of the 3-deoxy-2-oxo-D-gluconate-transport system in *E. coli* (Pouysségur & Lagarde, 1973; Lagarde *et al.*, 1973; Lagarde & Stoeber, 1974, 1975). In the present report we extend the above studies and describe the concomitant uptake of protons with both 3-deoxy-2-oxo-D-gluconate and D-glucuronate in cells synthesizing the 3-deoxy-2-oxo-D-gluconate-transport system constitutively.

## Materials and Methods

### *Bacterial strains*

The bacterial strains used were all *E. coli* K-12 derivatives requiring thiamin for growth. Strains CK101 (*metB*, *kdgR*1, *kdgK*; Pouysségur & Lagarde, 1973) and PA3K1 (*metB*, *kdgP*3, *kdgA*3, *kdgK*1; Pouysségur & Lagarde, 1973) carry respectively a regulator-constitutive (*kdgR*) and an operator-constitutive (*kdgP*) mutation that de-repress the synthesis of the 3-deoxy-2-oxo-D-gluconate-transport system. Strain PAUKT4 (*metB*, *argG*, *exuT*9, *kdgP*3, *kdgK*3, *kdgT*(*ts*-4), str$^R$; Lagarde & Stoeber 1976) is similar to strain PA3K1 but synthesizes a functional transport system at 30°C but not at 40°C as a result of a thermosensitive mutation in the structural gene for the transport system [*kdgT*(*ts*)]. Strain TH9 (*metB*, *exuT*9; Nemoz *et al.*, 1976) does not synthesize the 3-deoxy-2-oxo-D-gluconate-transport system and lacks the hexuronate-transport system (*exuT*). To prevent metabolic conversion of the sugars, the above-mentioned strains are deficient in 3-deoxy-2-oxo-D-gluconate kinase activity (EC 2.7.1.45) (*kdgK*) and in glucuronate isomerase (EC 5.3.1.12) (under non-induced conditions).

### *Growth conditions and preparation of cells*

Cells were grown aerobically at 37°C (except for strain PAUKT4, where growth was at 30°C or 40°C as indicated) in the mineral-salts medium described by Cohen & Rickenberg (1956) containing in addi-

tion glycerol (0.5%, w/v), thiamin (0.5 μg/ml), methionine (10 μg/ml) and arginine (10 μg/ml), in 2-litre baffled conical flasks each containing 625 ml of growth medium. Cells were harvested in the late-exponential phase of growth ($3\times10^8$–$6\times10^8$ cells/ml) by centrifugation at 4500 *g* for 15 min. Cells referred to as 'starved' were depleted of their energy source by incubating harvested cells, at their respective growth temperatures, for 2h with vigorous shaking in the basic growth medium without additions, supplemented with α-methyl glucoside (20 mM) and $NaN_3$ (40 mM) as originally described by Koch (1971). Sphaeroplasts from either 'starved' or 'unstarved' cells were prepared as described by Garland *et al.* (1975). Cells were finally washed twice with 0.3 M-sucrose/0.15 M-KCl/2.5 mM-$MgCl_2$/1.5 mM-glycylglycine, pH 7.5, referred to subsequently as incubation medium, and resuspended to a final protein concentration of 30–40 mg/ml. Stock cell suspensions were stored at 0–4°C for up to 5h.

*Assay techniques*

Protein was determined by the method of Lowry *et al.* (1951), with dry bovine serum albumin fraction V (BDH Chemicals Ltd., Poole, Dorset, U.K.) as standard. The experimental vessel, electrode and recording system for measuring pH changes were as described by Lawford & Garland (1972). The cell chamber (1.0 ml final volume) was filled with stock cell suspension diluted to give a final protein concentration ranging from 2 to 5 mg/ml in incubation medium, at 25°C. The pH was adjusted to between 7.15 and 7.25 with 0.1 M-KOH. The well-stirred suspension became anaerobic within a few minutes, but equilibration of the system was continued until the pH drift reached a minimum value (0.1 pH unit/min). The experiments were started by the addition of known volumes of anaerobic stock sugar solutions and the pH change of the outside medium was monitored. At the end of each experiment 1–2 μl of an anaerobic 5 mM-HCl solution was injected for calibration of the system.

*Stock solutions, standards and inhibitors*

Stock solutions of sugars (1 M) were made in 0.15 M-KCl and appropriately neutralized to pH 7.1–7.2 with 5 M-KOH. For pH titrations, standard HCl (5 mM) and KOH (0.1 M) were made in 0.15 M-KCl. Stock solutions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.4 μM), valinomycin (1.0 mg/ml) and nigericin (1.0 mg/ml) were made in ethanol. All solutions were freed from oxygen by bubbling with $O_2$-free $N_2$.

*Reagents*

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Valinomycin, lysozyme chloride (grave VI), gluconic acid (sodium salt), glucuronic acid and galacturonic acid were from Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, Surrey, U.K. Nigericin was a generous gift from Eli Lilly and Co., Indianapolis, IN 46206, U.S.A. 3-Deoxy-2-oxo-D-gluconic acid (potassium salt) was synthesized by the method of Pouysségur & Stoeber (1970). All other reagents were from BDH Chemicals Ltd. and were of the highest available purity.

## Results

*Proton influx elicited by 3-deoxy-2-oxo-D-gluconate and D-glucuronate*

Fig. 1 shows the pH recordings obtained with a $H^+$-sensitive electrode after the addition of an anaerobic solution of 3-deoxy-2-oxo-D-gluconate to an anaerobic suspension of *E. coli* strain CK101 previously starved and converted into sphaeroplasts according to the procedure detailed in the Materials and Methods section. Fig. 1(*a*) shows that the addition of the sugar caused an immediate alkalinization of the medium, which is interpreted as an effective proton uptake into the cells (or an equivalent outflux of $OH^-$ ions from the cells). No pH change was observed on the addition of sugar to buffer alone under the same conditions (results not shown). Proton uptake was not modified in the presence of valinomycin (Fig. 1*b*), but was completely abolished when either carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 1*c*) or nigericin (Fig. 1*d*) was present. Exactly the same responses were obtained on the addition of D-glucuronate in place of 3-deoxy-2-oxo-D-gluconate (results not shown). The effects of the above-mentioned ionophores were not elicited if the sphaeroplast-formation stage was omitted, and this can be ascribed to the impermeability of the *E. coli* cell wall to these ionophores. If the starvation stage was omitted during the preparation of the cells, proton uptake was still abolished by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and nigericin, but was slightly stimulated by the addition of valinomycin. Under these conditions the collapse of the electrical potential gradient across the membrane by valinomycin with $K^+$ ions is thought to enhance the pH gradient. These results suggest that the driving force for the accumulation of 3-deoxy-2-oxo-D-gluconate and D-glucuronate is the pH-gradient component of the total protonmotive force. That a protonmotive force still existed across the membrane before sugar addition is indicated by the re-equilibration of cations between the extracellular and intracellular compartments on the addition of any of the above-mentioned iono-

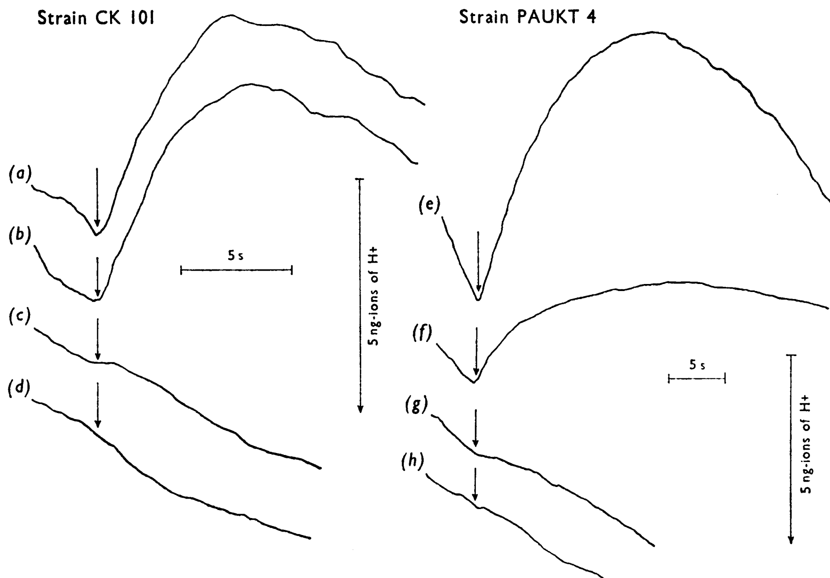


Fig. 1. *Time-course of extracellular pH changes on the addition of* 3-*deoxy*-2-*oxo*-D-*gluconate and* D-*glucuronate to E. coli strains CK*101 *and PAUKT*4

The electrode vessel contained in 1.0ml: 0.3M-sucrose, 0.15M-KCl, 2.5mM-$MgCl_2$, 1.5mM-glycylglycine and cells from strain CK101 (2.7mg of protein), PAUKT4 grown at 30°C (4.7mg of protein) and PAUKT4 grown at 40°C (3.9mg of protein). The cell suspensions were adjusted to pH7.25 and equilibrated at 25°C for 15min. At times indicated by the arrows, anaerobic solutions (2mM) if 3-deoxy-2-oxo-D-gluconate (*a, b, c, d, e, g*) or D-glucuronate (*f, h*) were injected. Cells from strain CK101 were starved and converted into sphaeroplasts and cells from strain PAUKT4 were only starved. Traces: (*a*), without further addition; (*b*), with valinomycin (1 μg/ml); (*c*), with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1.2nM); (*d*), with nigericin (1 μg/ml); (*e*), (*f*), growth occurred at 30°C; (*g*), (*h*), growth occurred at 40°C.

phores. This re-equilibration was still observed if potassium iodoacetate (1mM) was added to the incubation medium (results not shown) to inhibit glycolysis.

Evidence that the proton uptake observed on the addition of sugars is strictly dependent on a functional 3-deoxy-2-oxo-D-gluconate-transport system is given in Figs. 1(*e*)–1(*h*). Strain PAUKT4 (Lagarde & Stoeber, 1976) carries a thermosensitive mutation [*kdgT*(*ts*)] in the structural gene responsible for the synthesis of the carrier protein. When cells were grown at the permissive temperature (30°C), protons were taken up by the cells on the addition of 3-deoxy-2-oxo-D-gluconate (Fig. 1*e*) or D-glucuronate (Fig. 1*f*), whereas at the restrictive growth temperature (40°C) both sugars failed to elicit a pH change (Figs. 1*g* and 1*h*). In addition, if the synthesis of the 3-deoxy-2-oxo-D-gluconate-transport system is not derepressed, as in strain TH9 (Nemoz *et al.*, 1976), there was no proton uptake at any growth temperature (results not shown). Further, in all the strains tested there was no net proton accumulation in the presence of either D-galacturonate or D-gluconate; it has been shown previously that these two sugars are not substrates for the 3-deoxy-2-oxo-D-gluconate-transport system (Lagarde *et al.*, 1973).

*Kinetic parameters of the carrier-mediated proton uptake*

In the experiments summarized in Table 1, proton uptake was measured with various concentrations of 3-deoxy-2-oxo-D-gluconate or D-glucuronate, by using cells treated in different ways. Results clearly indicate that initial rates of $H^+$ uptake obey Michaelis–Menten kinetics with respect to the external concentration of sugars. Apparent $K_m$ values for proton uptake are remarkably similar to the $K_m$ values obtained for the uptake of labelled sugars as measured in previous studies (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1975), with mean values of 0.15mM for 3-deoxy-2-oxo-D-gluconate and of 1.5mM for D-glucuronate. The corresponding $V_{max.}$ values are more difficult to correlate, because the sugar-uptake data were not obtained under the same experimental conditions. However, $V_{max.}$ values for proton uptake do increase with the carrier concentration within the membrane,

Table 1. *Kinetic parameters of proton uptake elicited by substrates of the 3-deoxy-2-oxo-D-gluconate-transport system of E. coli*

Glycerol-grown cells were prepared as described in the Materials and Methods section. The electrode vessel contained in 1 ml: 0.3 M-sucrose, 0.15 M-KCl, 2.5 mM-$MgCl_2$, 1.5 mM-glycylglycine and cells (3–5 mg of protein). The suspensions were equilibrated anaerobically for 15 min at pH 7.25 ± 0.02. The experiments were started by injecting anaerobic solutions of 3-deoxy-2-oxo-D-gluconate (0.010–2.0 mM) or D-glucuronate (0.50–6.0 mM). Proton influx was measured during the first 4 s after sugar addition, over which time protein uptake was linear. Double-reciprocal plots were used to compute the kinetic parameters. Each experiment refers to separate batches of cells.

| Strain | Cell preparation | Substrate added | Expt. no. | $V_{max.}$ (ng-ions of $H^+$/min per mg of protein) | $K_m$ (mM) |
|---|---|---|---|---|---|
| CK101 | Starved | 3-Deoxy-2-oxogluconate | 1 | 35.7 | 0.200 |
| | | 3-Deoxy-2-oxogluconate | 2 | 41.6 | 0.143 |
| | Starved, sphaeroplasts made | 3-Deoxy-2-oxogluconate | 3 | 39.3 | 0.150 |
| | Unstarved, sphaeroplasts made | 3-Deoxy-2-oxogluconate | 4 | 75.5 | 0.175 |
| | | 3-Deoxy-2-oxogluconate | 5 | 154.0 | 0.143 |
| PA3K1 | Starved | 3-Deoxy-2-oxogluconate | 6 | 5.6 | 0.143 |
| | Unstarved, sphaeroplasts made | 3-Deoxy-2-oxogluconate | 7 | 30.6 | 0.182 |
| | | Glucuronate | 8 | 32.0 | 1.670 |

as suggested from a comparison of the results obtained with the regulator-constitutive strain CK101 and the operator-constitutive strain PA3K1. The results also suggest that $V_{max.}$ values depend on the magnitude of the protonmotive force at the beginning of the experiment, since the values are generally much lower in starved cells than in unstarved cells.

It should be noticed that the total extent of the external pH change, after sugar addition and attainment of the new steady state, also obeys Michaelis–Menten kinetics with respect to the external sugar concentration. This is in agreement with the finding that the overall proton-uptake kinetics fit a pesudo-first-order law with respect to the external proton concentration, whatever the external sugar concentration may be (results not shown). For instance, the first-order rate constant was about $0.07\,s^{-1}$ in strain PA3K1 (Expt. 4, Table 1).

## Discussion

From the results presented in this paper it may be concluded that proton uptake elicited by the addition of 3-deoxy-2-oxo-D-gluconate or D-glucuronate to anaerobic cell suspensions is mediated by the same carrier that has been shown previously to be responsible for the uptake of these sugars (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1976). As with the *scyllo*-inositol (Reber & Deshusses, 1976) and the pentitol (Höfer & Misra, 1976) $H^+$-symport systems, we found that the apparent $K_m$ for $H^+$ influx and for sugar influx are identical. The finding that the rate of proton uptake is proportional to the external $H^+$ concentration (or more likely related to the transmembrane pH gradient) remains difficult to reconcile with a carrier-mediated process unless it is assumed that the affinity of the 3-deoxy-2-oxo-D-gluconate-carrier protein for protons is well above the extracellular proton concentration (i.e. $\gg 0.1\,\mu M$).

The starvation procedure (Koch, 1971) used to deplete cells of their energy reserves does not collapse the protonmotive force, as demonstrated by the observation that, in the absence of sugars, re-equilibration of cations occurred across the membrane on the addition of various ionophores. A comparison of starved and unstarved cells indicates that the initial rate of proton uptake and the extent of external pH variation, on addition of the sugars, were different in the two cell preparations. Thus it is likely that the sugar-induced flux of $H^+$ is dependent, at least in part, on the magnitude of the protonmotive force existing at the start of the experiment. Even if the value of the total protonmotive force was indeed zero, net $H^+$ uptake could be observed if the magnitude of the pH gradient is equal and opposite to the electrical potential gradient (Mitchell, 1976).

Valinomycin plus $K^+$ ions failed to modify the rate or extent of proton uptake (Fig. 1*b*) on addition of the sugars, whereas, in contrast, compounds known to collapse the pH gradient abolished the response (Figs. 1*c*, 1*d*). This result suggests that the co-transport of $H^+$ with 3-deoxy-2-oxo-D-gluconate is electroneutral and is driven by the pH-gradient component alone. However, alternative interpretations are possible (Rottenberg, 1976) and the precise mechanism of sugar transport will require (*a*) elucidation of the stoicheiometry of $H^+$:sugar uptake; (*b*) knowledge of the net charge on the free carrier protein; and (*c*) determination of the relative magnitudes of the protonmotive force (including the separate assessment of the individual components) and the resulting sugar concentration gradient.

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