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Cation Transport and Electrogenesis by *Streptococcus faecalis*

II. Proton and Sodium Extrusion

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Summary. Glycolyzing cells of *Streptococcus faecalis* 9790 accumulate large amounts of lipid-soluble cations such as dimethyldibenzylammonium (DDA^+). We showed in the preceding paper that uptake of DDA^+ occurs in response to an electrical potential, interior negative, which arises by extrusion of H^+ and Na^+. The experiments described here deal with the mechanism of electrogenesis. Evidence is presented to indicate that extrusion of protons is an electrogenic, energy-linked process which can proceed against the electrochemical gradient for H^+. Proton extrusion is blocked by dicyclohexylcarbodiimide (DCCD), an inhibitor of the membrane-bound ATPase of *S. faecalis*. However, in a mutant whose ATPase is resistant to DCCD, proton extrusion is also resistant to the inhibitor. We conclude from these results that the membrane-bound ATPase is involved in proton extrusion. Extrusion of sodium can also occur against the electrochemical gradient, but we find no evidence for the existence of an electrogenic sodium pump. It rather appears, from studies with ionophorous agents and inhibitors of the ATPase, that the cells extrude Na^+ in exchange for H^+; the H^+ is then extruded by the proton pump. Evidence is presented for an influx of H^+ coupled to the efflux of Na^+.

Among the mutants known to be defective in K^+ accumulation one class is deficient in proton extrusion and another lacks the Na^+/H^+ exchange. Thus, proton extrusion and Na^+/H^+ antiport are essential elements in K^+ accumulation.

Glycolyzing cells of *Streptococcus faecalis* accumulate large amounts of the lipid-soluble cations dibenzyldimethylammonium (DDA^+) and triphenylmethylphosphonium ($TPMP^+$) by exchange for Na^+, but not by exchange for K^+. The characteristics of this accumulation were considered in the preceding paper (Harold & Papineau, 1972) in which we concluded that uptake of the cations is not due to active transport against the electrochemical gradient. Rather, the cells appear to generate a considerable electrical potential across the membrane, interior negative, and uptake of DDA^+ and $TPMP^+$ occurs in response to this potential.

The experiments described in the present paper constitute an attempt to analyze the mechanism of electrogenesis and to assess its role in K^+

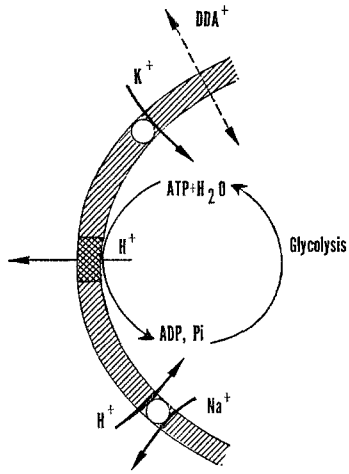

Fig. 1. Possible interrelationships of cation movements in *S. faecalis*. Symbols after Mitchell (1970), to designate the proton translocating ATPase and carrier-mediated Na^+/H^+ antiport and K^+ uniport. The carrier mediated processes may be independently coupled to metabolism, and are not freely reversible. Passive diffusion of DDA^+, indicated by the broken arrow, is reversible

uptake. The findings are consistent with the scheme shown in Fig. 1 which derives originally from Mitchell's chemiosmotic hypothesis (Mitchell, 1966, 1968, 1970). We propose that glycolyzing cells can extrude protons electrogenically against both the electrical and the concentration gradients. The membrane-bound ATPase is implicated in this process, which is a case of true active transport. Sodium can also be extruded against the electrochemical gradient, but there appears to be no electrogenic sodium pump. Instead, sodium efflux occurs by obligatory exchange for H^+ (Na^+/H^+ antiport) as originally envisioned by Mitchell (1966, 1968; Mitchell & Moyle, 1969); the protons are extruded by the pump. The electrical potential thus generated is a necessary condition, but perhaps not a sufficient one, for the accumulation of K^+ by the cells.

Materials and Methods

Organisms and Cell Preparations

In addition to the wild type *Streptococcus faecalis* 9790 we employed two mutants which require high levels of K^+ for growth. Mutant 687 A has a genetic defect in net uptake of K^+ by exchange for H^+, while mutant 7683 is deficient in Na^+ extrusion (Harold, Baarda & Pavlasova, 1970*a*).

Mutant DC-8 which is resistant to dicyclohexylcarbodiimide (DCCD) has recently been isolated by Abrams, Smith, and Baron (*in preparation*).

The preparation of KTY, NaTY, sodium-loaded and H^+-loaded cells was described in the preceding paper (Harold & Papineau, 1972). Some of the present experiments

were conducted with cells depleted of H^+ by alkaline washing: cells from either KTY or NaTY medium were washed three times at room temperature with 0.1 N Na_2CO_3 and then three times with water. Viability was not reduced by the carbonate wash but, as had been reported earlier by Chesbro and Evans (1960), the amino acid pool was largely lost from the cells. Moreover, part of the K^+ complement of the cells was replaced by Na^+ (this was especially pronounced in mutant 7683).

Analytical Methods

The H^+ content of the cells was determined as titratable acidity. A sample of cells (5 mg dry weight) was collected on a millipore filter, washed with 1 ml of water and the filter placed in a tube containing 2 ml of 9% (by volume) of *n*-butanol in water in a 40°C water bath. Disruption of the permeability barrier is complete in approximately 20 sec. After 5 to 10 min the tubes were cooled, 0.20 ml 0.5 N Na_2SO_4 were added and the pH was read to two significant figures. The readings were converted into changes of titratable H^+ content by means of the calibration curve illustrated in Fig. 2*A*. It should be recognized that the resulting value refers to the total available H^+ in the cells and not to the internal pH. The values obtained with carbonate-washed cells, pH 7.2 to 7.3 were taken as a titratable H^+ content of 0 μmoles/g cells.

To check the validity of the method, H^+-loaded *intact* cells were titrated on the pH-stat at pH 8.0 with standard alkali. Potassium, DDA^+ or $TPMP^+$ served as counterions. Release of H^+ as determined by alkali consumption agreed well with the loss of protons as determined after disruption with butanol (Fig. 2*B*).

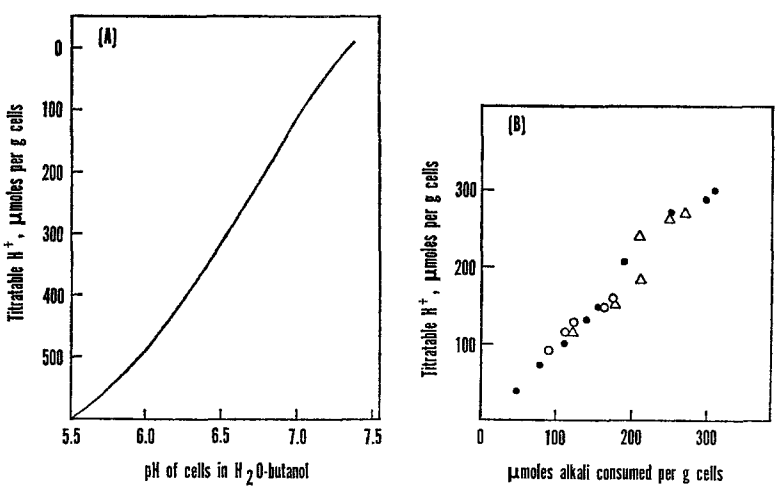

Fig. 2. Determination of the H^+ content of cells by titration. (*A*) Calibration curve. H^+-loaded cells (100 mg dry weight in 50 ml water) were disrupted by incubation with butanol and titrated with standard alkali. The alkali added is expressed as μmoles titratable H^+ per g cells and plotted against the pH of the suspension. (*B*) Suspensions of *intact*, H^+-loaded cells were titrated on the pH-stat at pH 8.0 with standard alkali (no glucose). Counterions were K^+, (•), DDA^+ (o) and $TPMP^+$ (Δ). Alkali consumption, per g dry weight of cells, is plotted on the abscissa. At intervals, samples were filtered and disrupted with butanol. The release of titratable H^+, calculated from the calibration curve, is plotted on the ordinate

Several sources of error must be kept in mind when using this method. The precision is generally no better than ± 15 μmoles H^+/g cells. Glycolysis continues during sampling and the lactic acid trapped in the cell samples can give a spurious increase in the titratable H^+ content (this is particularly annoying when glycolysis is initiated in the course of an experiment, or if its rate changes). In cells harvested after overnight growth, from media whose pH may be as low as 4.5, some of the titratable H^+ is external to the cell membrane (probably associated with the cell wall). Finally, disruption of the cells with butanol is not instantaneous. In a previous study (Harold *et al.*, 1970*a*) the cells were disrupted by boiling, but boiling was found to destroy DDA^+ and $TPMP^+$ with the generation of protons. The gentler butanol procedure is free of this objection.

Other analytical methods and general experimental conditions were described in the preceding paper.

Results

Uptake of DDA^+ and $TPMP^+$ by Exchange for Protons

Thus far we have considered only the uptake of DDA^+ and $TPMP^+$ by sodium-loaded cells, in which the overall process is the exchange of sodium for an equivalent amount of DDA^+ or $TPMP^+$ (Harold & Papineau, 1972). Fig. 3 documents that uptake of the lipid-soluble cations occurs equally well by exchange for protons. In the particular experiment illustrated here we employed NaTY cells, which contain both Na^+ and H^+; uptake of DDA^+ was equivalent to the sum of Na^+ and H^+ extruded, and there was no evidence for movement of cellular anions such as phosphate. Since uptake of DDA^+ indicates an excess of negative charges in the cell it follows that either the extrusion of protons, or of sodium, or both, must be an electrogenic process. In this section we shall focus on proton movements.

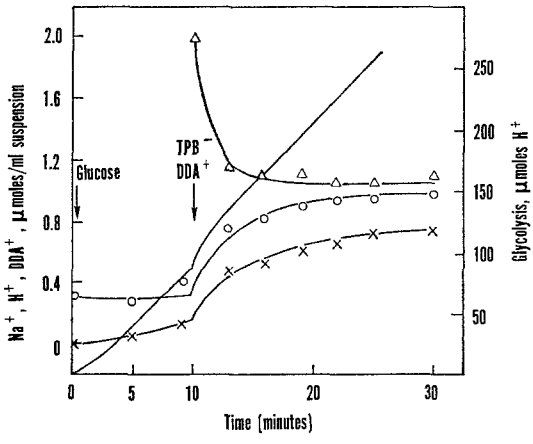

Fig. 3. Extrusion of H^+ and Na^+ by exchange for DDA^+. NaTY cells were suspended in water (60 ml, 2 mg cells/ml) and allowed to glycolyze at an external pH of 7.5. At 10 min, TPB^- (0.01 μmole/ml) and DDA^+ (2 μmoles/ml) were added. Samples of cells and filtrates were analyzed for Na^+ (○), DDA^+ (△) and H^+ (×)

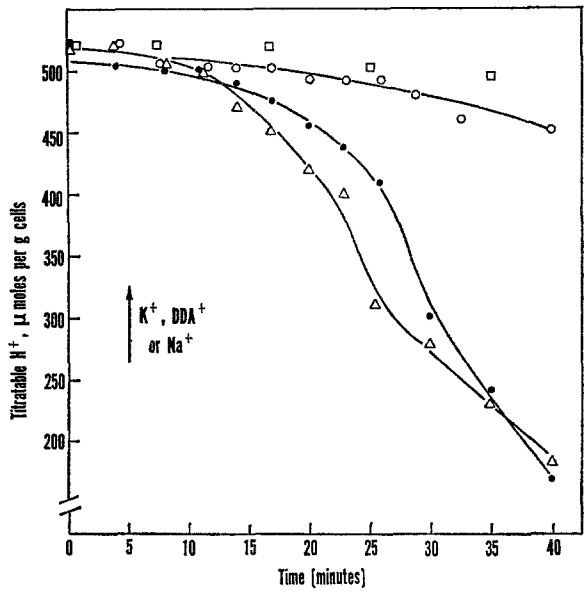

Fig. 4. Evidence that proton extrusion is an electrogenic process. Cells grown on KTY were protonated as extensively as possible by the monactin procedure, washed and resuspended in water at 2 mg cells/ml. Successive aliquots (60 ml) of the suspension were put on the pH-stat at pH 7.0. Glucose was added to each at 0 min, and the following additions were made at 5 min: No additions, ▫; K^+, 2 mM, •; DDA^+, 2 mM plus 10^{-5} M TPB^-, ▵; Na^+, 20 mM, ○. At intervals, samples of the cells were collected for determination of titratable H^+

Two modes of net proton loss from the cells were distinguished in these experiments. When the external pH was 7.5 or above, protons exchanged for K^+ or DDA^+ at a significant rate even in the absence of an energy source. Examples are illustrated in Fig. 2*B* and in Fig. 6. We attribute this passive loss of protons to titration of acidic groups external to the permeability barrier, together with diffusion of protons across the membrane. Of greater interest is net proton extrusion at pH 7.0 or below, which depends upon concurrent glycolysis.

To study metabolic proton extrusion in isolation, apart from Na^+ movements, we employed KTY cells that had been extensively protonated by the monactin procedure (*see* preceding paper). Approximately half the total K^+ complement was replaced by H^+. Such cells initiate glycolysis only after a lag of several minutes, presumably because of their low internal pH. Addition of 2 mM K^+ or DDA^+ stimulated the rate of glycolysis and induced net extrusion of protons from the cells (Fig. 4). Rb^+ and $TPMP^+$, not illustrated, likewise induced proton extrusion. By contrast 20 mM Na^+, as well as Li^+ or Mg^{++} (not illustrated) permitted little or no proton

extrusion. Analogous experiments were performed with NaTY cells, which had not been exposed to monactin; again, net proton extrusion was supported by K^+, DDA^+ and $TPMP^+$ but not by Na^+. We conclude from these experiments that proton extrusion is an electrogenic process which requires compensatory ion movements: K^+ and Rb^+, like DDA^+ and $TPMP^+$, can serve as counter-cations but Na^+, Li^+ and Cs^+ can not. There is evidently no *obligatory* coupling between the movement of H^+ and that of its physiological counterion, potassium.

Supporting evidence for the electrogenic nature of proton extrusion comes from experiments with ion-conducting antibiotics. Net proton extrusion by exchange for DDA^+ or K^+ was unaffected by valinomycin and monactin, which facilitate movement of K^+, but was blocked by the proton conductors tetrachlorosalicylanilide (TCS) (2×10^{-6} M) and carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP) (2×10^{-5} M).

Participation of ATPase in Metabolic Proton Extrusion

When the external pH was 7.0 or below, net extrusion of protons by exchange for DDA^+ (or for K^+) required concurrent glycolysis (Fig. 5).

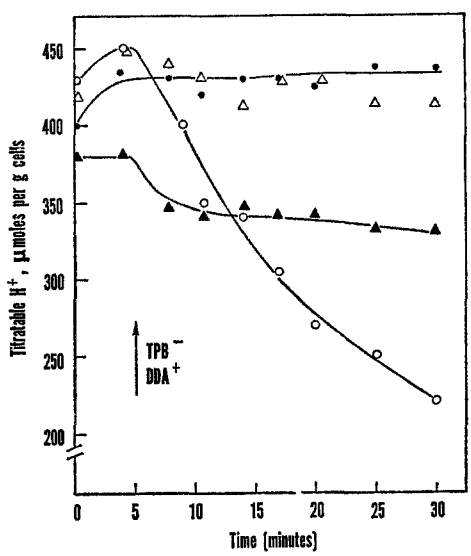

Fig. 5. Inhibition of proton extrusion by DCCD. NaTY cells were suspended in water at 2 mg cells/ml and placed on the pH-stat at pH 7.0. The following additions were made: (1) Glucose added at 0 min, DDA^+ (2 µmoles/ml) and TPB^- (0.01 µmoles/ml) at 5 min (○). (2) Cells preincubated with 2×10^{-4} M DCCD for 5 min. Glucose was added at 0 min, TPB^- and DDA^+ at 5 min (●). (3) Glucose at 0 min, no countercation (△). (4) No glucose; TPB^- and DDA^+ added at 5 min (▲)

Table 1. Effect of DCCD on the extrusion of H^+ by wild-type and mutant DC-8[a]

Minutes	Titratable H^+ (μmoles per g cells)			
	Wild-type		Mutant DC-8	
	Control	with DCCD	Control	with DCCD
0	490	500	500	500
15	270	480	260	300
30	260	470	220	240

[a] Wild-type and mutant DC-8 were grown on NaTY medium. The cells were washed, resuspended in water and allowed to glycolyze at pH 7.0 for 5 min. At this point, designated 0 min, a sample was taken. DDA^+ (2.0 μmoles/ml, together with TPB^-, 0.01 μmoles/ml) was added to serve as counterion for protons, and samples were taken at intervals thereafter for determination of titratable H^+ content. Control suspensions received no DCCD, experimental suspensions were preincubated with 1×10^{-4} M DCCD for 5 min.

This metabolic proton extrusion was abolished by N,N′-dicyclohexylcarbodiimide (DCCD), a reagent which had earlier been found to inhibit the membrane-bound ATPase of *S. faecalis* but not the generation of ATP (Harold, Baarda, Baron & Abrams, 1969). Passive extrusion of protons at alkaline pH was, of course, unaffected by DCCD. Inhibition by DCCD implicates the ATPase in the energy-linked extrusion of protons.

Further evidence comes from mutant DC-8, a strain whose ATPase is relatively resistant to inhibition by DCCD. As shown in Table 1, in this mutant extrusion of protons by exchange for DDA^+ was unaffected by 1×10^{-4} M DCCD; this concentration of DCCD completely blocked proton extrusion by the wild-type. The fact that a genetic modification which alters the ATPase also affects proton extrusion is strong evidence that these two are related.

A Genetic Defect in Proton Extrusion

In a previous study (Harold *et al.*, 1970*a*), mutant 687 A was shown to be deficient in net uptake of K^+ by exchange for H^+, particularly at pH below 7.0. Exchange of K^+ for K^+ or Rb^+, however, is normal. Moreover, the mutant can exchange K^+ for Na^+, at least to some extent. The use of DDA^+ as a nonphysiological counterion made it possible to define a genetic lesion in proton extrusion.

It will be recalled (Fig. 5) that, in the wild-type, proton extrusion was strongly stimulated by glycolysis. This was not the case in mutant 687 A: As shown in Fig. 6, net proton extrusion was unaffected by glucose, both

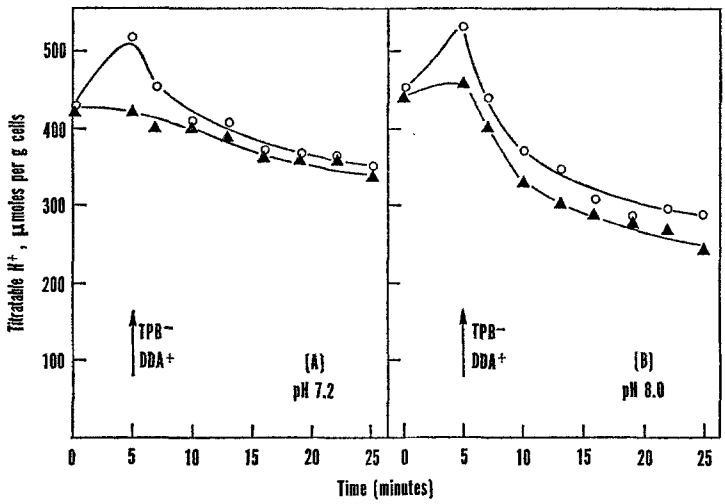

Fig. 6. Absence of metabolic proton extrusion in mutant 687 A. The organisms were grown on medium NaTY and resuspended in water at 2 mg cells/ml. (*A*) at pH 7.2; (*B*) at pH 8.0. (1) Glucose added at 0 min, TPB^- (0.01 μmoles/ml) and DDA^+ (2 μmoles/ml) at 5 min (○). (2) No glucose; TPB^- and DDA^+ were added at 5 min (▲)

Table 2. Uptake of DDA^+ by sodium-loaded cells of wild-type and mutants 687 A and 7683 [a]

Procedure	DDA^+ in filtrate (μmoles/ml)		
	Wild-type	Mutant 687 A	Mutant 7683
1. Initial DDA^+ in solution	1.08	1.07	1.1
2. Incubated for 10 min (without glucose)	0.74	0.81	0.69
3. Added glucose and incubated for another 20 min	0.21	0.62	0.60
4. Added monensin and incubated for 10 min	0.21	—	0.18

[a] Wild-type and mutants were grown on KTY medium. Sodium-loaded cells were prepared by the monactin procedure, resuspended in water at 2 mg cells per ml and incubated on the pH-stat at pH 7.5. At 0 min each suspension received about 1.1 μmoles DDA^+ and 0.01 μmoles TPB^- per ml. Samples were taken 10 min later. At this time, glucose was added to each suspension and a further set of samples was taken after 20 min of glycolysis. Monensin (2 μg/ml) was added and a final set of samples was taken.

at pH 8.0 and at pH 7.2, nor was it inhibited by DCCD (not illustrated). At pH below 7.0, net proton loss from the cells was negligible and glycolysis was severely inhibited. We conclude from these observations that mutant 687 A is deficient in the metabolic extrusion of protons; the mutant can lose protons only passively, provided the external pH is alkaline.

These experiments were done with DDA^+ as counterion. Proton extrusion is somewhat more efficient with K^+, though still much less than in the wild-type (Harold *et al.*, 1970*a*).

We shall argue below that accumulation of DDA^+ by sodium-loaded cells requires extrusion of protons. If mutant 687 A is deficient in energy-linked proton extrusion, it should also be unable to accumulate DDA^+. Table 2 documents that this is in fact the case. We showed in the preceding paper (Harold & Papineau, 1972) that uptake of DDA^+ consists of two phases: passive uptake, by ion exchange at the cell surface, and metabolic uptake in response to an electrical potential. In the mutant there was the usual passive uptake, but energy-linked uptake of DDA^+ was much less extensive than in the wild-type.

The Mechanism of Na^+ Extrusion

Most of our experiments, in this as in previous studies, were done in media containing little or no sodium. However, as illustrated in Fig. 7, glycolyzing *S. faecalis* can extrude Na^+ against a substantial concentration gradient. In the experiment illustrated here, the initial Na^+ concentration of the cytoplasm was about 0.2 N, and the external Na^+ concentration was 1.0 N, yet after addition of K^+ the level of Na^+ in the cytoplasm fell below 0.04 N. Overall, then, Na^+ moves against both the concentration gradient and the electrical gradient by what appears to be "active" transport.

Possible mechanisms of Na^+ extrusion will be considered in the Discussion. Suffice it to state here that the experiments discussed in this section

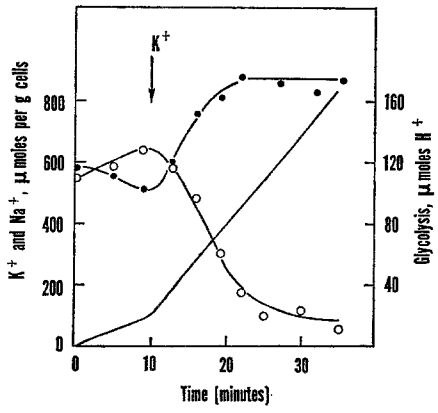

Fig. 7. Extrusion of Na^+ against a concentration gradient. Wild-type NaTY cells were resuspended in 1 N Na_2SO_4 at 1 mg/ml and allowed to glycolyze at an external pH of 6.0. Potassium to 10 mM was added at 10 min. At intervals, samples of the cells were filtered and analyzed for K^+ and Na^+. K^+, •; Na^+, ∘; —, glycolysis

were designed to discriminate between: (1) a sodium pump which actively extrudes Na^+ by an electrogenic process; and, (2) obligatory exchange of Na^+ for H^+ (Na^+/H^+ antiport). The latter hypothesis predicts that proton influx is necessarily and stoichiometrically coupled to Na^+ efflux and therefore, that *net* extrusion of Na^+ will be limited unless the cells are able to re-extrude those protons.

An early indication that sodium extrusion is linked to proton movements was the observation that both DCCD and proton conductors inhibit sodium efflux (Harold *et al.*, 1969; Harold *et al.*, 1970*a*). In an experiment in which DDA^+ served as counterion, glycolyzing cells lost 900 μmoles of Na^+ per g dry weight. When TCS was added the cells lost only 400 μmoles, whereas DCCD reduced Na^+ efflux to less than 200 μmoles/g. It should be noted that the Na^+ concentration in the cytoplasm was of the order of 0.4 M, whereas that in the medium was minimal. This large concentration gradient would be expected to favor electrogenic efflux of Na^+, by exchange either for DDA^+ or for H^+ from glycolysis. Inhibition of Na^+ efflux therefore suggests that the process is more complex than simple efflux down the concentration gradient.

Direct evidence for influx of protons coupled to the extrusion of Na^+ was obtained by the use of cells whose internal pH had been raised by washing with sodium carbonate. A typical experiment with such cells is shown in Fig. 8*A*. Carbonate-washed cells suspended in water leaked little Na^+ but Na^+ efflux began soon after addition of glucose and amounted to more than a third of the total Na^+ content of the cells. Concurrently, the titratable H^+ content of the cells increased, suggesting exchange of Na^+ for H^+. Sodium efflux considerably exceeded H^+ uptake, but it is hard to say how serious this objection is: H^+ uptake may be underestimated due to concurrent extrusion, while the Na^+ efflux may include a component of passive leakage. Sodium efflux ceased after 10 to 15 min; addition of K^+ at this point allowed the remainder of the Na^+ to be expelled together with the H^+ taken up.

Control experiments were performed with cells washed with *potassium* carbonate. Such cells glycolyzed rapidly but lost little K^+ and maintained a titratable H^+ content near 0 μmoles/g.

The role of glycolysis in Na^+/H^+ exchange is not entirely clear. Significant exchange of Na^+ for H^+ did occur in resting cells kept at pH 6.0. At more alkaline pH, (e.g. 7.5) Na^+ efflux required concurrent glycolysis. However, Na^+/H^+ exchange does not involve the ATPase. As shown in Fig. 8*B*, carbonate-washed cells preincubated with DCCD exchanged Na^+ for H^+ upon addition of glucose (albeit more slowly than the control cells). Sub-

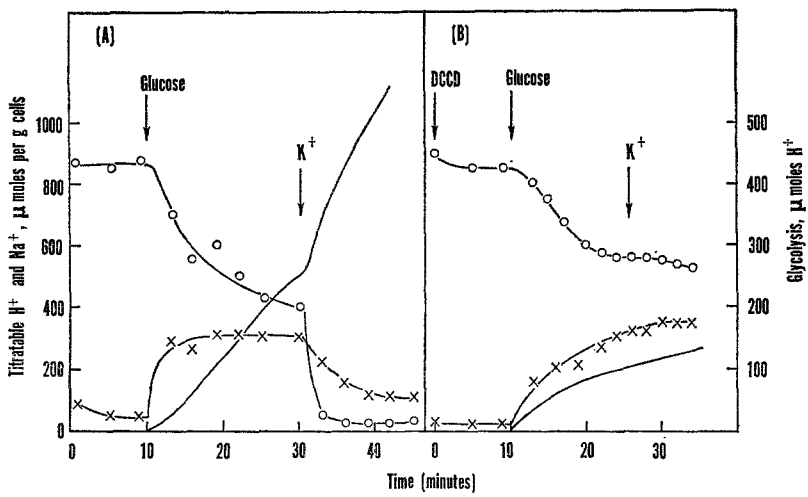

Fig. 8. Evidence for proton influx coupled to sodium efflux. *S. faecalis* was grown on medium KTY. The cells were washed with sodium carbonate and water as described in the text and resuspended in water at 2 mg cells/ml. (*A*) A suspension, 70 ml, was maintained on the pH-stat at pH 7.5. Glucose was added after 10 min. At 30 min, K^+ (2 mM) was added. (*B*) A similar cell suspension was incubated with 2×10^{-4} M DCCD. Glucose was added after 10 min, K^+ (2 mM) after 26 min. At intervals, samples of medium and cells were collected for determination of Na^+ and titratable H^+. ∘, Na^+; ×, H^+

sequent addition of K^+ induced neither reextrusion of the H^+ nor additional Na^+ efflux, indicating that the DCCD had indeed blocked the proton pump. It is noteworthy that, in the presence of DCCD, H^+ uptake was approximately equivalent to sodium efflux.

A Genetic Defect in Na^+/H^+ Antiport

In a previous paper (Harold *et al.*, 1970*a*), it was proposed that mutant 7683 is defective in Na^+ extrusion, but has a normal capacity to extrude H^+. In agreement with this conclusion, cells of the mutant that had been washed with sodium carbonate extruded little sodium when allowed to glycolyze and unlike the wild type, did not take up H^+ (Fig. 9). Thus the Na^+/H^+ antiporter appears to be missing or defective in this mutant. Experiments analogous to those shown in Fig. 4 confirmed that proton extrusion was normal.

The antibiotic monensin is known to mediate exchange of Na^+ and H^+ across biological and artificial membranes (for reviews *see* Pressman, 1968; Mueller & Rudin, 1970; Harold, 1970), and should therefore in some degree replace the defective antiporter in the mutant. Fig. 10 demonstrates that this is in fact the case: glycolyzing cells of mutant 7683, unlike those

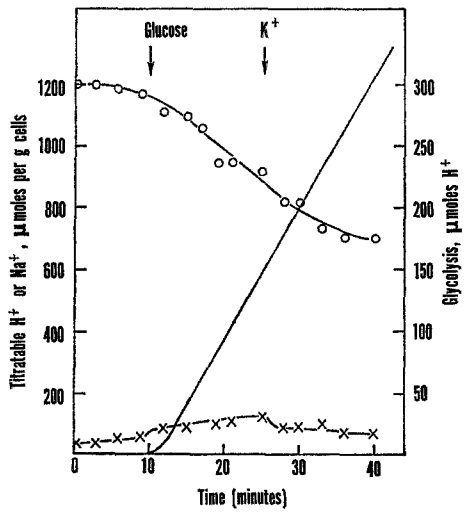

Fig. 9. Absence of Na^+/H^+ exchange from mutant 7683. Cells grown on KTY medium were washed with sodium carbonate as described in the text and resuspended in water at 2 mg cells/ml. The pH was maintained at 7.5. After 10 min glucose was added, and after 30 min K^+ (2 mM). Samples of filtrate and cells were collected at intervals for determination of Na^+ and titratable H^+ content. ∘, Na^+; ×, H^+

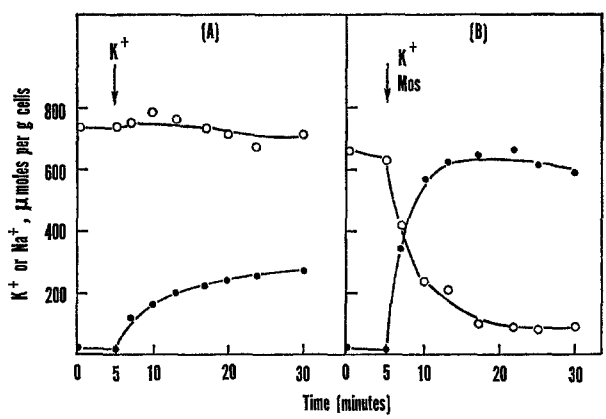

Fig. 10. Restoration of K^+ uptake in mutant 7683 by monensin. Sodium-loaded cells (1 mg/ml) of the mutant were allowed to glycolyze on the pH-stat at pH 7.5. (*A*) K^+, 1 mM, was added at 5 min. (*B*) K^+, 1 mM plus 4 µg/ml monensin were added at 5 min. At intervals, the K^+ and Na^+ content of the cells was determined. K^+, •; Na^+, ∘

of the wild type, took up little K^+ by exchange for Na^+, but uptake was markedly enhanced by addition of monensin. Analogous results were obtained with DDA^+ (Table 2).

Discussion

The point of departure for the present experiment is the finding, shown in Fig. 3, that glycolyzing cells of *S. faecalis* expel both H^+ and Na^+ and

accumulate an equivalent amount of the lipid-soluble cation DDA^+. We demonstrated in the preceding paper that uptake of DDA^+ occurs in response to an electrical difference across the membrane, interior negative. Consequently, either the extrusion of H^+, or that of Na^+, or both must be an electrogenic process. The simplest scheme capable of accommodating the results presently available is shown in Fig. 1: We propose that the primary process is the electrogenic extrusion of protons, mediated by the membrane-bound ATPase. Sodium ions are expelled by electroneutral exchange for protons (Na^+/H^+ antiport), a process dependent upon metabolism but not upon the ATPase.

Existence of an electrogenic proton pump was initially suggested by experiments with inhibitors. Glycolyzing cells maintain an internal pH considerably more alkaline than that of the medium. The pH gradient is collapsed by proton-conducting reagents and we therefore argued that the results are best attributed to active expulsion of H^+ (Harold, Pavlasova & Baarda, 1970*b*). The case is considerably strengthened by the present demonstration that proton expulsion leads to uptake of DDA^+ (Fig. 3), and does in fact not occur unless a suitable cation (K^+, DDA^+ or $TPMP^+$) is available to take the place of the H^+ (Fig. 4): If it is accepted that uptake of DDA^+ and $TPMP^+$ occurs in response to an electrical difference, as we argued in the preceding paper, it follows at the least that the cells *can* expel protons electrogenically. (Incidentally, it does not seem necessary to assume that obligatory exchange of H^+ for another cation is the *only* pathway of proton extrusion available to the cells. Under other experimental conditions, proton extrusion may well be balanced by concurrent secretion of anions, e.g., glycolytic H^+ is accompanied by lactate).

Under appropriate conditions, electrogenic proton extrusion is completely dependent upon concurrent glycolysis, and this metabolic proton extrusion is blocked by DCCD (Fig. 5). DCCD was previously shown to inhibit the membrane-bound ATPase of *S. faecalis*, but not the generation of ATP (Harold *et al.*, 1969). The inference that the ATPase is required for proton extrusion is substantially strengthened by the observation that proton extrusion in mutant DC-8 was resistant to this inhibitor (Table 1). Abrams, Smith, and Baron (1972) found that in this mutant growth, K^+ accumulation and the ATPase of isolated membrane preparations are all resistant to DCCD; the gene product is a component of the membrane itself, possibly the DCCD-receptor site. Taken together, these results are good, albeit circumstantial, evidence for the participation of the ATPase in proton expulsion as shown in Fig. 1. The alternative possibility, that proton extrusion and ATPase are independent yet both involve the DCCD-sensitive site, cannot be disproven but seems far-fetched.

The concept of the ATPase as a proton pump derives, of course, from Mitchell's chemiosmotic hypothesis (Mitchell 1966, 1968, 1970). This is not the place to review the arguments and controversies which exercise investigators of photosynthesis and oxidative phosphorylation (for recent reviews *see also* Greville, 1969; Liberman & Skulachev, 1970; Slater, 1971). Suffice it to recall that the ATPases of mitochondrial, chloroplast and chromatophore membranes are thought to couple translocation of one or more protons to the hydrolysis of ATP (Mitchell & Moyle, 1968; Scholes, Mitchell & Moyle, 1969; Grinius, Jasaitis, Kadziauskas, Liberman, Skulachev, Topali, Tsofina & Vladimirova, 1970; Isaev, Liberman, Samuilov, Skulachev & Tsofina, 1970; Mitchell, 1970). In many of its characteristics the ATPase of *S. faecalis* membranes resembles that of mitochondria (Harold *et al.*, 1969; Schnebli, Vatter & Abrams, 1970). However, the primary function of the *S. faecalis* ATPase would be the reverse of that of the mitochondrial enzyme: not the synthesis of ATP by the agency of the proton gradient, but the generation of a proton gradient by the hydrolysis of ATP. Proton extrusion takes place against a pH differential of up to one unit (Harold *et al.*, 1970*b*) and against an electrical potential of the order of -200 mV (Harold & Papineau, 1972) and thus appears to be a clear case of "active transport".

The evidence that sodium extrusion involves Na^+/H^+ antiport is somewhat less direct and bears reviewing in some detail. Glycolyzing cells extrude Na^+ by exchange for K^+ or for DDA^+. Indeed, Na^+ can be expelled against a substantial concentration gradient, some 20:1, as was first shown by Zarlengo and Schultz (1966) and confirmed here (Fig. 7). There is no evidence that the Na^+ is accompanied by an anion. On the contrary, *overall* the process of Na^+ extrusion is clearly electrogenic and "active" in the sense that Na^+ is expelled against both a concentration gradient and an electrical potential.

In mammalian cells, Na^+ extrusion is obligatorily linked to simultaneous uptake of K^+, and the ATPase of the mammalian cell membrane requires the simultaneous presence of both ions. But in *S. faecalis* (Harold *et al.*, 1970*a*), as also in *Escherichia coli* (Schultz & Solomon, 1961) there is no obligatory linkage between Na^+ and K^+: even nonphysiological ions such as DDA^+ or $TPMP^+$ can serve as counterions. We are left, then, with two alternative hypotheses: (1) An energy-linked, electrogenic Na^+ pump. (2) Exchange of Na^+ for H^+ by an antiport system, followed by extrusion of the H^+ by the proton pump. Movement of Na^+ against the concentration gradient (Fig. 7) would be facilitated by a pH difference across the membrane, interior alkaline, to provide a gradient for protons.

The idea of Na^+/H^+ antiport was favored early on by the discovery that TCS, which collapses a pH gradient, blocks exchange of Na^+ for K^+ (Harold *et al.*, 1970*a*); Mitchell (*personal communication*) suggested Na^+/H^+ antiport as a possible interpretation, and the experiments described here provide substantial support for this hypothesis. Accumulation of DDA^+ by sodium-loaded cells is inhibited both by DCCD and by proton conductors, which suggests that generation of an electrical potential by *S. faecalis* ultimately depends upon proton extrusion. Moreover, by the use of cells rendered alkaline and sodium-rich by washing with sodium carbonate, it was possible to detect an influx of protons coupled to the efflux of Na^+. This does not appear to be an experimental artefact, and we suggest with some reservations that the amounts of Na^+ and H^+ exchanged are stoichiometrically equivalent. Significantly, Na^+/H^+ antiport is not inhibited by DCCD and thus does not involve the ATPase.

These experiments are evidence that protons enter the cells when Na^+ is extruded, but it may appear that one could still attribute Na^+ extrusion to an electrogenic Na^+ pump: H^+ uptake would simply be due to the fact that, under the conditions of the present experiments, no other cation is available to take the place of Na^+. We believe this argument to be untenable, for the following reason. It is clear from Fig. 8*B* that DCCD slows, but does not block, exchange of Na^+ for H^+. If Na^+ extrusion were due to an electrogenic Na^+ pump, uptake of other cations such as DDA^+ or K^+ should likewise be resistant to inhibition. Yet we know that DCCD abolishes uptake of K^+ and of DDA^+ by sodium-loaded cells. Furthermore, the influx of protons seen in Fig. 8*B* was not reduced when K^+ or DDA^+ were present throughout the experiment. It therefore seems unlikely that there is an electrogenic Na^+ pump in *S. faecalis*. We conclude that efflux of Na^+ occurs via electrically neutral Na^+/H^+ antiport.

So far as we are aware, the only previous report of Na^+/H^+ antiport in biological membranes is that of Mitchell and Moyle (1969) in mitochondria. The concept is, however, well established for antibiotics: monensin and some others catalyze Na^+/H^+ antiport, whereas nigericin catalyzes preferentially K^+/H^+ antiport (Reviews: Pressman, 1968; Mueller & Rudin, 1970; Harold, 1970). However, unlike the Na^+/H^+ antiport mediated by the antibiotics, that observed in *S. faecalis* was strongly stimulated by glycolysis. It is not at all clear whether glycolysis is required as a source of H^+ (from lactic acid), as a source of energy (ATP?) or for other reasons.

The existence of both a proton pump and a Na^+/H^+ antiport system is supported by the identification of mutants genetically defective in these entities. Two classes of mutants were described in previous publications

from this laboratory (Harold *et al.*, 1970*a*, *b*); both were isolated by virtue of their requirement for relatively high concentrations of K^+ for growth, but neither now appears to be primarily defective in K^+ transport. (1) Mutant 687 A is representative of a class previously called Cn_{K6}^-; such mutants require high concentrations of K^+ for growth at pH 6.0. K^{42}/K^+ exchange is normal but net uptake of K^+ by exchange for H^+ is severely deficient; moreover, the cells tend to leak K^+ when incubated in high concentrations of sodium at pH 6.0. It appears from the experiments reported here that this mutant is deficient in the net extrusion of protons. Since failure to extrude protons would inhibit the Na^+/H^+ antiporter and also restrict generation of a membrane potential, this defect offers a sufficient explanation for the phenotype. The nature of the actual lesion remains unknown: it may be a defect in the ATPase, or perhaps a change in membrane structure which enhances its permeability to H^+ so that protons diffuse back into the cells. (2) Mutant 7683, previously found to be defective in Na^+ extrusion (Harold *et al.*, 1970*a*) appears to lack the Na^+/H^+ antiporter (Fig. 9). The proton pump is functional, and therefore the capacity for uptake of K^+ and DDA^+ could be restored by addition of the antibiotic monensin. (Absence of Na^+/H^+ antiport fails to account for the marked tendency of this mutant to leak K^+. Since the mutant was isolated after mutagenesis with nitrosomethylguanidine the possibility of multiple genetic lesions cannot be excluded.)

Let us now turn to the physiological role of proton extrusion, Na^+/H^+ antiport and the electrical potential, in the accumulation of K^+. Following the argument first formulated by Mitchell (1966, 1967), we proposed in previous publications (Harold *et al.*, 1970*a*, *b*) that K^+ accumulation occurs in response to the electrical potential generated during glycolysis. As a result of the present study, the case can be stated as follows: The cells do generate a potential of approximately the correct magnitude by extrusion of protons coupled with Na^+/H^+ antiport. Reagents which permit protons to flow back into the cell and thus collapse the potential difference, inhibit K^+ uptake. Mutations which affect either proton extrusion or Na^+/H^+ antiport also restrict K^+ uptake. Valinomycin, however, inhibits K^+ uptake only slightly: Indeed, *so long as valinomycin is present* we may regard K^+ as a permeant cation which distributes itself across the membrane according to the potential difference. Breaching of the permeability barrier to protons should prevent the cells from maintaining a potential difference, and indeed proton conductors let K^+ run out of the cells with concomitant uptake of H^+ (*see* Fig. 5 of Harold *et al.*, 1970*b*).

But it is quite clear that in normal cells, glycolyzing in the absence of valinomycin, K^+ is *not* in equilibrium with an electrical potential across

the membrane. Some of the experimental observations which bear on this are as follows: (1) Proton conductors stop net K^+ uptake but do not induce substantial efflux; only when valinomycin is present as well, providing a passive channel for K^+ movement, does massive K^+ efflux take place (Fig. 5 of Harold *et al.*, 1970*b*); (2) K^+ quickly and completely displaces DDA^+ from cells which had previously been allowed to accumulate this lipid-soluble cation, but DDA^+ does not displace K^+ (except in the presence of valinomycin); (3) There is virtually no uptake of DDA^+ at pH 6.0, as was shown in the preceding paper, yet accumulation of K^+ is perfectly normal. Even at pH 7.5, K^+ accumulation indicated a far higher potential than did that of DDA^+; (4) Mutant 687 A is virtually unable to accumulate DDA^+, yet exchanges K^+ for Na^+ moderately well (Harold *et al.*, 1970*a*). The implication of these discrepancies is that proton extrusion via the ATPase, the Na^+/H^+ antiporter, and the electrical potential which these can generate are necessary elements of K^+ transport by *S. faecalis* but are not sufficient to account for it. It is quite possible that the electrogenic proton extrusion described here is an artefact, or rather a partial reaction, seen only in the absence of external K^+. For the present, however, we prefer to envisage H^+ extrusion, Na^+/H^+ antiport and electrogenic K^+ uptake as separate vectorial processes mediated by genetically distinct carriers as shown in Fig. 1. There is some evidence that the latter two require glycolysis but not the ATPase, but nothing is known at present concerning their coupling to metabolism. Be this as it may, K^+ accumulation by bacteria is likely to be a more sophisticated process than the unphysiological uptake of K^+ by mitochondria. The recent identification of no fewer than nine genes concerned with K^+ transport in *Escherichia coli* (Epstein & Kim, 1971) is an indication of this complexity.

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