Evidence for an Electrogenic 3-Deoxy-2-oxo-D-gluconate–Proton Co-transport Driven by the Protonmotive Force in *Escherichia coli* K12

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Evidence is presented indicating that the carrier-mediated uptake of 3-deoxy-2-oxo-D-gluconate and D-glucuronate in *Escherichia coli* K12 is driven by the ΔpH and $\Delta\psi$ components of the protonmotive force. 1. Approximately two protons enter the cells with each sugar molecule, independent of the sugar and the strain used. 2. In respiring cells, the magnitude of the pH gradient alone, as measured by distribution of [^3H]acetate, appears to be insufficient to account for the chemical gradient of 3-deoxy-2-oxo-D-gluconate that is developed between pH 6.0 and 8.0. 3. If the external pH is varied between 5.5 and 8.0, 3-deoxy-2-oxo-D-gluconate uptake is gradually inhibited by valinomycin plus K^+ ions, whereas the inhibition caused by nigericin is concomitantly relieved, thus reflecting the relative contribution of ΔpH and $\Delta\psi$ to the total protonmotive force at each external pH. 4. 3-Deoxy-2-oxo-D-gluconate can be transiently accumulated into isolated membrane vesicles in response to an artificially induced pH gradient. The process is stimulated when the membrane potential is collapsed by valinomycin in the presence of K^+ ions.

Previous studies have demonstrated that 3-deoxy-2-oxo-D-gluconate and D-glucuronate can be concentrated several hundredfold inside whole cells (Pouysségur & Lagarde, 1973; Lagarde *et al.*, 1973; Lagarde & Stoeber, 1975, 1977) or inside isolated membrane vesicles at the expense of respiration (Lagarde & Stoeber, 1974). These monocarboxylic sugars exhibit pK values around 3, so that at physiological pH values they are fully dissociated. Given the polarity of the electrical potential gradient, inside negative, that is developed across the *Escherichia coli* membrane during respiration (Hamilton, 1975; Padan *et al.*, 1976; Ramos *et al.*, 1976; Collins & Hamilton, 1976; Harold, 1977), compensation of the anionic charge on the sugars is required for their translocation against the electrical barrier. Lagarde & Haddock (1977) showed that the addition of 3-deoxy-2-oxo-D-gluconate to anaerobic suspensions of *E. coli* sphaeroplasts containing the carrier specific for these sugars elicited proton uptake, or a thermodynamically equivalent hydroxyl-ion efflux. This result suggested that the mechanism of charge compensation could be mediated through a specific sugar–proton(s) symporter as shown for many nutrients in a variety of micro-organisms (for reviews see Hamilton, 1977; Harold, 1977).

Depending on the net electrical charge carried across the membrane by the presumed sugar–proton(s)–symporter complex, it is generally accepted that the co-transport may be either electroneutral and must therefore be driven strictly by the pH gradient,

or electrogenic, in which case the driving force must be the total protonmotive force, defined as the sum of the pH gradient (ΔpH) and of the membrane potential ($\Delta\psi$) (Mitchell, 1969, 1970, 1976). Several stoichiometrical associations between the carrier and its ligands could account for both situations (Rottenberg, 1976). Whatever the electrical charge of the free carrier may be, the electroneutral or electrogenic character of the co-transport should simply depend on the ratio of protons translocated per anionic sugar molecule.

The experiments reported in the present paper were undertaken to elucidate the electrical nature of the 3-deoxy-2-oxo-D-gluconate– H^+ co-transport. For this purpose I examined the following points: (i) the stoichiometry of the process and the possible involvement of other cations; (ii) the quantitative relationships between the sugar gradient and the magnitude of the pH gradient; (iii) the influence on 3-deoxy-2-oxo-D-gluconate uptake of ionophores that act in abolishing either the ΔpH or the $\Delta\psi$ component; (iv) the possibility of eliciting sugar uptake in isolated membrane vesicles by inducing an artificial pH gradient and/or an electrical potential gradient.

The results are consistent with the conclusion that, owing to a stoichiometry of two protons translocated per sugar molecule, the 3-deoxy-2-oxo-D-gluconate transport is electrogenic and does respond to the total protonmotive force.

Materials and Methods

Bacterial strains

The bacterial strains used were all *E. coli* K12 derivatives requiring thiamin for growth. Strain CK101 (*metB, kdgR1, kdgK*; Pouysségur & Lagarde, 1973) and PA3K1 (*metB, kdgP3, kdgA3, kdgK1*; Pouysségur & Lagarde, 1973) carry respectively a regulator-constitutive (*kdgR*) and an operator-constitutive (*kdgP*) mutation that de-represses the synthesis of the 3-deoxy-2-oxo-D-gluconate transport system. They are also deficient in 3-deoxy-2-oxo-D-gluconate kinase activity (EC 2.7.1.45) (*kdgK*) to prevent the metabolic conversion of the sugar.

Preparation of cells, spheroplasts and membrane vesicles

Cells were grown aerobically at 37°C in the mineral-salts medium described by Sistrom (1958) with glycerol (0.5%, w/v) as a carbon source as detailed previously (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1975). In the mid-exponential phase of growth, the cells were harvested by centrifugation, washed once and resuspended in a medium containing 0.25 M-sucrose and 2.5 mM-MgCl₂, to a final concentration of 20–30 mg dry wt./ml, and kept in ice. Spheroplasts were prepared as described by Garland *et al.* (1975) and resuspended into 0.25 M-sucrose/2.5 mM-MgCl₂ to a protein concentration of 15–20 mg/ml. Isolated membrane vesicles were prepared by the method of Kaback (1972), except that the osmotic shock and subsequent washings were done in 25 mM-potassium phosphate buffer, pH 8.0. They were stored frozen at a protein concentration of about 10 mg/ml and thawed once as required. Protein was determined by the method of Lowry *et al.* (1951) with dry egg albumin (five-times crystallized; Nutritional Biochemicals Corporation, Cleveland, OH, U.S.A.) as a standard.

Measurements of H⁺, Na⁺ and K⁺ fluxes

A thermostatically controlled water-jacketed glass vessel (20°C) was filled with 10 ml of buffer medium described below containing either bacterial cells (1.5–3.0 mg dry wt./ml) or spheroplasts (2.0–3.0 mg of protein/ml) and one of the following buffers: 0.15 M-KCl/1.5 mM-glycylglycine or 0.2 M-sucrose/2 mM-MgCl₂/75 mM-choline chloride/12.5 mM-CaCl₂/0.125 M-KCl/0.75 mM-glycylglycine. The medium was stirred magnetically and bubbled with O₂-free N₂. The pH of the medium was adjusted to 7.00 ± 0.05 with 50 mM-HCl, 0.5 M-KOH or saturated Ca(OH)₂. A period of 15–20 min was allowed for equilibrating the system, after which the pH drift was only 0.01 unit/min. Microlitre quantities of O₂-free solutions of 1 M-3-deoxy-2-oxo-D-gluconate or 0.5 M-D-glucuro-

nate were injected to give the desired final concentration. The pH of the medium was recorded until a new steady-state was reached, by using a combination pH electrode (no. 405.S0; Tacussel, Paris, France), connected to a Minisis pH-meter (Tacussel) and a recorder (no. LS 437700; Linseis G.m.b.H., Selb, West Germany) set at 1 mV full-scale deflexion and at 200–500 mm/min chart speed. The same equipment was used to follow Na⁺ and K⁺ movements, except that the pH-electrode was replaced by a pNa electrode (no. 209.085; Tacussel) or a pK electrode (no. 202.012; Tacussel) coupled to a reference electrode (no. 209.176; Tacussel). These electrodes were completely insensitive to pH changes in the range studied. The system was calibrated before and after each sugar injection by adding O₂-free solution of respectively 50 mM-HCl (5 µl), 0.1 M-NaCl (10 µl) or 0.1 M-KCl (10 µl). The decay portion of the corresponding pH trace was analysed as described by Mitchell & Moyle (1967) to evaluate the buffering capacity of the outer phase of the medium.

Measurement of the pH gradient

The assay for measuring the pH gradient across the membrane was based on the distribution of methylamine and acetate by the method of Nicholls (1974). Cells or spheroplasts were incubated with 10 µM-[¹⁴C]methylamine (0.1 µCi/ml) and 10 µM-[³H]acetate (0.5 µCi/ml) for various periods of time as specified for each experiment, and samples containing no more than 100 µg of protein were filtered on dried Sartorius membranes (cellulose nitrate, 0.45 µm pore size). The filters were immediately transferred into scintillation vials containing Triton X-100 (2 ml), toluene (4 ml), water (0.6 ml) and 2,5-diphenyloxazole (5 mg/ml). After leaving for 3 h at room temperature to allow dissolution, the homogeneous solution was counted for radioactivity of the two isotopes in a Packard spectrometer. Results were computed by calculating the apparent spaces for each radioisotope, exactly as described by Nicholls (1974). The internal volume for whole cells and membrane vesicles was taken as 2.7 µl/mg dry wt. (Collins & Hamilton, 1976) and 2.2 µl/mg of protein (Ramos *et al.*, 1976) respectively. No attempt was made to measure the possible acetate incorporation during the time course of the assays, but no significant difference in ΔpH values was found when using isotope concentrations in the range 2.5–20 µM. In addition, the kinetics of isotope distribution were steady after 3–5 min and ΔpH values remained constant for up to 30 min.

Assays for sugar uptake

The standard technique for assaying 3-deoxy-2-oxo-D-[¹⁴C]gluconate and D-[¹⁴C]glucuronate uptake in whole cells and spheroplasts was essentially the

same as that used previously (Lagarde *et al.*, 1973; Lagarde & Stoerber, 1974, 1975, 1977). Briefly, samples (100 μ l) were taken from the incubation medium at time intervals and filtered on wet Millipore filters (HAWP, 0.45 μ m pore size). Filters were washed twice with 2 ml of medium that served for uptake, dried and counted for radioactivity in a scintillation mixture consisting of 2,5-dimethyloxazole (5 g) and toluene (1 litre). To obtain information on the stoichiometry of the sugar-proton co-transport, the following technique, referred to as the 'fast technique' was used: 12 samples containing less than 150 μ g dry wt. of bacterial cells were filtered every 6–7 s on filters supported by a Millipore sampling manifold (no. 1225) under continuous suction. In such conditions, the filtration step takes about 2 s so that O_2 contamination was minimized. At the end of the experiment, the filters were washed twice with 2 ml of N_2 -bubbled buffer that served in the incubation medium, then dried and counted for radioactivity as described above. Such experiments were repeated routinely twice, since the accuracy of this method is less than that with the standard technique. A background radioactivity was subtracted from gross values by extrapolating the linear portion of the uptake kinetics to zero time.

Stock solutions, standards and inhibitors

Stock solutions of 3-deoxy-2-oxo-D-gluconate (1 M) and D-glucuronate (0.5 M) were appropriately neutralized to $pH 7.00 \pm 0.05$ with 5 M-KOH or saturated $Ca(OH)_2$. For pH, pNa and pK titrations, standard HCl (50 mM), NaCl (0.1 M) and KCl (0.1 M) were made in water. Stock solutions of valinomycin (1.0 mg/ml), nigericin (1.0 mg/ml) and gramicidin D (1.0 mg/ml) were made in ethanol. When required, solutions were freed from O_2 by bubbling with pure N_2 .

Chemicals

Valinomycin and gramicidin D were from Calbiochem (Laboratoires Eurobio, Paris, France). Nigericin was generously given by Eli Lilly and Co., Indianapolis, IN, U.S.A., and obtained through Dr. B. A. Haddock, University of Dundee. D-Glucuronic acid was from Sigma (Paris, France). 3-Deoxy-2-oxo-D-gluconic acid (potassium salt) was prepared as described by Pouyssegur & Stoerber (1970). 3-Deoxy-2-oxo-D-[U- ^{14}C]gluconic acid, potassium salt (5 mCi/mmol) was synthesized by the method of Pouyssegur (1973). Other radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K.: D-[U- ^{14}C]glucuronic acid (potassium salt; 76 mCi/mmol); [3H]acetic acid (sodium salt; 250 mCi/mmol); [^{14}C]methylamine hydrochloride

(56 mCi/mmol). All other reagents were of analytical grade.

Results

Stoichiometry of the 3-deoxy-2-oxo-D-gluconate-proton and D-glucuronate-proton co-transport

My preliminary approach to the study of the mechanisms involved in the active transport system catalysing 3-deoxy-2-oxo-D-gluconate uptake in *E. coli* K12 has provided convincing evidence that it may be mediated by a sugar-proton(s) symporter, as postulated by the chemiosmotic theory of Mitchell (1969, 1970, 1976). Indeed, protons disappeared from

Fig. 1. Time course of extracellular proton disappearance and 3-deoxy-2-oxo-D-gluconate uptake in *E. coli* strain CK101

The electrode vessel contained in 10 ml: 0.15 M-KCl, 1.5 mM-glycylglycine and cells (26.1 mg bacterial dry wt.). The cell suspension was adjusted to pH 7.05 and equilibrated at 20°C for 15 min with continuous bubbling of N_2 , after which the chart recorder was started. At zero time an O_2 -free solution of 3-deoxy-2-oxo-D-[^{14}C]gluconate (0.45 μ Ci/ μ mol) was injected to give 1 mM final concentration. Every 6–7 s, 50 μ l of the suspension was rapidly filtered as described in the Materials and Methods section. The system was calibrated by injecting 5 μ l of 50 mM-HCl in 0.15 M-KCl. The buffering capacity of the outer phase (about 144 ng-ions of H^+ /pH unit per mg bacterial dry wt.) was calculated as described by Mitchell & Moyle (1967). ○, 3-Deoxy-2-oxo-D-gluconate uptake; ●, proton disappearance.

the medium when 3-deoxy-2-oxo-D-gluconate or D-glucuronate were added to lightly buffered suspensions of *E. coli* containing the carrier molecule specific for these sugars (Lagarde & Haddock, 1977). To gain further information on this basic mechanism, I examined the stoichiometry of the process, i.e. the number of protons translocated per sugar molecule. A typical experiment, depicted in Fig. 1, consisted of measuring sugar uptake, by using the fast technique described in the Materials and Methods section, and recording the simultaneous movements of protons from the external medium, as monitored by a pH electrode, under strictly anaerobic conditions. As observed previously (Lagarde & Haddock, 1977), the alkalization of the outer phase follows pseudo-first-order kinetics, with a time for half-equilibration of 8 s. In contrast, 3-deoxy-2-oxo-D-gluconate uptake proceeds linearly at least during the first minute of incubation. At that time, it was estimated that the sugar is concentrated about 10-fold over the external concentration. Such a high concentration ratio is not surprising, since the cells were not previously starved and are able to develop, before the sugar addition, a pH gradient of -2.0 ± 0.2 pH units, as measured by [3 H]acetate distribution (results not shown).

The comparison of initial rates of sugar uptake and sugar-induced proton uptake clearly indicates that at least two protons (2.23 ± 0.30) are taken up per sugar molecule. Although some variations were registered between different batches of cells, the mean stoichiometry value is near 2, as shown by several independent experiments (Table 1). It is the same for the two sugars transported by the 3-deoxy-2-oxo-D-gluconate transport system (Lagarde *et al.*, 1973) and is independent of the carrier concentration (compare the regulator-constitutive strain CK101 and the operator-constitutive strain PA3K1). In addition, maximal rates of 3-deoxy-2-oxo-D-gluconate-induced

proton uptake, obtained by increasing the external sugar concentration, were approx. 61 and 14 ng-ions of H^+ /min per mg dry wt. in strains CK101 and PA3K1. They are about twice the values of maximal rates of 3-deoxy-2-oxo-D-gluconate uptake, measured previously under aerobic conditions (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1975). The sensitivity of the pH-measuring equipment did not permit enough accurate measurements of the stoichiometry at an external pH far from neutrality. However, a preliminary investigation showed that the pH-dependence of 3-deoxy-2-oxo-D-gluconate uptake roughly paralleled the pH-dependence of the sugar-induced proton in the pH range 6–8 (optimum pH 6.5). Therefore it seems reasonable to propose that the stoichiometry is constant in the pH range studied.

Movements and influence of cations

The co-transport of two protons together with a sugar carrying a single anionic charge strongly suggested that the process is electrogenic: in the situation where the translocation leads to the net transfer of one positive charge across the membrane, an electrical potential, interior positive, will develop and is expected to impede the further transfer of charges inside, unless it is exactly compensated by the influx of an anion or the efflux of a cation. As shown in Fig. 2(a), when the calcium salt of D-glucuronic acid is added to cells suspended in a medium containing a low concentration of K^+ and Na^+ ions, no movement of either cation was observed by using ion-specific electrodes. This negative result cannot be interpreted unequivocally for the following reasons: (a) Ca^{2+} ions present in the medium could play the role of a counter-ion since a Ca^{2+} - H^+ antiport system is operating in *E. coli* (Tsuchiya & Rosen, 1976); (b) Cl^- ions could be

Table 1. *Number of protons translocated per sugar molecule*

Experimental conditions were identical with that described in Fig. 1 except that the cell concentration was 1.80–2.40 mg dry wt./ml. Each experiment refers to separate batches of cells and was repeated twice. Proton influx was measured during the first 5 s after sugar addition. Sugar influx was linear with time for at least 60 s.

Strain	Substrate added	Expt. no.	Initial pH	Proton uptake (ng-ions of H^+ /min per mg dry wt.)	Sugar uptake (nmol/min per mg dry wt.)	Protons translocated/sugar molecule (ng-ions of H^+ /nmol)
CK101	3-Deoxy-2-oxo- D-gluconate (1 mM)	1	7.00 ± 0.02	35.9 ± 0.7	14.2 ± 3.0	2.54
		2	7.05 ± 0.03	62.7 ± 3.2	23.5 ± 1.5	2.66
		3	7.10 ± 0.05	42.5 ± 6.4	23.4 ± 2.5	1.81
		4	7.05 ± 0.03	42.4 ± 1.3	19.8 ± 1.8	2.14
	D-Glucuronate (1 mM)	4	7.05 ± 0.03	15.3 ± 1.9	7.0 ± 1.5	2.18
PA3K1	3-Deoxy-2-oxo- D-gluconate (2 mM)	5	7.03 ± 0.04	19.3 ± 1.0	10.3 ± 2.0	1.88
						mean=2.20

Fig. 2. pH, pK and pNa recordings after addition of D-glucuronate to whole cells and spheroplasts of strain CK101

The electrode vessel contained in 10 ml: 0.2 M-sucrose, 2 mM-MgCl₂, 75 mM-choline chloride, 12.5 mM-CaCl₂, 0.125 mM-KCl, 0.75 mM-glycylglycine and 12.6 mg dry wt. of cells (a) or 11.6 mg of spheroplast protein plus valinomycin (1.8 μ M) (b). The pH of the suspension was adjusted to 7.00 ± 0.02 with saturated Ca(OH)₂ solution. At times indicated by arrows, the Ca²⁺ salt of D-glucuronic acid was injected to give 5 mM final concentration. The pH, pK and pNa traces were recorded with ion-specific electrodes as detailed in the Materials and Methods section. The systems were calibrated by injecting 5 μ l of 50 mM-HCl, 10 μ l of 0.1 M-KCl or 10 μ l of 0.1 M-NaCl respectively.

passively translocated inside; (c) protons could be translocated outside through the (Mg²⁺ + Ca²⁺)-dependent adenosine triphosphatase (EC 3.6.1.3), owing to the hydrolysis of endogeneous ATP; (d) the technique was not sensitive enough to detect the movement of only one positive charge, that is required to maintain the electroneutrality. At least, this experiment (Fig. 2a) excludes the possibility that D-glucuronate, and by extrapolation 3-deoxy-2-oxo-D-gluconate, is co-transported with Na⁺ ions instead of H⁺ ions in response to an electrochemical gradient of Na⁺ that could have been generated through the specific Na⁺-H⁺ antiport system (West & Mitchell, 1972, 1974).

In contrast with whole cells, spheroplasts of *E. coli* are sensitive to ionophores, so that the counter-movement of a cation during sugar uptake can normally be observed, provided that a specific

Table 2. Influence of ionophores and K⁺ concentration on D-glucuronate-induced proton uptake (strain CK101)

Experimental conditions were identical with that described in Fig. 1, except that spheroplasts (2.12 mg of protein/ml) were suspended in either 0.25 M-sucrose/2.5 mM-MgCl₂/60 mM-choline chloride/10 mM-CaCl₂/0.1 mM-KCl/0.6 mM-glycylglycine (K⁺-free buffer) or 0.25 M-sucrose/2.5 mM-MgCl₂/60 mM-KCl/0.6 mM-glycylglycine (K⁺ buffer). In addition, the ionophores were present at the concentration indicated. The pH of the media was adjusted to 7.00 ± 0.03 with saturated Ca(OH)₂ and 0.5 M-KOH respectively; the calcium and potassium salt of D-glucuronic acid was injected respectively to give 5 mM final concentration. Initial rates of proton uptake were measured after 5 s of incubation.

	Proton uptake (ng-ions of H ⁺ /min per mg of protein)	
	K ⁺ -free buffer	K ⁺ buffer
Without addition	37.2	32.1
With valinomycin (1.80 μ M)	42.5	69.9
With nigericin (2.76 μ M)	0	0
With gramicidin (1.00 μ M)	40.9	25.8

ionophore is also present. As shown in Fig. 2(b), there is still no movement of K⁺ ions on the addition of the Ca²⁺ salt of D-glucuronic acid to spheroplasts suspended in a K⁺-free medium and treated with valinomycin. However, K⁺ efflux was observed on adding valinomycin to an equilibrated suspension of the same spheroplasts, so that the absence of K⁺ movements associated with D-glucuronate uptake cannot be ascribed to the sensitivity of the electrode. The result would therefore suggest that under these conditions the electrogenic influx of D-glucuronic acid with protons is not compensated. The discrepancy between such a conclusion and the results presented in Table 2 appears difficult to interpret. The action of several ionophores on D-glucuronate-induced proton uptake was tested in media containing a low (0.1 mM) and a high (60 mM) concentration of K⁺ ions. It is clear that whereas protons are taken up at about the same rate in both media without added ionophore, there is a 2-fold stimulation elicited by valinomycin, but only when the KCl concentration is high. In any case, nigericin has a drastic inhibitory effect, because it dissipates the pH gradient by exchanging protons with K⁺ ions. Although gramicidin is known to exhibit a rather broad selectivity towards cations (Pressman, 1976), it weakly inhibits uptake in the high-K⁺ medium.

Magnitude of the pH gradient and of the sugar gradient

When an anion carrying a single negative charge is transported together with *n* protons, one expects the

following relationship to hold at thermodynamic equilibrium (Mitchell, 1969, 1970):

$$\log [S^-_{in}]/[S^-_{out}] = -n \Delta pH + (n-1) \Delta \psi / z \quad (1)$$

where $[S^-_{in}]$ and $[S^-_{out}]$ are the internal and external concentration of the anion, ΔpH and $\Delta \psi$ are the pH gradient and the membrane potential across the membrane, $z = 2.303 RT/F$ (F , Faraday constant; R , gas constant; T , temperature).

For an electroneutral symport ($n = 1$), eqn. (1) simplifies to:

$$\log [S^-_{in}]/[S^-_{out}] = -\Delta pH \quad (2)$$

whereas for an electrogenic symport ($n = 2$), the following equation should be obeyed:

$$\log [S^-_{in}]/[S^-_{out}] = -2 \Delta pH + \Delta \psi / z \quad (3)$$

It has been reported by several authors that the magnitude of the membrane potential generated in *E. coli* cells during respiration remains approximately constant over external pH values ranging from 6.0 to 8.0, whereas the magnitude of the pH gradient is decreasing (Padan *et al.*, 1976; Ramos *et al.*, 1976; Collins & Hamilton, 1976). This property was used to compare the sugar gradient ($\log [S^-_{in}]/[S^-_{out}]$) with the magnitude of the pH gradient as measured by acetate distribution (Nicholls, 1974). As shown in Fig. 3, this last method leads to ΔpH values that appear significantly higher than those reported by Padan *et al.* (1976). In no case was $[^{14}C]$ methylamine found to be accumulated inside the cells, indicating that even at alkaline external pH, the polarity of the pH gradient (inside alkaline) is not reversed. The concentration gradient of 3-deoxy-2-oxo-D-gluconate is higher than the pH gradient, especially at pH 8.0 (Fig. 3), so that we may conclude that the results do not fit eqn. (2), which describes an electroneutral symport. In addition, the experimental situation that I described is far from being optimal with respect to the sugar concentration gradient, since the external 3-deoxy-2-oxo-D-gluconate concentration that I used ($[S^-_{out}] = 42.5 \mu M$) corresponds to about one-half the K_m for influx (Lagarde & Stoeber, 1975).

No attempt has been made to measure the membrane potential ($\Delta \psi$), but if one assumes a minimum value of +80 mV as given by Padan *et al.* (1976), concentration factors of 1.5×10^6 (pH 6.0), 6.0×10^3 (pH 7.0) and 1.2×10^3 (pH 8.0) can be predicted for the sugar, according to eqn. (3) and taking ΔpH values from Fig. 3. The fact that these values are much higher than the experimentally observed sugar concentration factors with 3-deoxy-2-oxo-D-gluconate or D-glucuronate (Lagarde & Stoeber, 1975) will be discussed below. However, the lack of correlation found between the experimental results (Fig. 3) and the theoretical eqn. (2) strongly implicates an electrogenic proton symport for 3-deoxy-2-oxo-D-gluconate.

Fig. 3. Magnitude of the pH gradient and of the 3-deoxy-2-oxo-D-gluconate gradient in strain CK101

Cells (0.28 mg bacterial dry wt./ml) were incubated for 60 min at 20°C in 50 mM-sodium phosphate buffer at the pH indicated, in the presence of 3-deoxy-2-oxo-D- $[^{14}C]$ gluconate ($42.5 \mu M$, $5 \mu Ci/\mu mol$) and 22 mM-glycerol. After that period, which corresponded to steady-state accumulation, cells were filtered and washed with the same buffer. The internal concentration of sugar ($[S^-_{in}]$) was calculated by assuming an internal volume of $2.7 \mu l/mg$ bacterial dry wt. (Collins *et al.*, 1976). The magnitude of the pH gradient was obtained under the same conditions by determining the distribution of $[^3H]$ acetate ($10 \mu M$) and $[^{14}C]$ methylamine ($10 \mu M$) after a 5 min period of incubation, by the method of Nicholls (1974). The pH gradient ($-\Delta pH$, ●) and the logarithm of the ratio of internal to external sugar concentration (□) were plotted as a function of external pH.

Influence of cations and ionophores on 3-deoxy-2-oxo-D-gluconate uptake

If only protons are participating in the translocation of 3-deoxy-2-oxo-D-gluconate, there should be no specific effect of cations on the uptake of the sugar in intact whole cells. This prediction was verified when care was taken to keep constant the osmotic pressure, the pH and the ionic strength of the incubation medium. For instance, absolutely no difference in sugar-uptake rates was registered at pH 5.8 when the external concentration of KCl or

NaCl was increased from 10 μM up to 150 mM, keeping a constant external salt concentration (150 mM) with choline chloride (results not shown). Other cations were not tested.

If the 3-deoxy-2-oxo-D-gluconate transport is electrogenic it must be driven by the pH gradient and also by the membrane potential as indicated by eqn. (3). As a consequence, uptake should be inhibited by agents that collapse ΔpH , $\Delta\psi$ or both. We showed previously that proton-conducting agents such as carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol and tetrachlorosalicylanilide were potent inhibitors of 3-deoxy-2-oxo-D-gluconate uptake (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1974, 1975). I here describe experiments using nigericin, which abolishes only ΔpH by exchanging H^+ for K^+ ions (neutral antiport), and valinomycin, which can decrease the electrical potential by transferring K^+ ions electrogenically (Henderson, 1971; Pressman, 1976). The action of these ionophores on 3-deoxy-2-oxo-D-gluconate transport was tested between pH 5.5 and 8.0 in media containing no KCl (replaced by 0.1 M-choline chloride) or 0.1 M-KCl (Fig. 4). In the

absence of external K^+ ions valinomycin exhibits no effect at all (Fig. 4a), whereas the inhibition caused by nigericin is gradually relieved between pH 5.5 and 8.0. In the presence of K^+ ions (Fig. 4b), the same inhibition profile was observed with nigericin, except that the inhibition at pH 8.0 was not completely relieved, presumably because it also collapsed part of the membrane potential under these conditions (Harold & Altendorf, 1974). Uptake is still poorly affected by valinomycin at acidic pH, but is strongly inhibited at pH 8.0. Additional observations were made at pH 7.0: (a) uptake was abolished in the presence of nigericin plus valinomycin (plus 0.1 M-KCl), in agreement with the fact that such a combination of ionophores is equivalent to the action of proton-conducting agents (Hamilton, 1975, 1977; Harold, 1977); (b) there is no inhibition by 4 mM-sodium acetate; (c) gramicidin D (1.0 μM) inhibits sugar uptake by 30%, 65% and 70% in media containing each 0.1 M of choline chloride, KCl and NaCl respectively; (d) valinomycin has no effect if KCl is replaced by 0.1 M-NaCl.

Our results are consistent with the expected action

Fig. 4. Inhibition of 3-deoxy-2-oxo-D-gluconate uptake by ionophores at various pH values

Spheroplasts from strain CK101 (3.23 mg of protein/ml) were incubated in a medium containing 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer at the pH indicated, 2.5 mM- MgCl_2 , 0.2 M-sucrose, 44 mM-glycerol and either 0.1 M-choline chloride (a) or 0.1 M-KCl (b), 3-deoxy-2-oxo-D-[^{14}C]gluconate (42.5 μM) in the presence of ethanol (2 μl), valinomycin (1.8 μM) or nigericin (2.76 μM). Samples were filtered and washed at time intervals over a 5 min period. Percentage inhibition of uptake was calculated by comparing initial rates (1 min incubation) in the absence and presence of added ionophore. $\square$, With valinomycin; $\blacksquare$, with nigericin.

Fig. 5. 3-Deoxy-2-oxo-D-gluconate uptake driven by an artificially induced pH gradient in membrane vesicles

Isolated membrane vesicles from strain CK101 (0.954 mg of protein/ml) were suspended in 25 mM-potassium phosphate buffer, pH 8.0, containing 10 mM-MgSO₄, 1.8 μ M-valinomycin and 42.5 μ M-3-deoxy-2-oxo-D-[¹⁴C]gluconate. (a) At zero time (indicated by the arrow) the pH of the medium was lowered to 4.76 by adding 10 μ l of 2M-HCl. At time intervals, samples were filtered and washed with 0.25M-sucrose/10mM-MgSO₄. The internal concentration of sugar ($[S^-_{in}]$) was calculated on a volume basis of 2.2 μ l/mg of membrane protein (Ramos *et al.*, 1976). A duplicate experiment was run under the same conditions except that 42.5 μ M-3-deoxy-2-oxo-D-

of nigericin and valinomycin between pH 5.5 and 8.0, due to the fact that the relative contribution of the pH gradient to the total protonmotive force is known to decrease within this pH range (Fig. 3) (Padan *et al.*, 1976; Ramos *et al.*, 1976). 3-Deoxy-2-oxo-D-gluconate uptake is therefore mostly driven by ΔpH at acidic pH and by $\Delta\Psi$ at alkaline pH.

3-Deoxy-2-oxo-D-gluconate uptake driven by an artificially induced pH gradient in membrane vesicles

The pH gradient and the membrane potential are normally generated in intact cells or organelles by respiration or ATP hydrolysis, both processes being catalysed by the membrane-bound respiratory chain and the adenosine triphosphatase complex (Mitchell, 1966, 1976). As a corollary from the chemiosmotic concept of active transport, one expects to produce the accumulation of metabolites by an artificially induced pH gradient or a membrane potential, under conditions where the physiological generators of protonmotive force are suppressed or rendered non-functional. Isolated membrane vesicles of *E. coli* proved to satisfy this last condition, since they are mostly of the right-side-out orientation, are devoid of endogenous energy reserves and consequently are unable to respire or synthesize ATP unless the suitable substrates are correctly supplied (Kaback, 1972). Vesicles from strain CK101 were previously shown to accumulate 3-deoxy-2-oxo-D-gluconate in the presence of added O₂ and respiratory substrates (Lagarde & Stoeber, 1974). Examples of artificially induced nutrient uptake and accumulation in micro-organisms are numerous (Kashket & Wilson, 1973; Ashgar *et al.*, 1973; Hirata *et al.*, 1973, 1974; Niven & Hamilton, 1974; Altendorf *et al.*, 1975; Flagg & Wilson, 1976).

The basic experiment for generating a pH gradient consisted of suspending vesicles from strain CK101 in 25 mM-potassium phosphate buffer, pH 8.0, and then adding a strong acid to lower the external pH quickly. The transient pH gradient that is created under these conditions is of the same polarity (inside alkaline) as that developed in whole cells or vesicles by respiration (Padan *et al.*, 1976; Ramos *et al.*, 1976;

[¹²C]gluconate was present together with 10 μ M-[³H]-acetate and 10 μ M-[¹⁴C]methylamine. The pH gradient was assayed by the method of Nicholls (1974). ●, ΔpH ; □, $\log[S^-_{in}]/[S^-_{out}]$. (b) Experimental conditions were the same as described in (a) with the following modifications: ●, no modification; ■, without valinomycin; △, with 1.8 μ M-valinomycin and 2.76 μ M-nigericin; ▲, with 2.76 μ M-nigericin alone; ○, 3-deoxy-2-oxo-D-[¹⁴C]gluconate was added 2 min after the HCl injection (indicated by the second arrow); □, the pH of the medium was lowered to 6.36 by injecting 5 μ l of 2M-HCl.

Collins & Hamilton, 1976), as evidenced by the fact that [^3H]acetate is accumulated (Fig. 5a). 3-Deoxy-2-oxo-D-gluconate is concomitantly concentrated inside the vesicles up to 14-fold over the external sugar concentration (Fig. 5a). For comparison, the same vesicles were found to accumulate the sugar about 6-fold in the presence of O_2 and 20 mM-D(-)-lactate (results not shown). The pH gradient is rapidly dissipated because of the carrier-mediated entry of protons and of the increase of the proton conductivity of the membrane (Mitchell & Moyle, 1967). If the sugar is added only 2 min after the acid transition was initiated, it is no longer taken up (Fig. 5b). If valinomycin is omitted, the sugar is accumulated to only one-half the amount found in its presence (Fig. 5b). This result is expected only in the case where the co-transport of protons with 3-deoxy-2-oxo-D-gluconate is electrogenic, because the transfer of one positive charge will develop a membrane potential, positive inside, that impedes the subsequent influx. By cycling K^+ ions out, valinomycin dissipates the diffusion membrane potential. Sugar accumulation is weaker if the magnitude of the acidic transition is lower (Fig. 5b). There is no sugar accumulation in the presence of nigericin, which immediately dissipates the pH gradient by exchanging external H^+ ions for internal K^+ ions (Fig. 5b). However, in the presence of both valinomycin and nigericin a small influx of 3-deoxy-2-oxo-D-gluconate is observed. The most likely interpretation is that valinomycin, in catalysing rapid efflux of K^+ ions in response to the electrogenic transfer of one positive charge mediated by the sugar carrier, decreases the probability for nigericin to catalyse the H^+ for K^+ exchange and therefore minimizes the dissipation of the pH gradient.

I tried without success a number of procedures intended to load membrane vesicles with a high concentration of K^+ ions: incubation of vesicles with 0.5 M-potassium phosphate buffer, pH 8.0, for 20 min at 36°C, for 30 min at 40°C (Hirata *et al.*, 1974), or for 30 min at 0°C in the presence of 1.8 μM -valinomycin. The failure to find the proper conditions, which could be related to the much higher leakiness of membranes from *E. coli* ML30 strains compared with K12 strains, prevented the demonstration that 3-deoxy-2-oxo-D-gluconate could be accumulated in response to an artificially induced membrane potential.

Discussion

Active transport is classically defined as the movement of a solute against its own electrochemical gradient. Such a process must be associated with energy dissipation. Non-equilibrium thermodynamics predicts that in a given system one diffusional flow may be coupled to either another diffusional flow or

alternatively to a spatially orientated chemical reaction (Katchalsky & Curran, 1967; Katchalsky & Oster, 1969; Caplan, 1971; Heinz, 1974). Example of the last-mentioned type of energy coupling is best illustrated in micro-organisms by the phosphorylation of certain sugars catalysed by phosphoenolpyruvate phosphotransferase (for a review see Postma & Roseman, 1976). We gave genetic evidence that such a mechanism does not operate in 3-deoxy-2-oxo-D-gluconate transport in *E. coli* K12 (Lagarde *et al.*, 1973). Therefore our interest was directed toward the possible coupling between the flux of the sugar and that of another chemical species. We were guided by the experimental observation that 3-deoxy-2-oxo-D-gluconate uptake is very sensitive to proton-conducting agents (Lagarde *et al.*, 1973; Lagarde & Stoeber, 1974, 1975), and also by the increasing evidence that proton circulation across the membrane plays an essential role in the energy cycle of mitochondria, chloroplasts and micro-organisms (Greville, 1969; Harold & Altendorf, 1974; Hamilton, 1975, 1977; Harold, 1977).

The two basic concepts that were put forward by Mitchell (1966, 1969, 1970, 1976) in the chemiosmotic theory of active transport were: (i) an electrochemical gradient of protons (inside alkaline and negative) is generated across the membrane by respiration or ATP hydrolysis; (ii) membrane-bound carrier molecules play the role of a coupling device in being able to translocate either neutral or negatively charged molecules together with protons. The occurrence of a 3-deoxy-2-oxo-D-gluconate-proton symporter was suggested by experiments showing protons movements linked specifically to the functioning of the transport system in the *E. coli* membrane (Lagarde & Haddock, 1977).

In examining the stoichiometry of the co-transport I found that about two protons are translocated for each 3-deoxy-2-oxo-D-gluconic or D-glucuronic acid molecule (Fig. 1, Table 1). Since these two compounds are monocarboxylic sugars, the electro-impelled transfer of one net positive charge inside was expected from such a high stoichiometry. As a consequence, the transport of 3-deoxy-2-oxo-D-gluconate was supposed to be driven not only by the pH gradient, but also by the membrane potential. Experimental findings that are consistent with such a view are: (a) the inadequacy of the magnitude of the pH gradient alone, developed across the membrane by respiring cells, to account for the sugar gradient, especially at alkaline pH values (Fig. 3); (b) the rather good correlation between the contribution of ΔpH and $\Delta\psi$ components to the total protonmotive force generated during respiration between pH 5.4 and 8.0 (Padan *et al.*, 1976; Ramos *et al.*, 1976) and the inhibition of 3-deoxy-2-oxo-D-gluconate uptake caused by nigericin and valinomycin, in accordance with the well-known

mode of action of these ionophores (Fig. 4); (c) the possibility to induce a transient accumulation of 3-deoxy-2-oxo-D-gluconate in non-respiring isolated membrane vesicles by generating an artificial pH gradient (Fig. 5). The stimulation obtained in the presence of valinomycin is taken as a further indication that sugar influx is electrogenic. The parallel demonstration of a membrane-potential-induced sugar accumulation requires further investigation because of technical difficulties. The fact that I failed to detect a counter-movement of K^+ ions during D-glucuronate uptake into spheroplasts (Fig. 2b) deserves additional analysis since it would contradict the electrogenic character of the sugar-proton symport and would be in contrast with the results obtained in the case of lactose symport (West & Mitchell, 1972; Collins *et al.*, 1976).

In the same way as neutral molecules may be transported with one proton (West & Mitchell, 1973), having a higher number of protons translocated per anion appears to be one of the possibilities to improve the efficiency of the accumulation process. Numerous anions seem to be transported in micro-organisms with a number of protons equivalent to the total negative charge of the anion, to give a strictly electroneutral process: gluconic acid in *E. coli* (Robin & Kepes, 1973) and *Clostridium pasteurianum* (Booth & Morris, 1975), glutamic acid in *Staphylococcus aureus* (Niven & Hamilton, 1974), hexose phosphates (Essenberg & Kornberg, 1975), D-(–)-lactic acid (Collins *et al.*, 1976) and dicarboxylic acids in *E. coli* (Gutowski & Rosenberg, 1975). In contrast, unusual super-stoichiometry has been observed in glycine transport in *Saccharomyces carlsbergensis* (Seaston *et al.*, 1976) and 1-deoxy-D-glucose transport in *Chlorella vulgaris* (Grüneberg & Komor, 1976). The general significance of such stoichiometries is unknown so far. Fundamental questions that remain to be explored concern the number and position of proton-binding sites on the 3-deoxy-2-oxo-D-gluconate carrier molecule, the electrical charge of the free carrier, the sequential order of binding of the protons and of the sugar anion.

The magnitude of the protonmotive force in *E. coli* (Padan *et al.*, 1976; Ramos *et al.*, 1976; Collins & Hamilton, 1976) appears sufficient to drive 3-deoxy-2-oxo-D-gluconate uptake, since eqn. (3) predicts a 1.5×10^6 -fold accumulation for the sugar at pH 6.0. However, the maximum concentration factor of 3-deoxy-2-oxo-D-gluconate in cells respiring glycerol cannot exceed 1.5×10^3 (Lagarde & Stoeber, 1975). Possible reasons that might explain such a marked difference may be relevant to a misuse of thermodynamics. It seems reasonable to assume, as predictable from irreversible thermodynamics (Kedem & Caplan, 1965), that the degree of coupling between two linked processes such as uptake of H^+ and sugar does not have a constant value, but must

depend in part on state parameters such as concentrations of the chemical species on both sides of the membrane and kinetic parameters of the carrier protein. It is well known, for instance for 3-deoxy-2-oxo-D-gluconate transport (Lagarde & Stoeber, 1975), that the sugar concentration gradient is a decreasing function of the external sugar concentration, an increasing function of the carrier concentration and also of the affinity of this carrier for its substrates. The degree of coupling (Kedem & Caplan, 1965) is a composite function of the conductance coefficients that relate the net fluxes to their driving forces, which in the present case should be both the electrochemical gradient of protons and the electrochemical gradient of sugar. In the general case these coefficients depend on state parameters (Lagarde, 1976) and as a consequence the stoichiometric coefficients appearing in the equation relating the sugar gradient to the components of the driving force (eqn. 1) have no fixed values. This seems to be the case for the calcium conductance in rat liver mitochondria inner membrane (Heaton & Nicholls, 1976). I suggest that future work dealing with the comparison between the driving force(s) and the driven gradient catalysed by a symport mechanism should be directed toward the evaluation of the degree of coupling. Such an approach has been adopted for Na^+ -dependent transport systems in eukaryotic cells (Geck *et al.*, 1972; Heinz, 1974; Heinz & Geck, 1974; Lahav *et al.*, 1976).

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