Proton/Sodium Ion Antiport in *Escherichia coli*

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In anaerobic suspensions of *Escherichia coli*, after H^+ ions have been translocated outwards across the plasma membrane by a respiratory pulse, re-equilibration is catalysed by Na^+ . The sudden addition of a Na^+ salt causes the effective outward translocation of H^+ by an electroneutral process. We conclude that the plasma membrane of *E. coli* contains a Na^+/H^+ antiport system that normally translocates Na^+ outwards under the influence of an inwardly directed H^+ -activity gradient.

The high K^+/Na^+ ratio in the cytoplasm of *Escherichia coli* has generally been attributed more to inward pumping of K^+ than to outward pumping of Na^+ , and this may perhaps explain why much less work has been done on the sodium metabolism than on the potassium metabolism of *E. coli* (see Rothstein, 1972). However, in view of the evidence for a large trans-membrane electric potential negative inside the cells (Harold, 1972), both K^+ and Na^+ would be expected to be drawn into the cytoplasm. It is therefore interesting to look at the exergonic reactions, which may be used to pump out the cations. Mitchell (1966) suggested that the accumulation of intramitochondrial (or intracellular) cations, resulting from the membrane potential, would be prevented by the operation of tightly coupled exchange-diffusion systems, catalysing, for example, the exchange of Na^+ for H^+ . The specific proposal is that the flow of Na^+ in one direction on the antiporter is strictly coupled to the flow in the opposite direction of either Na^+ or H^+ . The well-studied artificial K^+/H^+ antiporter, nigericin, provides a model for such a mechanism (see Pressman, 1968).

A system catalysing Na^+/H^+ antiport in the cristae membrane of mammalian mitochondria has been detected by pH measurements that show catalysis of H^+ equilibration by Na^+ (Mitchell & Moyle, 1967), and by osmotic swelling experiments that show the electroneutral entry of Na^+ in exchange for H^+ in iso-osmotic sodium acetate or other analogous media (Brierley *et al.*, 1968; Mitchell & Moyle, 1969b). Harold & Papineau (1972) provided evidence for a Na^+/H^+ antiporter in *Streptococcus faecalis* by showing that the extrusion of Na^+ was inhibited by proton-conducting uncouplers, and they isolated a mutant which appeared to lack the Na^+/H^+ antiporter. This phenotypic defect could be partially compensated for by adding monensin, an antibiotic which, like nigericin, catalyses the strictly coupled exchange of uni-

valent cations, though with a higher specificity for Na^+ and H^+ than for K^+ (Pressman, 1968).

In the present paper, two experimental procedures have been used to detect the activity of a Na^+/H^+ antiporter in *E. coli*. One is similar to the pH method used in the experiments on mammalian mitochondria mentioned above, and the other method corresponds to that used to demonstrate β -galactoside- H^+ symport in *E. coli* (West, 1970).

As before (West & Mitchell, 1972), we describe the movement of H^+ in one direction and the movement of OH^- in the opposite direction as 'effective' movements of H^+ , as they cannot be distinguished in this type of study.

Materials and Methods

Reagents

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine was a gift from Dr. P. G. Heytler of E. I. du Pont de Nemours and Co. (Wilmington, Del., U.S.A.), and carbonic anhydrase was obtained from Sigma (London) Chemical Co. Ltd. (Kingston-upon-Thames, Surrey, U.K.). The other chemicals were of AnalaR grade, where available, and were obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.).

Organism

The bacterial strain used in this work was *E. coli* ML 308-225 (i^- , z^- , y^+), constitutive for β -galactoside accumulation but lacking β -galactosidase, which was kindly provided by Dr. T. H. Wilson, Harvard Medical School, Boston, Mass., U.S.A.

Culture and preparation of bacteria and membrane vesicles

Stock cultures were maintained on solid medium [medium C of Roberts *et al.* (1955), containing 0.4 g of

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glucose and 1 g of agar/100ml]. The liquid culture medium used was that of Mager & Magasanik (1960), with succinate as carbon source, and contained (in 1 litre): 7.0 g of KH_2PO_4 , 3.2 g of Na_2HPO_4 , 2.0 g of $(\text{NH}_4)_2\text{SO}_4$, 0.41 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg of CaCl_2 and 2.5 g of succinic acid. The medium was adjusted to pH 7.0 with KOH. A small inoculum from the agar slope was grown for 24 h at 25°C in 4 ml of liquid medium, which was then added to 200 ml of medium in a 1-litre flask. This was incubated at 37°C for 17 h under aerated conditions. The organisms were harvested by centrifugation at 4°C for 5 min at 15000g 1 h after the stationary phase of growth (0.5–0.7 mg dry wt. of organisms/ml) was reached, when the E_{700} was about 1.0. Under these conditions, the harvested cells were depleted of carbohydrate reserves, and the rate of endogenous glycolysis was very low. The organisms were washed twice at 4°C with 150 mM-KCl buffered with 3.0 mM-glycylglycine (adjusted to pH 7.0 with KOH), and suspended at a density of 40–50 mg dry wt./ml in the same KCl-glycylglycine medium. This stock bacterial suspension was stored at 4°C, and was used for experiments for up to 5 h.

Stock solutions, standards and inhibitors

The stock solution of the proton-conducting uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was made up at a concentration of 1 $\mu\text{mol}/\text{ml}$ in ethanol. For pH titrations, standard HCl (50.0 mM) and KOH (50.0 mM) were made up in 100 mM-KCl. Aqueous solutions were freed of O_2 , where required, by evacuating and flushing with O_2 -free N_2 . The ethanolic solution of uncoupler was freed of O_2 by bubbling a stream of O_2 -free N_2 , saturated with ethanol vapour, through it for 10 min.

Method for measuring pH changes

The experimental vessel, electrodes, electrometers and recording systems were as described previously (Mitchell & Moyle, 1969a). The closed, temperature-controlled, electrode vessel (final volume 4.0 ml, temperature 25°C) was filled with anaerobic incubation medium, which was dispensed from a glass syringe through a narrow inlet tube. Stock cell suspension was added and the pH was adjusted to 7.0–7.1. Air was excluded by directing a stream of O_2 -free N_2 at the mouth of the inlet tube, and residual O_2 in the well-stirred suspension was consumed by the bacteria within 1–2 min. Anaerobic HCl and KOH standards, anaerobic solutions of proton conductor, and anaerobic salt solutions were injected into the electrode vessel from Agla micrometer syringes (Burroughs Wellcome and Co., Dartford, Kent, U.K.).

Results and Discussion

Na⁺ catalysis of pH equilibration

Cells were suspended in anaerobic medium in the electrode vessel, as described above. After 40 min incubation, to allow the intracellular and extracellular media to equilibrate (the pH being adjusted occasionally with standard acid or alkali to compensate for the slow pH drift), 50 μl of air-saturated, CO_2 -free, unbuffered medium was injected to cause a brief pulse of respiration. The incubation medium included the permeant anion SCN^- (Mitchell & Moyle, 1969b) to allow a rapid ejection of H^+ ions to occur without the development of an appreciable membrane potential.

Fig. 1(a) shows the rapid acidification of the suspension medium, caused by the respiratory pulse, and the gradual return of the trace to the baseline in the absence of Na^+ . The acceleration of the collapse of the pH difference by 10 mM- Na^+ is shown in Fig. 1(b).

Fig. 2 shows semi-logarithmic plots of the effective quantity of H^+ translocated during the decay of the pH difference induced by a brief respiratory pulse. In the absence of Na^+ , the decay was exponential, and in this experiment the half-time of decay was 130 s. The presence of Na^+ at 5 and 10 μg -ions/ml increased the rate of decay, but a higher concentration of Na^+ (75 mM) decreased the rate of decay. In another set of experiments, 135 mM- Na^+ gave a result almost identical with that given by 75 mM- Na^+ . These results are consistent with the operation of a Na^+/H^+ antiporter catalysing net H^+ diffusion in the presence of Na^+ , and catalysing Na^+/Na^+ antiport rather than Na^+/H^+ antiport when saturated with Na^+ at high Na^+ concentrations. This result is like that of the

Fig. 1. Time-course of change in extracellular pH of anaerobic *Escherichia coli* suspensions caused by a brief pulse of respiration

The electrode vessel contained in 4 ml: (a) 100 mM-KCl, 50 mM-KSCN, 2 mM-glycylglycine, 16.9 mg dry wt. of cells; (b) 90 mM-KCl, 10 mM- NaCl , 50 mM-KSCN, 2 mM-glycylglycine, 16.9 mg dry wt. of cells. The suspension was brought to pH 7.1 and equilibrated anaerobically at 25°C for 40 min. The oxygen was injected as 50 μl of air-saturated solution having the same composition as the suspension medium, but omitting glycylglycine.

Fig. 2. Semi-logarithmic plots of the time-course of the quantity of H^+ translocated during the decay of the pH difference induced by a respiratory pulse

Details are as in Fig. 1. The Na^+ concentration (μg -ions/ml) were: (a) 0; (b) 5; (c) 75; and (d) 10. The K^+ concentration was varied so that the sum of the Na^+ and K^+ concentrations was $150 \mu g$ -ions/ml. The oxygen was injected as in Fig. 1, but at zero time.

Fig. 3. Strip-chart recordings of responses of H^+ ion-sensitive electrode on adding anaerobic salt solutions to anaerobic suspensions of *E. coli*

The electrode vessel contained in 4ml: 100mM-KCl, 50mM-KSCN, 0.4mM-glycylglycine, 23.0mg dry wt. of cells. The suspensions were equilibrated anaerobically at pH 7.1 as in Fig. 1. (a) $10 \mu l$ of 2M-NaCl was injected at the arrow; (b) $10 \mu l$ of 2M-choline chloride was injected at the arrow; (c) as in (a) but without cells, with 2.0mM-glycylglycine in place of 0.4mM-glycylglycine, and 2M-NaCl was injected at the arrow.

Fig. 4. Strip-chart recordings of response of H^+ ion-sensitive electrode on adding anaerobic NaCl solutions to anaerobic suspensions of *E. coli*: effects of proton conductor and permeant anion

Cells were suspended at a density of 4.9mg dry wt./ml in the media described below, and were equilibrated anaerobically at pH 7.1 as in Fig. 1. Anaerobic injections of $10 \mu l$ of 2M-NaCl or $8 \mu l$ of 1mM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were made as indicated. The composition of the suspending media was as follows: (a) and (b) 100mM-KCl, 50mM-KSCN, 2mM-glycylglycine; (c) as (a) and (b) with addition of $2 \mu M$ -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; (d) 150mM-KCl, 2mM-glycylglycine.

analogous experiment on the lactose- H^+ symporter of *E. coli* (West & Mitchell, 1972).

Effective outflow of H^+ caused by Na^+ inflow

Cells were suspended anaerobically, and were equilibrated for 40min, as in the previous experiments. A small volume ($10 \mu l$) of anaerobic 2M-NaCl was then injected and the pH of the suspension was measured for the subsequent few minutes (Fig. 3). There appeared to be a rapid initial acidification of the extracellular medium followed by a slower, but more extensive, acidification. This second slower phase was not observed when 2M-choline chloride replaced the 2M-NaCl (Fig. 3b), and it may therefore be attributed to the addition of Na^+ , and not of Cl^- .

The initial rapid electrode response is evidently due, in part, to an effect of the salt on the buffer or glass, as can be seen from the small electrode response on adding 2M-NaCl in the absence of cells (Fig. 3c). The larger, but still rapid, response with cells present (Fig. 3b) is perhaps attributable to an effect of the

added salt on the ionization of protonated groups of the cell exterior. The subsequent slower appearance of H^+ ions in the extracellular medium (Fig. 3a), on the other hand, has the kinetic characteristics expected of an H^+ outflow coupled to an inflow of Na^+ across the plasma membrane.

As shown in Fig. 3(b), choline chloride did not elicit this effective H^+ outflow. There was some effective H^+ outflow with KCl, though it was slower than that induced by NaCl, whereas that induced by LiCl was more rapid than the NaCl-induced outflow.

It was important to rule out the possibility that an electrogenic inward movement of Na^+ might be causing a passive leakage of H^+ out of the cells down the electrochemical potential gradient. Fig. 4(a) shows the effect of adding a proton-conducting uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine) to a suspension of cells in a medium containing 50 mM- SCN^- during the effective outflow of H^+ caused by the addition of NaCl. After a small acidification, possibly due to residual oxygen in the ethanolic solution of the uncoupler, there was a very rapid inflow of H^+ , and the pH trace returned to the original baseline. However, the addition of the uncoupler alone caused a small effective inflow of H^+ (Fig. 4b), whereas the addition of NaCl after the uncoupler (Fig. 4c) caused a small net acidification of the suspension. In the absence of SCN^- (Fig. 4d), the inflow of H^+ , after addition of the uncoupler, was much slower. These experiments show that when NaCl is added to the suspension, H^+ is effectively forced out of the cells against its electrochemical potential gradient. When the proton-conducting uncoupler is added, H^+ is able to flow back in again electrogenically, this process being greatly facilitated when the permeant anion SCN^- is present to compensate for the inflow of positive charge. If, instead of being forced out against the electrochemical potential gradient, the H^+ had been effectively leaving the cells passively down the gradient, the uncoupler should not have decreased, but should have increased, the pH change observed on adding the NaCl.

The rate of acidification on addition of Na^+ to the suspension was very little affected by the presence of 50 mM- SCN^- (Fig. 4a and 4d), and it therefore seems likely that Na^+ entry is part of an electroneutral process, as would be the case for the postulated strict exchange of Na^+ for H^+ . This conclusion is further supported by the observation that the rapid H^+ inflow (Fig. 4a) required a mobile counter-ion (SCN^-), implying that the electrogenic movement of Na^+ across the membrane was not nearly as rapid as that of H^+ in the presence of the proton conductor or of SCN^- . We attempted to use osmotic-swelling experiments analogous to those used successfully to demonstrate Na^+/H^+ antiport in mitochondria (Mitchell & Moyle, 1969b), both with whole cells of *E. coli* and with osmotically sensitive membrane

vesicles prepared as described by Kaback (1971), but the results were unfortunately ambiguous, because we were not able to obtain complete inhibition of the respiratory chain of *E. coli* under the aerobic conditions used for these experiments.

Conclusions

Two of the predicted consequences of the operation of a strictly coupled, electroneutral, Na^+/H^+ antiport (or exchange) are demonstrated with suspensions of *E. coli*. First, Na^+ ions catalyse the equilibration of H^+ ions displaced from the cell by a respiratory pulse. Secondly, addition of Na^+ ions to an anaerobic cell suspension causes the effective expulsion of H^+ ions against their electrochemical potential gradient by an electroneutral process. Such a Na^+/H^+ antiport mechanism, found also in mitochondria and in *Streptococcus faecalis*, probably plays an important part in regulating the internal Na^+ concentration of the cell (Mitchell, 1970).

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